Development of a standardised methodology for the isolation and culture of murine mesenchymal stromal cells from different tissues and comparison of their migratory, leukocyte recruitment and immunomodulatory potential *in vivo*

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Abstract

Since the discovery of mesenchymal stromal cells (MSCs) ability to repair craniofacial defects and immunomodulatory properties, they became focus of research due to their cellular therapeutic potential. The Advanced Therapeutics Department within the Scottish National Blood Transfusion Service (SNBTS) has developed standardised methodologies for the isolation of MSCs from pancreatic islets, visceral adipose tissue, liposuction aspirate, bone marrow and umbilical cord. However, studying and comparing their *in vivo* function and the immunomodulatory potential is essential prior to their use within a clinical setting. The immunomodulatory abilities of human MSCs have been studied using mouse models that lack a control for mismatched major histocompatibility complex molecule expression. For this reason, this study aimed to objectively compare the phenotype and potential immunomodulatory functions *in vivo* of murine MSCs isolated from the bone marrow (BM MSCs), islets of Langerhans (Is MSCs) and adipose tissue (Ad MSCs) in a stringent, standardised manner, without any species or gender mismatch that could lead to both cell-mediated and humoral immune responses.

First, for use within a clinical settings MSCs need to be infused and home and engraft into the target tissue. However, most cells get entrapped in the lung and only a small percentage home and remain in the target tissue. Size could be essential to avoid lung entrapment and this study has described that murine BM MSCs are slightly smaller than Is and Ad MSCs, suggesting that they could be a better source of MSC if being delivered intravenously. Moreover, chemokine receptor expression targets immune cells into specific tissues. Comparison of chemokine receptor transcription showed that Ad MSCs have a greater transcription of *CXCR4* combined with a very low transcription of other chemokine receptors, suggesting that they will more likely suffer from lung entrapment compared to BM and Is MSCs. BM MSCs have the highest transcription of *CCR7* and *CXCR6*, and therefore, we hypothesise that BM MSCs will be more successful to reach lymphoid organs. Is MSCs have a greater potential to migrate towards the kidneys due to higher transcription of *CCR1* and *CXCR3*. Ad MSCs, on the contrary, transcribed statistically significantly more *CCR3* than Is and BM

MSCs and therefore, they have a greater potential to migrate towards the skin if they avoid entrapment within lungs.

Under resting conditions, MSCs from the three tissue sources were able to secrete chemokines at similar levels; however, this secretion did not produce a recruitment of leukocytes above control levels. Inflammatory stimulation led to increased secretion of chemokines where Is MSCs secreted the highest levels of CCL2, CCL5, CXCL1 and CXCL10, while CXCL12 was secreted at higher levels by BM MSCs. CCL2, CCL5 and CXCL1 are strong chemoattractans, but despite the higher secretion by Is MSCs under inflammatory conditions, Ad MSCs were able to recruit significantly more leukocytes *in vivo* than BM and Is MSCs. More importantly, Ad MSCs were the only MSCs able to produce the recruitment of T cells. Recipient cytotoxic cells are considered detrimental in clinical settings, but they are essential to initiate MSC-mediated immunosuppression; thus, we could hypothesise that Ad MSCs have a greater immunosuppression potential than BM and Is MSCs.

Chemokines not only have leukocyte recruitment properties, as examples, CXCL1, CXCL2 and CXCL12 have angiogenic properties while CXCL10 has angiostatic potential. Chemokines are not the only molecules secreted by MSCs with the potential to regulate angiogenesis. Ad MSCs secreted the most IL-6, which can promote VEGF secretion, but Is MSCs secreted the most VEGF under resting and stimulatory conditions, which combined with the increased secretion of CXCL1, CXCL2 and CXCL12, we hypothesise that Is MSCs could have greater re-vascularisation potential.

Altogether, this study highlights that MSCs from different sources differ in their ability to recruit and immunomodulate surrounding immune cells *in vivo*. These differences have the potential to influence their clinical performance.

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Author's Declaration

I declare that the work described in this thesis is original and was generated as a result of my own efforts. None of the data submitted as part of this thesis has been submitted for any other degree, either at the University of Glasgow, or at any other institution.

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Abbreviations

#		
	7TM	Seven-transmembrane
A		
	ACKR	Atypical chemokine receptor
	Ad MSC	Adipose derived mesenchymal stromal cell
	AIDS	Acquired immunodeficiency syndrome
	ATP	Adenosine triphosphate
B		
	B2M	Beta-2 microglobulin
	Bcl2	B cell lymphoma 2
	BCR	B cell receptor
	BM	Bone marrow
	BM MSC	Bone marrow derived mesenchymal stromal cell
	bp	Base pair
C		
	C5a	Complement component 5a
	CAF	Cancer associated fibroblasts
	cAMP	Cyclic adenosine monophosphate
	CD	Cluster differentiation
	CFH	Complement factor H
	CFU-Fs	Colony-forming unit fibroblasts
	CIA	Collagen induced arthritis
	CLP	Common lymphoid progenitor
	CLR	C-type lectin receptors
	CMP	Common myeloid progenitor
	COX2	Cyclooxygenase 2
	CSC	Cancer stem cell
	CT	Cycle threshold
	CTLA4	Cytotoxic T lymphocyte-associated protein 4
	CTLs	Cytotoxic T lymphocytes
D		
	DAG	Diacylglycerol
	DAMPS	Damage associated molecular pattern
	DC	Dendritic cell
	ddH_2O	Double distilled H_2O
	DMEM	Dulbecco's modified eagle medium
	DMT1	Diabetes mellitus type 1
	DPBS	Dulbecco's phosphate-buffered saline
E		
	ECP	Eosinophil cationic protein
	EDN	Eosinophil derived neurotoxin
	EDTA	Ethylenediaminetetraacetic acid

EPO	Eosinophil peroxidase
F	
FABP4	Fatty acid binding protein 4
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FMO	Fluorescence minus one
FO	Follicular
G	
G	Gauge
GAG	Glycosaminoglycans
GDMSCs	Granulocytic derived myeloid derived suppressor cells
GDP	Guanosine diphosphate
GMCSF	Granulocyte macrophage colony stimulating factor
GMDSCs	Granulocytic myeloid derived suppressor cells
GMP	Granulocyte/ monocyte precursor
GMP	Good manufacturing practice
Gp	Glycoprotein
GPCRs	G-protein coupled receptors
GTP	Guanosine triphosphate
GVHD	Graft versus host disease
H	
HGF	Hepatocyte growth factor
HIF1α	Hypoxia inducible factor 1α
HIV	Human immunodeficiency virus
HKG	Housekeeping gene
HLA	Human leukocyte antigen
HMGB	High mobility group box
HSC	Haematopoietic stem cell
HSCT	Haematopoietic stem cell transplant
I	
ICSs	Intermediate cell states
IDO	Indoleamine 2, 3 dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-1ß	Interlukin-1 beta
ILC	Innate like lymphocytes
iNOS	Inducible nitric oxide synthase
IP	Intraperitoneal injection
IP-10	Interferon-γ-inducible protein 10
IP3	Inositol (1,4,5) trisphosphate
IPMSC	Induced pluripotent stem cell derived mesenchymal stromal cell
IPSC	Induced pluripotent stem cell
Is MSC	Islet derived mesenchymal stromal cell
ISCT	International society for cellular therapy

	IV	Intravenous
K		
	KC	Keratinocyte chemoattractant
	KIR	Killer immunoglobulin-like receptors
L		
	LN	Lymph node
	LPS	Lipopolysaccharide
	LTA	Lipoteichoic acid
M		
	MBP	Major basic protein
	MC	Mast cell
	MCP	Monocyte chemoattractant protein
	MEF	Mouse embryonic fibroblasts
	MEP	Megakaryocyte/ erythrocyte progenitor
	MHC	Major histocompatibility complex
	MIP1α	Macrophage inflammatory protein 1 α
	MMP9	Matrix metallopeptidase 9
	MS	Multiple sclerosis
	MSC	Mesenchymal stromal cell
	MZ	Marginal zone
N		
	NETs	Neutrophil extracellular DNA traps
	NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
	NK	Natural killer
	NLR	Nucleotide-binding oligomerization domain like receptors
	NO	Nitric oxide
	Nod	Nucleotide-binding oligomerization domain
	NOS2	Nitric oxide synthase 2
P		
	P	Passage
	PAMPs	Pathogen associated molecular patterns
	PBS	Phosphate-buffered saline
	PD1	Programmed cell death protein 1
	PGE2	Prostaglandin E2
	PIP2	Phosphatidylinositol 4,5-biphosphate
	PKA	Protein kinase A
	PLC	Phospholipase C
	PPC	Positive PCR control
	PRRs	Pattern recognition receptors
	PVDF	Polyvinylidene difluoride
R		
	RA	Rheumatoid arthritis
	RAGE	Receptor for advanced glycation end products
	RBC	Red blood cell
	RBCLB	Red blood cell lysis buffer

	RIG-1	Retinoic-acid-inducible gene 1
	RIPA	Radioimmunoprecipitation assay
	RLR	Retinoic-acid-inducible gene I like receptors
	ROS	Reactive oxygen species
	RT	Room temperature
	RTC	Reverse transcription control
S		
	SC	Subcutaneous
	SCF	Stem cell factor
	SDF-1α	Stromal derived factor 1α
	SIV	Simian immunodeficiency virus
	SNBTS	Scottish national blood transfusion service
	SOT	Solid organ transplantation
	STAT	Signals transducers and activators of transcription
	STZ	Streptozotocin
T		
	T1DM	Type 1 diabetes mellitus
	TACE	Tumour necrosis factor-α converting enzyme
	TAM	Tumour-associated macrophages
	TCR	T cell receptor
	Tfh	T follicular helper
	Th	T helper cell
	TLRs	Toll like receptors
	Tm	Melting temperature
	TNF	Tumour necrosis factor
	Treg	T regulatory cell
U		
	UC MSCs	Umbilical cord derived mesenchymal stromal cells
V		
	Va MSCs	Visceral adipose derived mesenchymal stromal cell
	VEGF	Vascular endothelial growth factor
W		
	WBCs	White blood cells

Chapter 1

Introduction

1 Introduction

This study arises from the lack of consensus in understanding the immunomodulatory roles of mesenchymal stromal cells (MSCs), which has contributed to the controversial results in the literature as a consequence of the different isolation, enrichment and culture protocols, as well as the intrinsic variability among MSCs derived from different donors and tissues. For this reason, the aim of this study was to isolate, study and compare through a stringent set of standardised techniques murine bone marrow (BM), islets of Langerhans (Is) and adipose tissue (Ad)-derived MSCs to better understand the role of MSCs in inflammation and to study the effect of tissue source of isolation in the orchestration of inflammation. Given the nature of this work, this introductory section will provide an extensive overview of the immune system and MSCs. Firstly, the cellular and molecular mechanisms involved in the response to an inflammatory agent will be detailed. Secondly, the chemokine family will be reviewed due to its role in the migration of immune cells under homeostatic and inflammatory conditions. Lastly, I include a broad review of MSCs discussing topics such as the impact of MSC tissue of origin in the phenotype and function, the role of these cells within the immune response and the clinical uses of MSCs.

1.1 The immune system

Inflammation can be defined as a localised protective response process after injury or destruction of tissues to eliminate the injurious agent. Inflammation is characterised by four classical signs described by Celsus: rubor (redness), calor (increased heat), tumour (swelling) and dolor (pain). However, in the nineteenth century Virchow added a fifth sign, functio laesa (loss of function) (Rather, 1971).

The inflammatory response is driven by the immune system, a complex network of cells and organs that protect the body by recognition and elimination of nonself components. It is extremely important for the immune system to achieve a good self-nonself discrimination to avoid an overactive and misguided immune response against components normally present in the body, as this would lead to chronic inflammation and autoimmune diseases. The immune system has two

major arms working together to protect the host, the innate and the adaptive immune responses.

1.1.1 The innate immune response

The innate immune response is not specific to particular pathogens or damage, but it is of extremely high relevance the first hours and days of exposure to a new pathogen. The first barriers to prevent infection are skin and other epithelial surfaces that act as physical barriers to microorganisms. The inner part of these epithelial surfaces is covered with a mucus layer that protects against chemicals, physical damages and infections (Nochi and Kiyono, 2006).

Damage of these barriers gives microorganisms access to the body. Microorganisms have pathogen-associated molecular patterns (PAMPs), which must be rapidly recognised as nonself components by the immune system to neutralise the invading pathogen. PAMPs are of various types and they are usually molecules with essential functions for the pathogens and thus, molecules with low genetic variability. As an example, prokaryotic organisms use formylmethionine for mRNA translation initiation instead of the methionine residue used by eukaryotic organisms; this is easily recognised by the immune system. Moreover, microorganisms usually have outer surface molecules not present in humans that act as immunostimulants, such as lipopolysaccharide (LPS) on gram negative bacteria and lipoteichoic acids (LTA) on gram positive bacteria (Abraham and Medzhitov, 2011). Bacterial and viral genomes can also act as PAMPs due to the presence of CpG unmethylated dinucleotides flanked by two 5′ purine residues and two 3′ pyrimidines (Abraham and Medzhitov, 2011). In the case of viruses, viral DNA or RNA can also be recognised as PAMPs, leading to the activation of the immune response (Luecke and Paludan, 2016).

To recognise PAMPs, cells of the immune system express pattern recognition receptors (PRRs). However, the immune system does not only produce a response to microbial pathogens, but also to haemorrhagic shock, tissue injury, cell necrosis and reperfusion injury (Raymond et al., 2017). In the presence of damage or stress, cells release damage-associated molecular patterns (DAMPs), which are also able to activate the immune response via PRR-mediated recognition (Gallucci and Matzinger, 2001). PRRs include: toll-like receptors

(TLRs); C-type lectin receptors (CLRs); nucleotide-binding oligomerization domain (Nod) like receptors (NLRs); retinoic-acid-inducible gene-I (RIG-I) like receptors (RLRs); and receptor for advanced glycation end products (RAGE)(Raymond et al., 2017).

TLRs were the first PRRs to be discovered and are the most widely studied. There are 10 receptors in humans (TLR1-TLR10), and despite some overlapping ligands for the receptors, each TLR has specific molecular recognition patterns. TLRs recognise PAMPs both within and outwith the cell as TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are expressed on the cell membrane while TLR3, TLR7, TLR8 and TLR9 are expressed within intracellular vesicles of innate immune cells (Raymond et al., 2017). TLR1 recognises triacylated lipopeptides via dimerization with TLR2 (Takeuchi et al., 2002). TLR2 on its own is involved in gram positive bacteria recognition by interaction with peptidoglycans and LTA on their surface (Schwandner et al., 1999). Moreover TLR2, in combination with TLR6, is able to recognise diacylated bacterial lipoproteins (Takeuchi et al., 2001). TLR3 recognises retroviruses by interaction with double stranded RNA (Termeer et al., 2002). TLR4 is involved in the recognition of LPS from gram negative bacteria (Termeer et al., 2002), structural proteins from viruses (Del Cornò et al., 2016), mannan (a cell wall polysaccharide) from fungi (Figueiredo et al., 2012), glycoinositolphospholipids from *Trypanosoma* (Dos-Santos et al., 2016) and endogenous high mobility group box (HMGB) nuclear proteins from distressed cells (Yu et al., 2006). TLR5 is the cognate receptor for flagellin, an essential protein of the flagella of bacteria (Hayashi et al., 2001). TLR7 and TLR8 are both involved in the recognition of single stranded viral RNA (Heil et al., 2004). TLR9 is involved in the recognition of the CpG regions (Hemmi et al., 2000). The function of TLR10 remains unknown but it has been associated with the B cell lineage as antibody mediated engagement of TLR10 on primary human B cells suppresses B cell proliferation, cytokine production, and signal transduction (Hess et al., 2017).

CLRs are expressed on the surface of dendritic cells (DCs) and are able to recognise bacterial, fungal and viral PAMPs and DAMPs (Yamasaki et al., 2008). Intracellular PAMPs are mainly recognised due to NLRs, even if NLRs can also recognise DAMPs (Fritz et al., 2006). Interaction with NLRs leads to the

formation of inflammasomes, which activates caspase 1 and produces interleukin-1ß (IL-1ß), promoting and amplifying the immune response (Pétrilli et al., 2007). Moreover, NLRs are also able to produce pyroptosis, an inflammatory form of cell death, due to the activation of caspase 1 (Fitzgerald, 2010). RLRs are the cognate receptors for RNA viruses (Loo et al., 2008) while RAGE acts as a receptor for the products of nonenzymatic glycation and oxidation of proteins/ lipids (AGEs) (Wautier et al., 2001), HMGB1 (Rouhiainen et al., 2004) and S100 proteins (Hofmann et al., 1999).

1.1.1.1 Cells of the innate immune system

Every single cellular component from the blood is derived from a common progenitor haematopoietic stem cell (HSC) in the bone marrow. These HSCs are multipotent as they can engender all types of blood cells (Orkin, 2000). Multipotent cells, such as stem cells, have the ability of both self-renew and differentiate; HSCs can differentiate into two lineages of lower potential, the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP). CLPs give rise to lymphocytes and natural killer (NK) cells, while CMPs differentiate into granulocyte/ monocyte precursors (GMPs) and megakaryocyte/ erythrocyte precursors (MEPs). GMP is the precursor of granulocytes, macrophages, mast cells and myeloid DCs, while MEPs differentiate into platelets and erythrocytes. Granulocytes can further differentiate into neutrophils, eosinophils, basophils and monocytes. Figure 1-1 provides an overview of this system.

Cells of the immune system recognise pathogens or results of trauma, become activated and react against them. These cells are activated in a precisely regulated order, starting with tissue resident macrophages, mast cells, neutrophils, monocytes and recruited macrophages and later on, with cells of the adaptive immune system, T helper cells, cytotoxic T cells and B cells (Metz and Maurer, 2009). The activation of these cells leads to a fast recruitment of immune cells to sites of trauma due to the secretion of cytokines and chemokines. Further information about these molecules will be provided in Section 1.2.

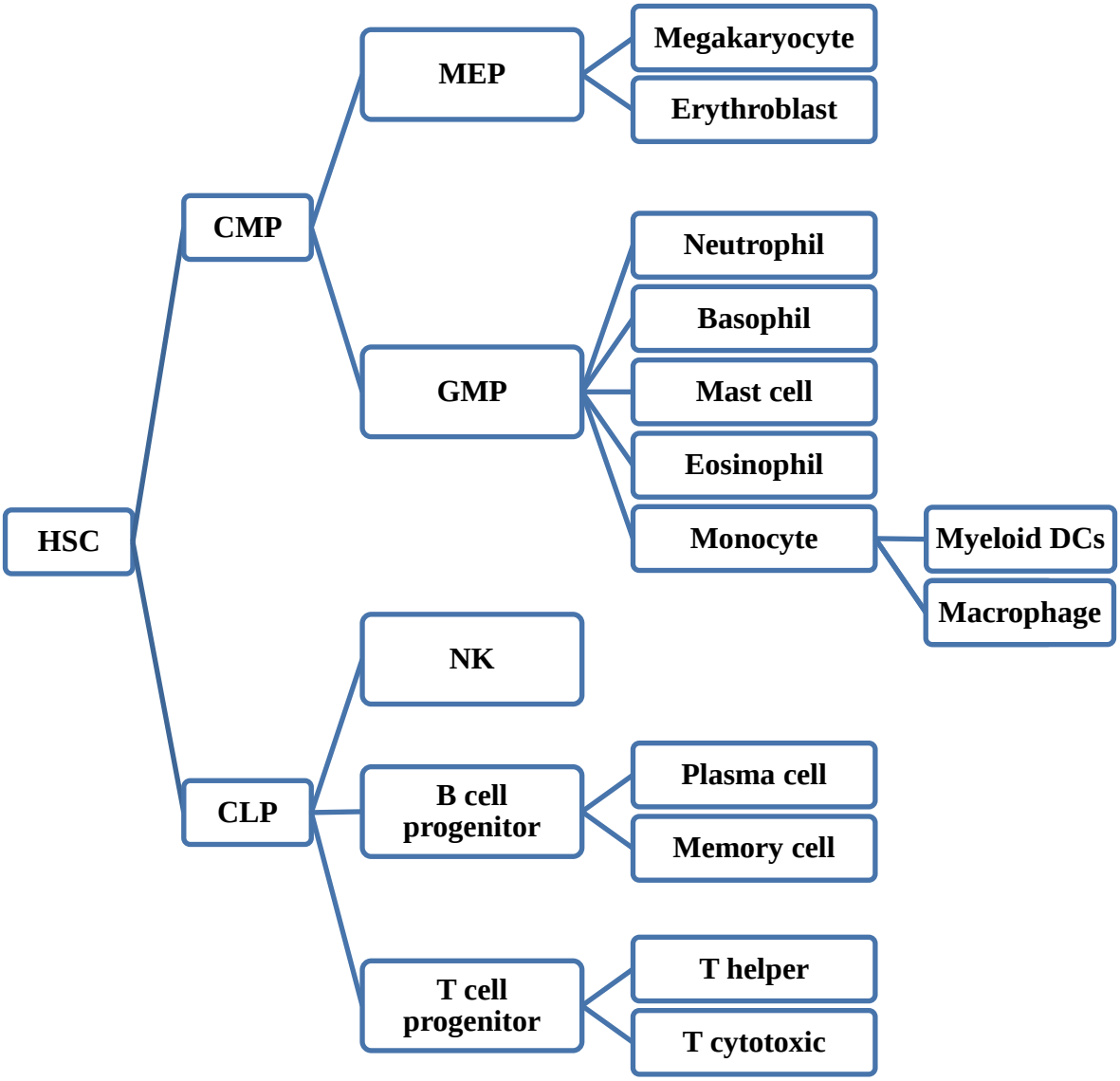


Figure 1-1. Schematic representation of the immune cell development and differentiation processes.
HSCs can differentiate into common myeloid progenitors (CMP) or common lymphoid progenitors (CLP). CMPs can then further differentiate into megakaryocyte/ erythrocyte precursors (MEP) or granulocyte/ monocyte precursor (GMP). MEPs can further differentiate into megakaryocytes and erythroblasts while GMPs can further differentiate into neutrophils, basophils, mast cells, eosinophils and monocytes. Monocytes can engender myeloid DCs and macrophages. CLPs can differentiate into natural killer (NK) cells, B cell progenitors and T cell progenitors. B cell progenitors can further differentiate into plasma and memory cells while T cell progenitor can give rise to T helper and T cytotoxic cells (Orkin, 2000).

1.1.1.1.1 Mast cells

Mast cells (MCs) are large, long-lived cells that are developed from $CD34^+$ pluripotent progenitor cells in the bone marrow and circulate in blood and lymphatic vessels in an immature state. These cells migrate to connective tissues and perivascular sites, where they undergo maturation upon interaction

with stem cell factor (SCF) and IL-3 (Haig et al., 1994). Mature MCs are usually found in tissues interfacing external environments and near to blood vessels to behave as a first line defence. Morphologically, the main feature of these cells is the presence of cytoplasmic granules; MCs are not a homogenous population and their heterogeneity is based on their granule content.

MCs can be activated via immunoglobulin (Ig) E-dependent (Rivera et al., 2008) and IgE-independent manners (Gilfillan and Rivera, 2009), leading to the secretion of their cytoplasmic granule contents. Allergens interact with high affinity IgE receptors on the surface of MCs, leading to the subsequent cross linking (Rivera et al., 2008). On the other hand, MCs contain other receptors on their surfaces able to recognise and become activated by cytokines, neurotransmitters and anaphylatoxins (Gilfillan and Rivera, 2009). Among the mediators secreted by MCs, histamine, serotonin and proteases are found at high concentrations. MCs also secrete newly synthesised mediators, such as tumour necrosis factor (TNF) (Olszewski et al., 2007), leukotrienes, prostaglandins and platelet-activating factors (Boyce, 2007). Moreover, MCs are also able to secrete cytokines and chemokines, transmit microRNAs and secrete exosomes (Skokos et al., 2003). Altogether, MCs are involved in the regulation of permeability, secretion, peristalsis, nociception, angiogenesis and innate and adaptive immunity (Lee and Lee, 2016). MCs deregulation is involved in allergic responses (Stelekati et al., 2007), multiple sclerosis (Krüger, 2001), bowel disease (Lee and Lee, 2016), arthritis (Nigrovic and Lee, 2005) and cancer (Ribatti, 2016).

1.1.1.1.2 Neutrophils

Neutrophils are short-lived phagocytes that account for 40-70% of white blood cells in mammals. Neutrophils have a multi-lobed nucleus, making their identification very easy by histological staining. Neutrophils are essential in innate immunity as they are the first leukocytes to be recruited to sites of trauma upon inflammation (Nourshargh and Alon, 2014); their role is so pivotal that individuals with congenital neutrophils deficiencies can die due to opportunistic infections (Keszei and Westerberg, 2014).

The granulocyte-colony stimulating factor (G-CSF)/ IL-17 axis is involved in the homeostatic regulation of neutrophil production, mobilization and clearance,

which is modulated by the circadian rhythm, the presence of microbiota and lipid mediators (Tan and Weninger, 2017). Under the influence of signals such as CXCL8, CXCL2, or vascular endothelial growth factor (VEGF), neutrophils are recruited to tissues (Pignatti et al., 2005). However, to arrive at the specific tissue, circulating neutrophils must undergo a multi-step process known as the "leukocyte adhesion cascade", where neutrophils need to extravasate the vascular endothelium and breach the basement membrane to arrive to the interstitial space. Once at the site of injury, neutrophils have several mechanisms to attack microorganisms. First, neutrophils are able to engulf microorganisms by a mechanism called phagocytosis leading to their degradation in lysosomes (Lee et al., 2003, Faurschou and Borregaard, 2003). Neutrophils secrete neutrophil extracellular DNA traps (NETs) that immobilise and target pathogens (Brinkmann et al., 2004). Lastly, neutrophils secrete cytokines and chemokines, and in this way, induce the recruitment of immune cells to amplify the inflammatory response.

Neutrophils have also been described to be involved in angiogenesis (Aldabbous et al., 2016), inflammation resolution and wound healing (Hahn et al., 2016). In mice, CXCL2 attracts proinflammatory neutrophils, which express CD11b and GR-1 and have a low expression of CXCR4, while proangiogenic neutrophils are recruited by VEGFa and secrete matrix metallopeptidase 9 (MMP9) (Christoffersson et al., 2012). In humans, neutrophils capable of suppressing T cells are $CD16^{BRIGHT}$ $CD62L^{DIM}$ (Pillay et al., 2012). Moreover, acute systemic inflammation lead to the discovery of two distinctive neutrophil populations with opposing roles, $CD16^{DIM}$ $CD62L^{BRIGHT}$ and $CD16^{BRIGHT}$ $CD62L^{BRIGHT}$ (Kamp et al., 2012). These findings have made immunologists consider the existence of two different neutrophil populations that could be recruited independently of one another.

Due to the inflammatory functions of neutrophils, over activation of neutrophils leads to extensive tissue damage in pathologies as chronic obstructive pulmonary disease (Hoenderdos and Condliffe, 2013), rheumatoid arthritis (Wright et al., 2014) and cancer (Spicer et al., 2012) among others.

1.1.1.1.3 Monocytes

Monocytes are circulating white blood cells that account for 2% and 8% of nucleated cells in the blood in mice and humans, respectively. Monocytes develop from the granulocyte/ macrophage progenitor in the bone marrow and circulate in blood; CCR2 is required for monocytes to leave the bone marrow and reach the bloodstream (Tsou et al., 2007). There are three main subsets of monocytes in humans, which are termed as classical, intermediate and non-classical monocytes. CD14 is a marker of human monocytes and each group expresses it at different levels. Classical monocytes are $CD14^{HIGH}$ $CD16^{-}$, have proinflammatory roles and give rise to inflammatory macrophages in tissues (Yona et al., 2013). Classical monocytes produce, and secrete, reactive oxygen species (ROS), TNF-α and IL-1ß. Moreover, they express CCR2, which allows them to leave the bone marrow and mediate increased monocyte attraction during inflammation (Zawada et al., 2012). Early in development, classical monocytes can differentiate into intermediate monocytes, which can then differentiate into non-classical monocytes. The non-classical monocytes are $CD14^{LOW}$ $CD16^{HIGH}$, have an anti-inflammatory role and give rise to profibrotic and anti-inflammatory macrophages (Yona et al., 2013). The so called intermediate monocyte population is $CD14^{HIGH}$ $CD16^{LOW}$ and gives rise to monocyte derived DCs (Ziegler-Heitbrock et al., 2010). Non-classical monocytes express low levels of CCR2 but high levels of CX3CR1, which allows them to patrol vessels wall and invade by interaction with CX3CL1 (Yang et al., 2014).

It is important to mention that there is a lack of homology between mice and humans regarding phenotypic markers in monocytes and monocyte-derived cells, which hindered the identification of homologous populations between species. Mice possess two subsets of monocytes according to their expression of Ly6C, CCR2 and CX3CR1. The use of transcriptomics, metabolomics, proteomics, and epigenomics has enabled the identification of homology between $Ly6C^{HIGH}$ $CCR2^{HIGH}$ $CX3CR1^{LOW}$ and $CD14^{++}$ $CD16^{-}$ and between $Ly6C^{LOW}$ $CCR2^{LOW}$ $CX3CR1^{HIGH}$ and $CD14^{+}$ $CD16^{+}$ monocytes (Reynolds and Haniffa, 2015). The $Ly6C^{LOW}$ $CCR2^{LOW}$ $CX3CR1^{HIGH}$, in mice, and $CD14^{+}$ $CD16^{+}$ monocytes, in human, have a patrolling role along the vascular endothelium where they are involved in tissue repair. On the other hand, $Ly6C^{HIGH}$ and $CD14^{++}$ monocytes perform pro-inflammatory functions (Reynolds and Haniffa, 2015, Yang et al., 2014). These populations do

not have a perfect overlap with the human monocyte subsets even if the roles of monocytes observed in the immune response look similar (60).

1.1.1.1.4 Macrophages

Macrophages are a subset of phagocytic cells involved in the engulfment and digestion of cellular debris, microorganisms, cancer cells and anything unhealthy for the host. Macrophages were thought to emerge only from circulating monocytes, which had been recruited to tissue. However, Schulz et al. showed that macrophages could emerge independently of HSCs as they found yolk sac-derived precursors during embryonic development (Schulz et al., 2012). Moreover, some tissue macrophages solely emerge from embryonic precursors (Van Gassen et al., 2015). Macrophages can be found in every tissue of the body; however, their nomenclature is based upon their tissue of origin, as an example, osteoclasts in bone, Kupffer cells in the liver and microglia in the brain. Tissue of residency comes together with functional specialization e.g. bone resorption by osteoclasts, breakdown of red blood cells by Kupffer cells or neural network maintenance and development by microglia (Reynolds and Haniffa, 2015). As previously mentioned, there is a lack of homology between mice and humans regarding phenotypic markers in the mononuclear phagocyte system. Murine macrophages are characterised by the expression of CD11b, CD68, CSF1R and F4/80 (Wynn et al., 2013), phenotypic markers shared by human macrophages with the exception of F4/ 80, which is expressed on human eosinophils (Hamann et al., 2007). To my knowledge, few comparative analyses between murine and human macrophages have been performed and cross species comparisons in health and disease have not been rigorous. There are inter-species variations in response to inflammation between human and mouse *in vitro* derived macrophages; upon LPS-mediated stimulation murine macrophages upregulate *iNOS* while human macrophages induce the transcription of *CCL20*, *CXCL13*, *IL-7R*, *P2RX7* and *STAT4* (Schroder et al., 2012).

Macrophages are not only phagocytes, but also release effector molecules involved in the recruitment of other immune cells upon damage. Macrophages can recognise both DAMPs and PAMPs, which makes them one of the first lines of response to damage. According to their functional phenotype, macrophages can be subdivided into three populations: classically activated or proinflammatory

M1 macrophages, alternatively activated macrophages and regulatory or anti-inflammatory macrophages. Alternatively activated macrophages and anti-inflammatory macrophages are usually referred as M2 macrophages. M1 macrophages are induced by interferon (IFN)-γ and are involved in T helper (Th) 1-related immune responses, while anti-inflammatory macrophages are activated by TLR agonists and are involved in Th2-related immune responses. Alternatively induced macrophages are activated by IL-4, IL-10 and IL-13 and are involved in the resolution of inflammation and in development and tissue repair (Van Gassen et al., 2015). Due to the ability of macrophages to secrete both pro- and anti-inflammatory molecules and attract other immune cells, they are involved in the development of cancer (Olefsky and Glass, 2010, Fuentes-Duculan et al., 2010, De Palma and Lewis, 2011). Tumour cells secrete several chemoattractants, including CCL2, CCL3, CCL4, CCL5, CCL8 and VEGF, among others. The release of these chemoattractants leads to the migration of monocytes through the circulatory system towards the tumour, and once they leave the circulatory system to infiltrate the tissue, monocytes differentiate into macrophages. Pro-inflammatory macrophages can exert anti-tumour responses via the secretion of inflammatory cytokines like IFN-γ, IL-12 or TNF-α. However, secretion of cytokines and growth factors, including IL-4, macrophage colony-stimulating factor and granulocyte-macrophage colony-stimulating factor by tumour cells promotes the differentiation of monocytes towards an anti-inflammatory phenotype, where macrophages secrete TGF-ß, IL-10, VEGF, IL-17 and IL-23, dampening the anti-tumour response and promoting angiogenesis, tumour growth and metastasis (Noy and Pollard, 2014, Dandekar et al., 2011).

1.1.1.1.5 DCs

DCs are mononuclear professional phagocytes that emerge from haematopoietic bone marrow progenitor cells. However, unlike neutrophils, they do not fully digest the phagocytosed material, they process it into peptide fragments, and present self and nonself antigens to $CD4^+$ and $CD8^+$ T cells using Major Histocompatibility Complex (MHC) Class I and MHC Class II molecules respectively. In this way, DCs can activate the adaptive immune response; therefore, they connect the innate and adaptive immune responses (Steinman, 2006).

DCs are found in an immature state circulating in the blood. These cells express low levels of MHC Class II molecules but do express pattern recognition receptors to recognise PAMPs and DAMPs and CD40L. Upon interaction with pathogens, PPRs stimulate DC maturation, and PPRs and CD40 signalling increase the expression of MHC Class II and co-stimulatory molecules CD80/ CD86 and CCR7 expression (Caux et al., 1994, Sozzani et al., 1998). Matured DCs are then able to migrate through the blood stream to the spleen, or through the lymphatic vessels to the lymph nodes, were they interact with T cells in T zones. Upon interaction with T cells, DCs can induce the activation of both naïve and memory T cells or they can also induce T-cell tolerance, depending on the expression of co-stimulatory molecules on their surface (Heath and Carbone, 2001).

1.1.1.1.6 Eosinophils

Eosinophils are a subset of granulocytes that emerge from GMPs in the bone marrow and leave to the periphery, where they are found in several different tissues including the thymus, spleen, ovary, uterus, mammary glands and lower gastrointestinal tract.

Eosinophils interact with PAMPs, DAMPs, cytokines and chemokines via the expression of their cognate receptors, which leads to their activation and recruitment to sites of inflammation (Kita, 2011). Once activated, eosinophils undergo non-cytotoxic degranulation to release chemical mediators. Human eosinophil granules contain major basic protein (MBP), MBP2, eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), eosinophil derived neurotoxin (EDN), and ß-glucuronidase, which are proinflammatory molecules (Kita, 2011). Moreover, eosinophils also secrete immunomodulatory enzymes (indoleamine 2, 3 dioxygenase [IDO]), lipids (leukotriene and prostaglandin), cytokines (IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-16 and IL-18) and chemokines (CCL3, CCL5 and CL11). The release of these mediators upregulates the vascular adhesion system as well as increases the vascular permeability, allowing the recruitment of immune cells. This mechanism is so powerful that it can be harmful for the host if not properly regulated; eosinophil mediated inflammation is associated with allergic inflammation. Eosinophils are most popularly known for their roles in allergic and parasitic infections but they also play roles in post-natal mammary

gland development (Gouon-Evans et al., 2000, Shi, 2004, Andersson et al., 2014, Tani et al., 2014).

1.1.1.1.7 Basophils

Basophils are large circulating white blood cells that account for less than 1% of circulating white blood cells. Basophils are developed from the granulocyte/ macrophage progenitors in the bone marrow and circulate in blood.

Basophils contain histamine in their granules and express the high affinity IgE receptor FcεRIα. Basophils can be activated by several signals including cytokines, Igs, proteases and antigens; even if the most studied mechanism is the IgE mediated one (Siracusa et al., 2011). Upon stimulation, basophils secrete histamine, cytokines (IL-3, IL-4, IL-6, IL-9 and IL-13 among others) and chemokines (CCL3 and CCL5), promoting vascular permeability and increasing the recruitment of immune cells. Moreover, basophils are also able to act as antigen presenting cells for small molecules (haptens) (Otsuka et al., 2013).

1.1.1.1.8 NK cells

NK cells are lymphocytes that were originally thought to emerge from a common lymphoid progenitor in the bone marrow. Nowadays, it is known that NK cells can differentiate and mature not only in the bone marrow, but also in the thymus, lymph nodes, liver, spleen, tonsils, uterus and mucosa associated lymphoid tissue, where they then enter into the circulation (Cooper et al., 2009). NK cells emerge from a lymphoid progenitor but they differ from T and B cells due to their inability to produce a somatic rearrangement of their surface Ig and the lack of T cell receptors.

NK cells are an essential defence against virus and emerging tumours. NK cells are highly cytotoxic and can mediate their activity via tightly regulated mechanisms. NK cells express in their surface Killer-cell Ig-like receptors (KIRs), which are able to recognise MHC Class I molecules. This interaction avoids the activation of NK cells, promoting self-tolerance. A common survival mechanism shared between virally infected and tumour cells, is based on the downregulation of MHC Class I molecules to avoid the recognition of nonself-antigens by the immune system. However, the absence of MHC Class I molecules

makes them a good target for NK cells; in the absence of MHC Class I, NK cells become activated and thus, cytotoxic (Raulet, 1992).

Once activated, NK cells can be cytotoxic in several ways. First, NK cells induce apoptosis by secreting cytoplasmic granules with perforins and granzymes. Perforins promote the disruption of the membrane generating a pore by which granzymes are able to enter into the targeted cell promoting apoptosis (Warren and Smyth, 1999). NK cells take part in antibody-dependent cell-mediated cytotoxicity as they express on their surface FcϒRIII (CD16) receptors. Infected cells become opsonised with antibodies, which can be recognised by FcϒRIII receptors leading to NK cell activation and the subsequent release of granules (Smyth et al., 2002). Moreover, NK cells are able to induce caspase-dependent apoptosis in cells expressing death receptors as Fas/ CD95 (Caligiuri, 2008). NK cells can act quickly as they can become activated by cytokines (IL-2, IL-12, IL-15, IL-18 and CCL5) and interferons, allowing them to contain viral infections while the adaptive immune system generates a more specific response (Smyth et al., 2002).

1.1.1.2 Humoral immunity

The immune response is not only orchestrated by cells, but also by macromolecules present in extracellular fluids such as complement proteins and secreted antibodies.

The complement system is a complex network of proteins involved in the clearance of damaged cells and defence against invading microorganisms. These proteins are synthesised in the liver and secreted into the blood where they circulate in an inactive form. Activation of these molecules can take place via three biochemical pathways; the classical, the alternative and the lectin pathway (Figure 1-2).

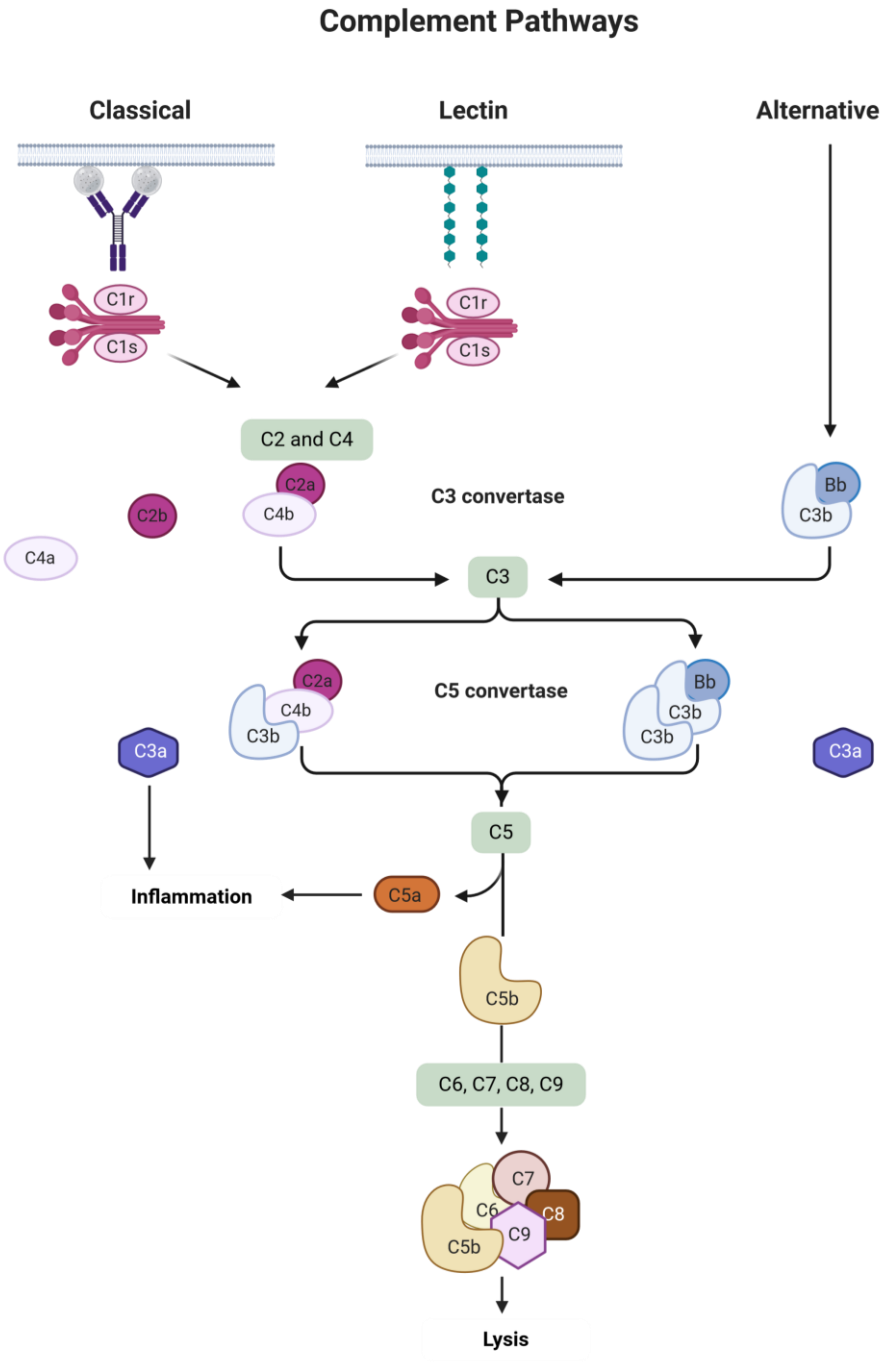


Figure 1-2. Schematic representation of the complement cascade.
There are three pathways of complement activation: the classical pathway, which is activated by the binding of antibodies to the C1q component of the complement system; the lectin pathway, which is triggered by the interaction of microbial carbohydrates bound to surface molecules on bacteria with the C1q component of the complement system; and the alternative pathway, which is constitutively active.

The classical pathway becomes activated via antigen-antibody immune complexes while the lectin pathway becoming activated by microbial carbohydrates bound to surface molecules on bacteria. The alternative pathway

does not need to become activated as it is constitutively active; however, the activation of the other pathways leads to the amplification of its activity (Reddy et al., 2017). Activation of these pathways produces the protease-mediated cleavage of C2 and C4 proteins leading to the formation of C3 convertase (C4bC2a). C3 convertase produces the cleavage of C3 to give rise to C3a, a potent anaphylatoxin, and C3b, which binds to the C3 convertase complex to generate C5 convertase. This converts C5 into C5a, another anaphylatoxin, and C5b, which makes a complex with C6, C7, C8 and C9 proteins. This complex, known as the membrane attack complex, promotes lysis of microbes. However, the complement pathway is not only involved in membrane attack, but also in phagocytosis and inflammation. C3b interacts with the surface of the pathogens promoting phagocytosis by opsonisation. The release of the anaphylatoxins leads to macrophage and neutrophil attraction and mast cell degranulation, generating an inflammatory environment.

1.1.2 The adaptive immune response

The innate immune system protects the host against pathogens, but it lacks immunological memory and what is more important, it is not antigen specific. The adaptive immune response is restricted to vertebrates and cartilaginous fish and it requires between 4-7 days before it begins while it also generates immunological memory towards specific antigens.

The adaptive immune system relies on somatic hypermutation and V(D)J recombination, a site-specific recombination process in the Ig and T cell receptor (TCR) genes, to generate a huge variety of antigen receptors. The heavy chain of the Ig and the ß chain of the TCR have a variable (V), a diversity (D) and a joining segment (J) as well as a constant domain (C); while the light chain of the Ig and the α chain of the TCR have the V and J segments as well as the constant domain. As examples, the locus for the ß chain is formed by 42 gene segments for the V region, 2 for D, 12 for J and 2 for C while the locus for the α chain includes 43 gene segments for the V region and 58 J segments (Turner et al., 2006). Each of the V, D, J and C segments are flanked by conserved recombination signal sequences. In the heavy chain of the Ig and the ß chain of the TCR, V(D)J recombination initiates by the introduction of double stranded DNA breaks between one D and one J segment, followed by the

rearrangement of the D and J segments and the removal of the DNA between those two segments (Figure 1-3). The DJ recombination is followed by the introduction of double stranded DNA breaks in the V segments, leading to the VDJ rearrangement; this is the first step in the recombination of the light chain of the Ig and the α chain of the TCR. In this manner, the different combinations of the V, D and J segments by the different chains of the Ig and the TCR leads to the generation of thousands of different receptors from a single DNA molecule. These rearrangements produce an irreversible change in the DNA that is passed down to the progeny. Upon exposure to the proper antigen, clonal expansion of the specific antigen receptor-containing cells takes place. This mechanism generates immunological memory, promoting a more efficient clearance of the pathogen in subsequent encounters.

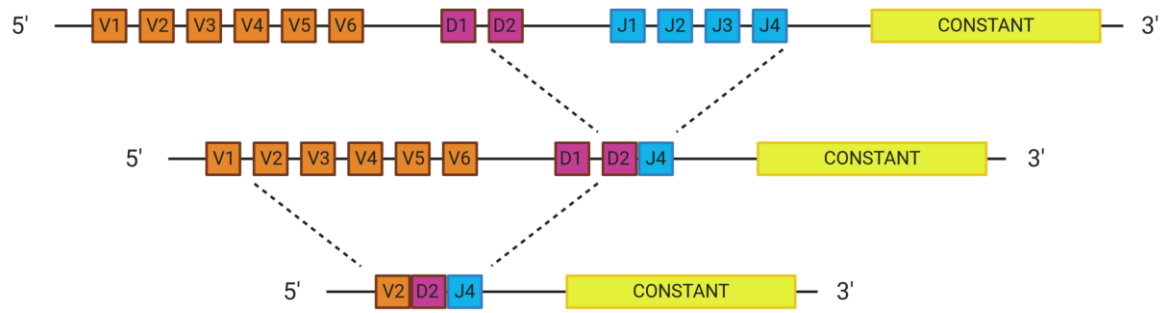


Figure 1-3. Schematic representation of V(D)J recombination.
V(D)J recombination initiates by the introduction of double stranded DNA breaks between one D and one J segment, followed by the rearrangement of the D and J segments and the removal of the DNA between those two segments. DJ recombination is followed by the introduction of double stranded DNA breaks around one V segment, leading to the VDJ rearrangement.

1.1.2.1 T cells

T cells are a type of white blood cell that emerge from the CLP in the bone marrow. They are known as T cells because they leave the bone marrow in order to reach the thymus, where they become mature. Immature T cells (thymocytes) undergo a selection where they become gradually reprogrammed into helper ($CD4^+$), cytotoxic ($CD8^+$) or regulatory T (Treg) cells.

Within the thymic cortex, thymocytes lack the expression of both CD4 and CD8 and they undergo TCR gene rearrangement. Upon the expression of the ß chain

of TCR, immature T cells simultaneously express CD4 and CD8, becoming double positive cells, which is the largest population of cells within the thymus. At this stage of maturation, the double positive cells undergo a process of positive selection where they need to prove the functionality of their TCR by interacting with self-peptides presented by MHC molecules with intermediate affinity. Double positive cells unable to bind MHC Class I or MHC Class II molecules undergo apoptosis while the rest persist by survival signals. In this manner, positive selection enables the differentiation of double positive cells towards single positive cells; cells that recognise MHC Class I will differentiate towards $CD4^-$ $CD8^+$ cells, while cells that interact with MHC Class II differentiate towards $CD4^+$ $CD8^-$ cells. Single positive cells are now able to enter the medulla of the thymus where they undergo a negative selection. Immature T cells are exposed to the presence of self-antigens and those cells binding to MHC Class I or MHC Class II with high affinity are targeted for apoptosis to avoid the proliferation of autoreactive T cells. However, a small percentage will survive negative selection and differentiate towards regulatory T cells. Cells that do not have a high affinity towards self-peptides are now able to mature and become naïve T cells, leave the thymus and migrate towards secondary lymphoid organs, where they will be able to interact with their cognate antigen and differentiate into effector cells (Germain, 2002), (Caramalho et al., 2015).

T helper cells (TH cells) are essential in the adaptive immune system as they regulate the antibody class switching in B cells, activation and growth of cytotoxic T cells, phagocytosis by macrophages and even suppression of the immune response. Naïve $CD4^+$ cells become activated upon exposure to peptide antigens by MHC Class II molecules expressed on the surface of antigen presenting cells. Th cells can differentiate into different subsets, Th1, Th2, Th9, Th17, T follicular helper (Tfh) and inducible Treg, according to the signals they receive from the surrounding environment and the type of immune response that is required in each specific situation (Abbas et al., 1996). Cytotoxic T cells, also known as T killer cells, are involved in the destruction of cancer cells, virus infected cells and even in transplant rejection, as well as in maintenance of immune tolerance. Unlike their $CD4^+$ counterparts, they recognise antigens presented by MHC Class I molecules, which leads to their activation and the subsequent release of perforins, granzymes and interferons, leading to the

target cell's death (Andersen et al., 2006). Regulatory T cells, also known as suppressor T cells, are essential for self-immunological tolerance and autoimmune diseases prevention. Moreover, they have immunosuppressive roles as they downregulate T cell mediated immune responses as well as suppress autoreactive T cells (Vignali et al., 2008). Tregs express on their surface the CD4 and CD25 biomarkers, are $FOXP3^+$, and have been postulated to emerge from the same progenitor as naïve $CD4^+$ T cells (Curiel, 2007).

Upon activation of both $CD4^+$ and $CD8^+$ lymphocytes, clonal expansion takes place and a small proportion of cells will become life-long memory cells, in order to produce a fast response after subsequent infections (Farber et al., 2014).

1.1.2.2 B cells

B cells are a type of white blood cell that emerge from the CLP in the bone marrow. B cells express B cell receptors (BCRs) on their surface, which have to undergo V(D)J recombination to enable the interaction with specific antigens (Brack et al., 1978). Each pre-B cell has the potential to express different BCR chains, however, each B cell can only express a single type of BCR, which is achieved by allelic exclusion, where the activation of one allele inhibits the activation of the rest. Developing B cells in the bone marrow undergo a selection process to ensure the capability of these BCRs to interact with antigens and at the same time, to avoid interaction with self-antigens (LeBien and Tedder, 2008). After this selection, immature B cells leave the bone marrow and go to the spleen, where they differentiate into naïve B cells, that can be follicular (FO) or marginal zone (MZ) B cells according to the signals they receive.

Naïve B cells are able to circulate in blood and lymph, as well as to go to secondary lymphoid organs such as the spleen, lymph nodes, tonsils and Peyer's patches. B cell activation begins when they interact with specific antigen presented by antigen presenting cells in the lymph nodes. Activated B cells are now able to migrate to the border between the T cell zone and the follicle within the lymph node to interact with the proper antigen-specific helper T cell (Okada et al., 2005). Upon interaction with the cognate T cell, clonal expansion

of the B cell takes place, giving rise to short-lived plasma cells, long-lived plasma cells or memory cells (Noelle and Snow, 1990).

Plasma cells, also called effector cells, are antibody-secreting white blood cells. It is important to consider that there are five different types of antibodies (IgM, IgG, IgA, IgE and IgE), but each plasma cell produces only one type of antibody, as once differentiated, plasma cells are no longer able to switch antibody classes. The production of antibody secreting B cells is a two-step process. In the first step, immediate protection is provided by the activation of B cells by an antigen receptor-dependent signal, which promotes differentiation towards short-lived plasma cells that secrete antibodies. This is a fast response but the affinity towards the antigen is moderate. In the second step, activated B cells are able to re-enter the B cell follicle and proliferate to generate a germinal centre with the help of follicular helper T cells. Germinal centres promote proliferation and somatic hypermutation to generate high-affinity antigen receptors. Affinity maturation enables the proliferation of B cells with high affinity towards antigen receptors and in this way, those highly specific B cells exit the germinal centre and differentiate into memory B cells or long-lived plasma cells that secrete antibodies with the highest affinity for the antigen (Nutt et al., 2015). The secreted antibodies will be circulating in the bloodstream as well as permeating other fluids to find cognate foreign antigens and lead to the destruction of the pathogen producing them. Antibody-antigen binding can lead to neutralisation, complement activation or phagocytosis by immune cells expressing Fc receptors, which interact with the Fc region of antibodies.

Memory B cells are dormant B cells that circulate through the blood to protect the host against subsequent infections. These cells have the same B cell receptor as the cells that were activated in the first infection and are programmed to rapidly differentiate into antibody secreting cells; for this reason, they promote a faster and stronger antibody response upon interaction with the antigen than that which gave rise to the activation of their parent B cell (Kurosaki et al., 2015).

1.2 Chemokines

As already described, the immune system is a complex network of cells that need to orchestrate their interactions to generate a proper defence response. Upon injury, cells of the immune system are induced to move to sites of inflammation. Chemically induced cell movement can be classified in terms of direction of the movement. In the case of chemokinesis, the induced movement is random and undirected while in chemotaxis, a directed movement towards the chemical takes place. In order to communicate, the immune system uses chemokines, small heparin-binding proteins that induce chemotaxis of circulating immune cells (Charo and Ransohoff, 2006).

The term chemokines derives from the combination of the words chemotactic and cytokines. Chemokines are a family of small (~8-14 KDa) homologous proteins involved in the regulation of cell migration under both inflammatory and physiological conditions that are highly conserved throughout vertebrate evolution (Zlotnik and Yoshie, 2000b). Since the identification of the first chemokines in the late 1980s, many chemokines have been identified due to the development of expressed sequence tag databases and bioinformatics, leading to a confusing nomenclature. For this reason, a standardised nomenclature was established based on the structure of the molecules (Zlotnik and Yoshie, 2000b).

1.2.1 Structural classification

Chemokines were classified taking into account their amino acid sequence, more specifically, the presence of a conserved tetra cysteine motif expressed at the amino terminus of the peptide, which is considered to be chemokines' molecular signature (Baggiolini et al., 1997). The relative position of the N-terminal first two consensus cysteine residues provides the basis for the classification of chemokines (Rot and von Andrian, 2004) (Table 1-1). This motif is of high relevance as the cysteines included on it form two covalent disulphide bonds pairing the first with the third and the second with the fourth cysteines (Zlotnik and Yoshie, 2012); these bonds are important for achieving the tertiary structure of the protein and therefore, for forming the functional protein.

Table 1-1. Chemokines classification.
Chemokine classification according to the relative position of the N-terminal first two consensus cysteine residues and the components of each subfamily.

Chemokine subfamilies	Cysteine residues configuration			Components
CC	CC	C	C	CCL1-CCL28
CXC	CXC	C	C	CXCL1-CXCL17
XC	XC		C	XCL1-XCL2
CX_3C	CXXXC	C	C	CX_3CL1

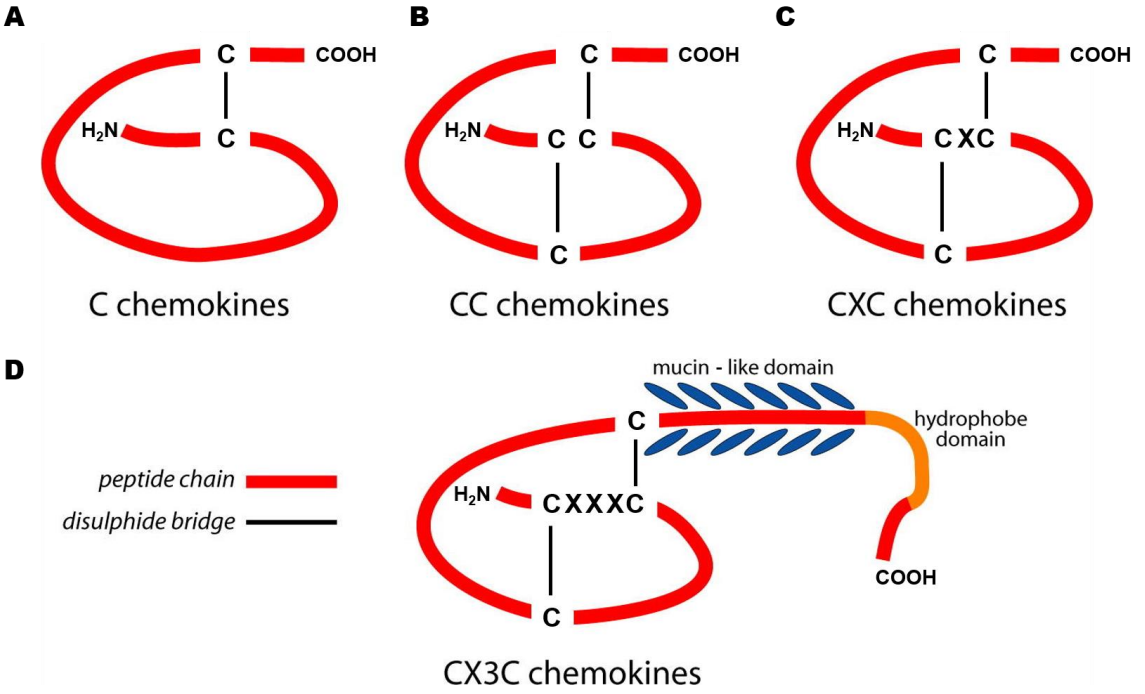


Figure 1-4. Highly conserved molecular signature of the chemokine subfamilies.
Chemokines are classified into 4 families according to the cysteine residues close to the amino terminus of the protein and the disulphide bonds originated due to these residues. XCL1 and XCL2 have just one cysteine residue near the amino terminus that enables the generation of a disulphide bond (A), while CC chemokines have two consecutive cysteine residues in the amino terminal (B) and CXC chemokines have two cysteine residues separated by only one non-conserved amino acid residue "X" (C). Fraktalkine, the only known member of the CX3C chemokine family, has two cysteine residues separated by three non-conserved amino acid residues "X" (D). The presence of two cysteine residues in the amino terminal of the protein enables the generation of two disulphide bonds. CX3CL1 and CXCL16 contain a mucin-like domain linked to a hydrophobic, and therefore transmembrane, domain and an intracellular tail that makes them be expressed as cell surface bound chemokines. However, these chemokines can be found in soluble forms too. [Image modified from Panda et al. (Panda et al., 2016)].

1.2.1.1 CC chemokines

CC chemokines, also known as the β chemokines, are the first and largest subfamily and are so named because the N-terminal first two consensus cysteine residues are adjacent to each other. There are currently 28 chemokines in this

family (CCL1-CCL28), which were numbered according to the order in which they were discovered. CC chemokines tend to attract mononuclear cells, such as monocytes, lymphocytes and some granulocytes to sites of inflammation (Alam et al., 1992a). Many of the genes for the CC chemokines are clustered on human chromosome 17 (Weber et al., 1996) or on murine chromosome 11 (Zlotnik et al., 2006).

The best characterised CC chemokines are monocyte chemoattractant protein 1 (MCP1) and macrophage inflammatory protein 1 α (MIP1α), called CCL2 and CCL3 respectively according to the systemic nomenclature. CCL2 is the most potent histamine releasing factor for basophils, while CCL3 has modest histamine releasing activity (Alam et al., 1992b, Alam et al., 1992a). Both chemokines are potent agonists for monocytes (Wolpe and Cerami, 1989) and basophils (Alam et al., 1992a) during the acute immune response. CCL2 is a potent agonist for neutrophils (Wolpe and Cerami, 1989) and both $CD4^+$ and $CD8^+$ T cells (Taub et al., 1993). RANTES, currently known as CCL5, is chemotactic for monocytes (Alam et al., 1994), $CD4^+$ T cells (Schall et al., 1990) and eosinophils (Kameyoshi et al., 1992). Some chemokines such as CCL2, CCL3 and CCL5 are produced by several cells in large amounts, while others are very specifically produced by particular tissues or cell types, such as CCL25, CCL27 and CCL28 which are respectively specific for thymus and intestine, skin keratinocytes and certain mucosal epithelial cells.

1.2.1.2 CXC chemokines

CXC chemokines, also known as the α chemokines, are the second major subfamily of chemokines and are so named because the N-terminal first two consensus cysteine residues are adjacent to each other with the addition of a single amino acid residue interposed between them. There are currently seventeen chemokines in this family (CXCL1-CXCL17). CXC chemokines can be further categorised into Glu-Leu-Arg $(ELR)^+$ and ELR^- CXC chemokines, according to the presence or absence of the motif ELR at the NH_2 terminus. CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8 belong to the ELR^+ subfamily (Murphy et al., 2000). Many of the genes for the CXC chemokines are clustered on human chromosome 4 (Weber et al., 1996) or on murine chromosome 5 (Zlotnik et al., 2006).

When trying to address the function of these chemokines, it was observed that some CXC chemokines, such as the platelet factor 4 (CXCL4), were inhibitors of angiogenesis (Maione et al., 1990) while others, such as interleukin-8 (IL-8 or CXCL8), have potent angiogenic properties (Koch et al., 1992). Even if these two chemokines have a significant homology, their roles are completely opposite. When comparing their sequences, the main difference was the presence of an N-terminal tripeptide motif glutamate (E)-leucine (L)-arginine (R) called ELR motif nearby to the CXC motif. CXC chemokines with the ELR motif promote angiogenesis upon interaction with endothelial cells, and the addition of this motif to the N-terminal domain of CXCL4 resulted into a molecule with potent angiogenic properties (Hébert et al., 1991, Clark-Lewis et al., 1993). For this reason, CXC chemokines are functionally divided based on the presence of the ELR motif nearby to the CXC motif; ELR positive CXC chemokines promote angiogenesis (Strieter et al., 2005), cell proliferation (Mockenhaupt et al., 2003) and survival during development (Li et al., 2003), while ELR negative CXC chemokines tend are more angiostatic.

CXCL8 is the archetype of the CXC chemokines and is the most potent attractor of neutrophils to sites of trauma in humans. Moreover, CXCL8 enhances cell survival and proliferation as well as angiogenesis. CXCL8 precursors are kept within endothelial cells in Weibel Palace bodies to be released after injury and recruit neutrophils to sites of acute inflammation. Due to the differences in pathogen driven evolutionary experiences between mouse and human, some inflammatory chemokines are not present in both species (Zlotnik and Yoshie, 2000b). Mice lack CXCL8, but they have CXCL1 and CXCL2, both neutrophils chemoattractants (Hol et al., 2010).

1.2.1.3 XC chemokines

XC chemokines, also known as the γ chemokines, are so named because they lack one of the first two consensus cysteine residues in the N-terminal region and retain the fourth one. There are just two chemokines in this subfamily XCL1 (lymphotactin α) and XCL2 (lymphotactin ß). The gene that encodes lymphotactin maps to chromosome 1 (Kelner et al., 1994).

XCL1was first detected in activated $CD8^+$ T cells from thymus and spleen and in activated $CD4^-$ $CD8^-$ $TcR\alpha\beta^+$ thymocytes (Yoshida et al., 1995); therefore, it was associated with the Th1 response (Dorner et al., 2002). Dorner et al. found that lymphotactin has a relevant role in the development of efficient cytotoxic immunity *in vivo* (Dorner et al., 2009). 8 to 36 hours after antigen recognition by DCs, when T cell and DCs interact, $CD8^+$ T cells secrete high amounts of XCL1, which increased the survival and differentiation of $CD8^+$ T cells towards IFN-γ-secreting effectors and leading to an increase of the pool of antigen-specific $CD8^+$ T cells *in vivo*. However, XCL1 depletion avoids the development of cytotoxicity to antigens cross-presented by $CD8^+$ DCs (Dorner et al., 2009). Moreover, XCL1 mediates the accumulation and the interaction with thymic DCs, which are key for the proper development of natural regulatory T cells (Lei et al., 2011).

1.2.1.4 CX3C chemokines

The last chemokine subfamily is a single entity, rather than a group, and the last one to be discovered (Bazan et al., 1997). CXXXCL1 or CX3CL1 chemokine, also known as fractalkine, is so named because the N-terminal first two consensus cysteine residues are adjacent to each other with the addition of three amino acid residues interposed between them. The gene encoding CX3CL1 maps to chromosome 16 (Bazan et al., 1997).

Unlike the majority of the chemokines, CX3CL1 can be found in two forms, either as a ligand anchored to the membrane or as a soluble ligand (Bazan et al., 1997). CXCL16 is the only other chemokine which seems to have a transmembrane conformation (Matloubian et al., 2000). Under normal conditions, CX3CL1 is synthesised as an intracellular precursor, which is transported to the membrane where it is anchored to the membrane by an extended mucin-like stalk (Garton et al., 2001). This cell-surface-bound conformation allows it to act as an adhesion molecule to cells with the cognate receptor; for example, it promotes strong adhesion of leukocytes on activated primary endothelial cells (Bazan et al., 1997). Once CX3CL1 is on the surface, the release of the extracellular domain takes place via metalloproteinase-dependent cleavage. Tumour necrosis factor-α converting enzyme (TACE) is the protease involved in the generation of the soluble ligand (Garton et al., 2001).

The soluble CX3CL1 chemokine works as a T cell and monocyte chemoattractant (Bazan et al., 1997).

1.2.2 Functional classification

Chemokine nomenclature is based on their structural classification; however, they can also be sorted out into two functional categories. Chemokines can be homeostatic or proinflammatory depending on when they are expressed and their *in vivo* function. However, some chemokines are multifunctional and they can have a dual function; therefore, some inflammatory chemokines can have homeostatic roles under certain conditions while some homeostatic chemokines can be upregulated under certain injury conditions.

1.2.2.1 Inflammatory chemokines

Inflammatory chemokines were the first ones discovered as they are upregulated after tissue damage. In the early 1990s, activated cells of the immune system were studied, allowing the identification of many chemokines due to the abundance of their transcripts in these cells (Zlotnik and Yoshie, 2012). Those chemokines that are expressed by a variety of cells only after injury are classified as inflammatory.

Inflammatory chemokines are essential as they become upregulated and induce the recruitment of leukocytes in the presence of infection, inflammation, tissue injury and tumours. Inflammatory chemokines can be expressed in any place in the body; as an example, all nucleated cells are able to express CCL2 to induce the attraction of cells to site of injury. These chemokines are said to be promiscuous and their receptors unfaithful, as a single ligand has broad receptor selectivity and a single receptor has a broad number of agonists (Bachelerie et al., 2014). Even if this mechanism seems to lack specificity (and makes this field a challenge to understand), it facilitates the fast recruitment of effector cells into almost any tissue to restore the injury and protect the host.

The most relevant inflammatory chemokines are clustered, in the case of CC chemokines, many are localised on chromosome 17 in humans and on chromosome 11 in mice, while many CXC chemokines are clustered on chromosome 4 in humans and on chromosome 5 in mice (Zlotnik et al., 2006).

The explanation for the existence of these clusters is based on gene duplication, which produces two copies of a gene that can evolve independently and develop specialised functions (Wagner, 2001, Zlotnik et al., 2006). For this reason, clustered genes share characteristics that do not apply to the non-cluster chemokines, such as promiscuous ligand-receptor relationships (Figure 1-5) and a poor correlation between species, as happens between human and mouse (Yoshie et al., 2001, Zlotnik et al., 2006, Hughes and Nibbs, 2018).

1.2.2.2 Homeostatic chemokines

Even if the first chemokines to be identified were the inflammatory ones, the high degree of conservation among chemokines and the development of expressed sequence tag databases and bioinformatics allowed the identification of new chemokines (Zlotnik and Yoshie, 2000b). Unlike inflammatory chemokines, the expression of homeostatic chemokines is constitutive and cell or tissue specific (Zlotnik and Yoshie, 2012). Moreover, they are involved in the recruitment of cells involved in adaptive immunity, such as lymphocytes or DCs. Homeostatic chemokines are involved in the organization of the immune system and regulate the movement of lymphocyte and DC subsets during normal processes of tissue maintenance or development (Zlotnik and Yoshie, 2000a). A good example is CXCL12, which is essential for the migration of HSCs in embryonic development and in lymphocytic circulation and immune surveillance in the postnatal life (Kabashima et al., 2007, Zlotnik and Yoshie, 2000a).

In both human and mouse, homeostatic chemokines are encoded by non-clustered genes; thus, the limited gene duplication makes these chemokines much less promiscuous than their counterparts (Figure 1-5). Actually, many of these chemokines have very restrictive ligand-receptor relationships (Zlotnik and Yoshie, 2000b).

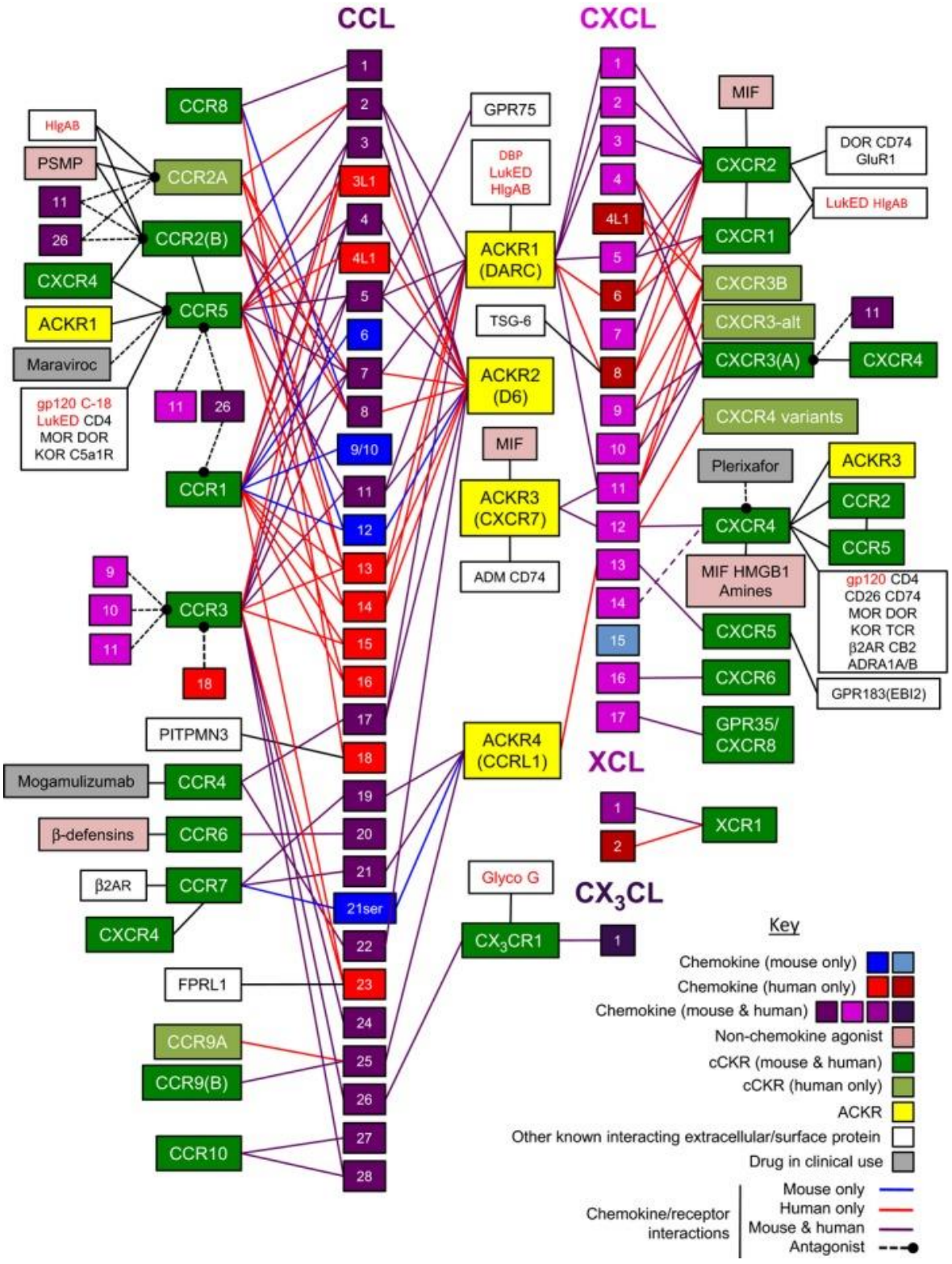


Figure 1-5. Mammalian chemokine receptors and their known interactions with chemokines and other molecules.
The above diagram shows the complexity of the relationship between chemokine receptors and chemokine ligands. Chemokine receptors interact with several different chemokines and at the same time, a chemokine molecule can interact with several receptors. Chemokines are arranged numerically in columns and a colour-code was used to represent whether they are in humans and mice, humans only, or mice only (see Key). Chemokine receptors are colour-coded and are linked to their known ligands (see Key). The colour of the linking key indicates the species in which the interaction has been demonstrated (see Key). Non-chemokine proteins, including drugs, known to interact with the chemokine receptors are also represented (see Key). Image from Hughes et al.

1.3 Chemokine receptors

1.3.1 Structure

Chemokine ligands must interact with chemokine receptors and glycosaminoglycans (GAGs) in order to induce cell migration. GAGs can be part of proteoglycans on cells or can be part of the extracellular matrix and they act by promoting chemokine immobilization to induce the migration of chemokine receptor expressing cells.

Chemokine receptors are seven-transmembrane spanning proteins between 340 and 370 amino acids long that constitute the largest division of the γ subfamily rhodopsin-like seven-transmembrane receptors (Griffith et al., 2014). Chemokine receptors can be classified into two groups: G protein-coupled chemokine receptors, also known as classical receptors, and atypical chemokine receptors, which lack the motif that enables coupling to G proteins. The chemokine receptor genes were also generated via gene duplication as a large cluster in human chromosome 3. However, as happens with chemokine ligands, chemokine receptors are well conserved among species; they have conserved structural, and therefore, functional, properties (Clark-Lewis et al., 1993, Clark-Lewis et al., 1995). One of the key motifs conserved among classic receptors is the DRYLAIV motif (D: Aspartate; R: Arginine; Y: Tyrosine; L: Leucine, A: Alanine; I: Isoleucine; V: Valine), also known as DRY motif. This motif is located on the second intracellular loop of the receptors and is essential for the G protein-coupled intracellular response. The presence of this motif allows the production of a calcium flux following the chemokine ligand-chemokine receptor interaction (Graham et al., 2012).

Chemokine receptors have the N-terminus on the extracellular side of the cell membrane, as this region is involved in ligand recognition and initial binding. The ligand binding process has been described as a two-step mechanism (Monteclaro and Charo, 1996, Pease et al., 1998). In the initial step, the N-terminus of the chemokine receptor interacts with the chemokine core domain through a high-affinity bond producing the immobilization of the chemokine ligand. This arrest allows the formation of low-affinity interactions between the ligand and the receptor (Monteclaro and Charo, 1996, Pease et al., 1998). Once

the ligand has completely interacted with the receptor, the receptor becomes activated leading to down-stream signalling cascades. The chemokine receptor contains several serine and threonine residues that can be phosphorylated, providing additional regulatory mechanisms.

The elucidation of the three-dimensional structures of the chemokine receptors allowed confirmation of the structural characteristics of these receptors and confirmed the ability of some receptors, such as CXCR4, to homodimerise (Wu et al., 2010). Different approaches has been used to solve the three-dimensional structures of these chemokines, including X-ray, crystallography for CXCR4 (Wu et al., 2010) and CCR5 (Tan et al., 2013) and nuclear magnetic resonance spectroscopy for CXCR1 (Park et al., 2012). The discovery of the three-dimensional structure is of high relevance as it allows a better understanding of chemokine ligand-receptor interactions and therefore, it provides the first step for the rational design of antagonist for clinical use.

1.3.2 CC chemokine receptors

Classical chemokine receptors are divided into 4 subfamilies in relation to the subfamily of their major chemokine ligands (Zlotnik et al., 2006). CC chemokine receptors are the first family of chemokine receptors and they are involved in the interaction with CC chemokines. There are 10 chemokine receptors in this family (CCR1-CCR10). The first receptors to be identified were those involved in inflammatory responses. In fact, CCR1, CCR2, CCR3 and CCR5 are considered to be the classical inflammatory CC chemokine receptors.

CCR2 has been identified on the surface of a subset of inflammatory cells, such as monocytes, activated memory T cells, B cells and basophils in humans. CCR2 is essential for the recruitment of inflammatory monocytes into tissue. For example, CCR2 is crucial after acute myocardial infarction, as it supports monocyte recruitment that are able to differentiate into matrix metalloproteinases and TNF-α secreting macrophages that promote ventricular remodelling (Kaikita et al., 2004). However, it has been shown that CCR2 is not the only chemokine receptor involved in leukocyte recruitment into tissue, as CCR2 knockout mice were able to support effective macrophage infiltration via CCR1 and CCR5 (Dagkalis et al., 2009). This is an example of the redundancy

that takes place in organisms, and more specifically in this context, in the immune response (Rot and von Andrian, 2004). Redundancy is of high relevance as it ensures the efficiency of the most relevant functions; however, it also means that it is complex to target these receptors therapeutically as they create a complex network that is not fully understood.

CCR5 is expressed on peripheral DCs, $CD34^+$ haematopoietic progenitor cells and activated Th1 lymphocytes and is of high significance as it works as a major coreceptor for human immunodeficiency virus-1 (HIV-1) infection (Bleul et al., 1997).

CCR7 is an archetypic homeostatic receptor as it is involved in the cellular organisation of secondary lymphoid organs; CCR7 is crucial as it guides the patrolling immune cells into secondary lymphoid organs (Förster et al., 1999). The migration of lymphocytes is impaired in CCR7-deficient mice, leading to alterations in all secondary lymphoid organs. Moreover, the antibody response is delayed as activated DCs and T cells fail to exit from the peripheral tissue into the lymph nodes in CCR7 deficient mice. CCR7, as expected due to its homeostatic role, has a quite restrictive ligand-receptor relationships as it just has two ligands, CCL19 and CCL21 (Vander Lugt et al., 2013).

1.3.3 CXC chemokine receptors

CXC chemokine receptors are involved in the interaction with CXC chemokines. There are 8 chemokine receptors in this family (CXCR1-CXCR8) and most are found on neutrophils and lymphocytes. CXCR1-3 are inflammatory receptors while the rest are homeostatic. All ELR^+ CXC chemokines interact with CXCR1 and CXCR2, which are both involved in the migration of neutrophils during the inflammatory response (Ludwig et al., 1997). In addition, ELR^+ CXC chemokines also promote angiogenesis (Strieter et al., 2005), cell proliferation (Mockenhaupt et al., 2003) and survival during development (Li et al., 2003). CXCR1 interacts with high affinity with CXCL8, which is released at high concentrations after trauma to stimulate cell migration and to enhance endothelial cell survival, proliferation, angiogenesis and the reconstruction of the matrix (Levashova et al., 2007, Li et al., 2003). Rodents lack an equivalent CXC chemokine ligand to

CXCL8; however, they have two homologous receptors, CXCR1 and CXCR2, able to coordinate cell migration during inflammation (Levashova et al., 2007).

CXCR3 is mainly expressed on Th1 lymphocytes and some B cells and NK cells. This receptor is highly upregulated following cell activation and its ligands are CXCL9, CXCL10 and CXCL11. There are two isoforms of this receptor, CXCR3a and CXCR3b, which differ in their interaction partners, as CXCR3b can also interact with CXCL4 (Struyf et al., 2011). CXCR3 is involved in several inflammatory and autoimmune diseases such as insulitis and type 1 diabetes mellitus (Frigerio et al., 2002) and rheumatoid arthritis (Patel et al., 2001).

CXCR4 is a very important homeostatic chemokine receptor expressed in both the immune and the central nervous system. Upon interaction with its cognate ligand CXCL12, CXCR4 induces the migration of resting leukocytes and haematopoietic progenitors. CXCL12 is important as the ligand-receptor interaction is responsible for the retention of HSCs in the bone marrow niche (Zou et al., 1998). It is such an important receptor that mice lacking this receptor die perinatally due to haematopoietic and cardiac defects. CXCR4 is upregulated during the implantation window, therefore, the prenatal death could also take place due to an inappropriate implantation (Dominguez et al., 2003). Inhibition of CXCR4 using a small molecule inhibitor, AMD3100 (plerixafor), induces the mobilisation of haematopoietic progenitors from the bone marrow into peripheral tissues (Liles et al., 2003). This discovery was a compelling advance in the stem cell transplantation field. CXCR4 is also relevant as it is a major coreceptor for HIV-1 (Feng et al., 1996, Donzella et al., 1998) and AMD3100 has been shown to block viral entry of HIV-1 into some host cells (Donzella et al., 1998).

CXCR5, like CCR7, is involved in the organization of secondary lymphatic tissues. The only cognate ligand of CXCR5 is CXCL13, which is produced by DCs of B cell zones in the lymph nodes to induce the migration of B cells expressing CXCR5 (Yu et al., 2002). Moreover, T helper cells upregulate CXCR5 to migrate to B cell zones in the lymph nodes and in this way, contributes to the adaptive immune response. CXCR5 is of relevance in disease as it is specifically expressed in Burkitt's lymphoma (Gunn et al., 1998). Moreover, CXCR5 upregulation has a

positive correlation with lymph node metastases in breast cancer patients as this axis regulates epithelial to mesenchymal transition (Biswas et al., 2014).

CXCR6 has also been identified as an entry coreceptor for viral infection. In the case of HIV-1, it is a minor coreceptor as almost every strand of the virus infects cells using CXCR4 and/ or CCR5 (Liao et al., 1997). However, CXCR6 is important for simian immunodeficiency virus (SIV) as it acts as a major coreceptor for infection (Elliott et al., 2015).

1.3.4 XC chemokine receptor

XC chemokine receptor is involved in the interaction with XC chemokines; there is just one receptor in this subfamily (XCR1) and it is known as lymphotactin receptor (Zlotnik and Yoshie, 2000b). XCR1 is expressed by a subset of DCs having roles in antigen cross-presentation and immunity against viruses and cancer (Ohta et al., 2016). The XCR1-XCL1 axis is involved in the modulation of both the localization and function of T cells and DCs; thus, this axis is involved in several autoimmune diseases such as rheumatoid arthritis (Blaschke et al., 2003) or Crohn's disease (Middel et al., 2001). Moreover, XCR1 is involved in the progression of several cancers as it is positively correlated with bone metastasis in non-small cell lung cancer (Wang et al., 2015); it promotes cell migration and proliferation in epithelial ovarian carcinoma (Kim et al., 2012); and it is expressed in diffuse large B cell lymphoma initially manifesting in the bone marrow (Yamashita et al., 2011).

1.3.5 CX3C chemokine receptor

The last family of chemokine receptors is also a single entity and is known as CX3CR1 or fractalkine receptor. Expression of this receptor has been found on T cells, NK cells and monocytes and microglial cells of the central nervous system. CX3CR1 plays a major role on the survival of monocytes (Landsman et al., 2009), while CX3CR1 signalling pathway modulates microglial activation and therefore, interactions with neurons and synapses (Paolicelli et al., 2014). In addition, CX3CR1 is relevant for macrophage mediated apoptotic cell engulfment as CX3CL1 is released from apoptotic lymphocytes (Truman et al., 2008).

1.3.6 Chemokine receptor signalling

It is important to remember that classic chemokine receptors are G protein-coupled receptors; therefore, the binding of a chemokine ligand to its receptor leads to changes in the G proteins. Before ligand interaction, the receptor is bound to a heterotrimeric G protein complex, formed by α, ß and γ subunits, which has associated a guanosine diphosphate (GDP) molecule to its α subunit, making the heterotrimeric G protein inactive. However, binding of an agonist to the receptor produces a conformational change in the receptor, which leads to the allosteric activation of the G_α subunit. The activated G_α subunit releases GDP and exchanges it for guanosine triphosphate (GTP), triggering the dissociation of the G protein from the receptor as well as from each other, yielding a G_α-GTP monomer and a $G_{\beta\gamma}$ heterodimer, which are now free to interact and modulate other intracellular proteins. The receptor is now internalised and the cell is desensitised.

As shown in Figure 1-6, ligand interaction can lead to a multitude of downstream pathways determined by the ligand, the receptor and the physiological context in which the binding takes place (Rot and von Andrian, 2004). There are multiple different G proteins and the binding of an agonist can lead to the activation of different G proteins depending on the capability to induce the stabilization of specific guanine-nucleotide exchange factors.

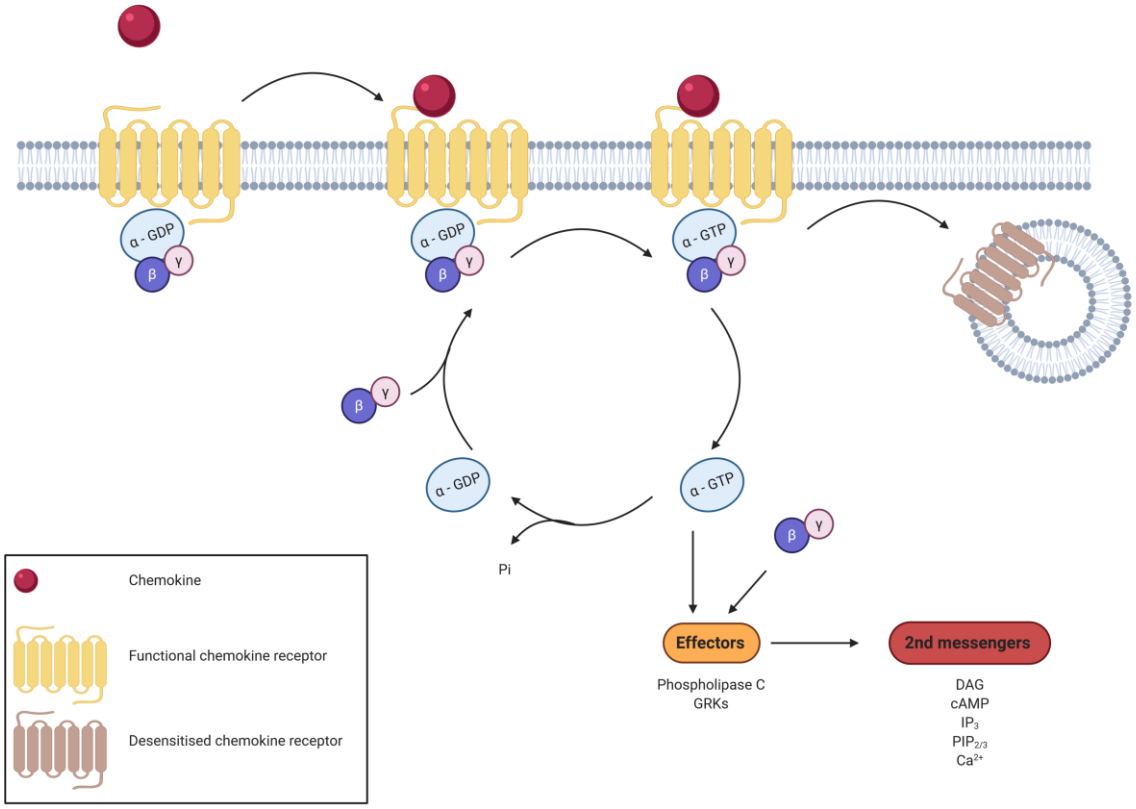


Figure 1-6. Schematic summary of chemokine-mediated G protein-coupled receptor signalling.
Chemokine ligand interaction with its cognate receptor can lead to a multitude of downstream pathways determined by the ligand, the receptor and the physiological context in which the binding takes place. Before ligand interaction, the receptor is bound to a heterotrimeric G protein complex, formed by α, β and γ subunits, which has associated a guanosine diphosphate (GDP) molecule to its α subunit, making the heterotrimeric G protein inactive. However, binding of an agonist to the receptor produces a conformational change in the receptor, which leads to the allosteric activation of the G_{α} subunit. The activated G_{α} subunit releases GDP and exchanges it for guanosine triphosphate (GTP), triggering the dissociation of the G protein from the receptor as well as from each other, yielding a G_{α}-GTP monomer and a $G_{\beta\gamma}$ heterodimer, which are now free to interact and modulate other intracellular proteins. The receptor is now internalised and desensitised.

Several G_{α} proteins are able to regulate the calcium ion flux (Wu et al., 1993, Rollins et al., 1991). $G_{\alpha s}$ and $G_{\alpha i}$ both regulate adenylate cyclase, $G_{\alpha s}$ subunit induces its activity while $G_{\alpha i}$ acts as an inhibitor. Adenylate cyclase catalyses the conversion of adenosine triphosphate (ATP) to cyclic-adenosine monophosphate (cAMP). Cytosolic cAMP levels regulate the activity of ion channels, such as calcium, as well as serine/ threonine protein kinase A (PKA), which can also produce a calcium influx. $G_{\alpha q/11}$ regulates phospholipase C-ß (PLCß), which produces inositol trisphosphate (IP3) and diglyceride (DAG) by catalysing the cleavage of the membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP2). IP3 produces the release of calcium ions from the endoplasmic reticulum while DAG activates protein kinase C (PKC), leading to further downstream signalling.

G_{α} proteins are also responsible for cytoskeleton regulation (Gu et al., 2003). $G_{\alpha 12/13}$ allosterically activates three members of the cytosolic small GTPase Rho family (p115-RhoGEF, PDZ-RhoGEF, and LARG). Once it is activated Rho can activate Rac1, promoting lamellipodia formation at the leading edge of cells and its subsequent movement.

Moreover, the $G_{\beta}\gamma$ heterodimer by itself is able to modulate the activity of potassium and calcium channels, as well as adenylate cyclase and PI3K. In this manner, the mitogen-activated protein kinase pathway gets activated leading to cell polarisation and movement towards the highest concentration of chemokines (Patel et al., 2013).

1.3.7 Atypical chemokine receptors (ACKRs)

ACKRs, like the classical chemokine receptors, are seven-transmembrane spanning proteins; however, ACKRs have a missing or modified DRYLAIV motif in the second intracellular loop, which disables the coupling to G-proteins and therefore, the classical chemokine receptor mediated cell signalling (Ulvmar et al., 2011).

There are currently five receptors in this family (ACKR1-ACKR5) which can interact with a wide range of chemokine ligands. These receptors were thought to be silent because they fail to induce cell migration; nevertheless, they are not, as upon interaction with ligands ACKRs are internalised. After internalisation, two outcomes can take place. On the one hand, ligands can be internalised and targeted for lysosomal degradation. On the other hand, internalisation can be followed by transcytosis, a process allowing the transport of chemokines across biological barriers in order to take them to less approachable microanatomical domains (Graham et al., 2012). The main goal of these receptors is to regulate chemokine availability within tissues.

1.3.8 Chemokines and chemokine receptors in disease

1.3.8.1 Inflammatory diseases

As has already been discussed, it is essential for the immune system to achieve self-nonself discrimination to avoid the development of chronic inflammation

and autoimmune disease. Tissues of patients with inflammatory conditions show upregulated levels of specific chemokines and their receptors. In inflammatory rheumatic diseases, CXCL8 (Seitz et al., 1992) as well as XCL1 (Blaschke et al., 2003) have been shown to be present in synovial fluid. Furthermore, CXCL8 is also upregulated in patients suffering active ulcerative colitis (Mahida et al., 1992). Rheumatoid arthritis patients not only have increased XCL1 and CXCL8 levels in synovial fluid, but also have CCL2, CCL3, CCL5 and CXCL12 levels increased in their joints, which leads to the recruitment of pro-inflammatory cells into synovial tissues (Shadidi et al., 2003). CCR5, CCR6, CCR7, CXCR3, CXCR4 and CXCR5 have also been found to be involved in B cell synovial cytokine production and activity (Nanki et al., 2009). Chemokines are also involved in psoriasis pathophysiology; CXCR3 has been found to induce lymphocyte recruitment to the skin leading to inflammation (Flier et al., 2001).

1.3.8.2 Human Immunodeficiency Virus (HIV)

The human immunodeficiency virus is a lentivirus discovered in 1983 that targets and destroys immune system cells of the host leading to the development of acquired immunodeficiency syndrome (AIDS) (Barré-Sinoussi et al., 1983). Due to the failure of the immune system, AIDS allows the appearance of life-threatening opportunistic infections as well as cancer development. HIV has a huge propensity for genetic variation, mainly within the envelope gene, making it very difficult to develop a vaccine capable of targeting all the variants and therefore, preventing infection (Berkower et al., 1989). However, the virus cannot mutate at sites related with crucial functions.

The entry of the virus into host cells takes place through glycoproteins in the envelope of the virus, more accurately, glycoprotein (gp) 120. CD4 gp is able to induce $CD4^+$ T cell specific antigen responses after interaction with MHC Class II molecules; however, gp120 is also able to interact with CD4 leading to conformational changes in gp120 that promote the exposure of chemokine receptor binding regions on the surface of the virus (Silberman et al., 1991).

Once the virus is showing the chemokine receptor binding regions, it needs to interact with the corresponding receptors, CCR5 and CXCR4, as they act as coreceptors promoting the entry of the virus into the host cells (Bleul et al.,

1997). In the initial stage of the infection, HIV uses CCR5 as a coreceptor to target monocytes, macrophages and $CCR5^+$ Th1 cells. For this reason, it is known as M-tropic HIV at this stage. To infect lymphocytes, the virus uses CXCR4, which is known as the T-tropic or X4-tropic stage of the infection. At this stage, the immune system is already much weakened leading to AIDS.

There have been several approaches to target CD4, CCR5 and CXCR4 to reduce the ability of HIV to target the immune system. Among these three coreceptors, CCR5 seems to be most relevant as individuals with a defective expression of CCR5 are resistant to infection with HIV-1 (Liu et al., 1996). Moreover, the usage of natural CCR5 ligands *in vitro* produces a decrease in the infection rate due to competition events between the virus and the natural ligands (Cocchi et al., 1995). In addition, Clerici et al. showed that patients with increased CCL5 levels seem to be refractory to the progression of HIV infection to AIDS (Clerici et al., 1996). According to studies done in 1996, approximately 1% of people of northern European descent have a non-functional CCR5 receptor activity and are known to have the CCR5Δ32 mutation. This mutation is characterised by the absence of 32 base pairs of the second extracellular loop impairing the receptor activity. CCR5Δ32 homozygous individuals are highly resistant to HIV-1 infection, while Δ32 heterozygotes can become infected but the progression of the disease is much slower, taking longer to develop AIDS (Huang et al., 1996).

On the one hand, CCR5Δ32 is not only protective for HIV-1, but also for graft rejection (Fischereder et al., 2001) and inflammatory disorders such as asthma (Srivastava et al., 2003) and rheumatoid arthritis (Pokorny et al., 2005). On the other hand, CCR5Δ32 produces a more apparent disease in patients with pulmonary sarcoidosis and an increased requirement for corticosteroids (Petrek et al., 2000). Moreover, CCR5Δ32 mutation increases the risk of suffering a lethal encephalopathy after infection with West Nile virus (Glass et al., 2006).

1.3.8.3 Cancer

It is important to consider that even if cancer appears as a result of the accumulation of genetic and epigenetic changes (Joyce, 2005), those alterations are not enough to provide malignant properties to cancer cells (Sounni and Noel, 2013). Tumours must develop essential properties to progress and develop; these

are induced by the tumour microenvironment. The tumour microenvironment provides the necessary signals that turn on transcription factors from development or embryology programs allowing the necessary mesenchymal phenotypes to invade distant tissues and establish a new environment (Sounni and Noel, 2013).

Tumour cells work in close interaction with the extracellular matrix and with genetically stable cells that constitute the tumour microenvironment (Fang and Declerck, 2013). The tumour microenvironment describes the non-cancerous cells present in the tumour, which includes fibroblasts, myofibroblasts, neuroendocrine cells, adipose cells, immune and inflammatory cells and the blood and lymphatic vascular networks (Chen et al., 2015). The interactions between tumour cells and the tumour microenvironment have a deep influence on cancer development and progression and contributes to almost all of the hallmarks of cancer (Hanahan and Weinberg, 2011). The stromal cells that interact with the tumour are not only from neighbouring tissues, but also endothelial progenitor cells, myeloid and lymphoid inflammatory cells, and mesenchymal cells that have been recruited from the bone marrow (Fang and Declerck, 2013). Once these cells are within the tumour microenvironment, the signals originating from the tumour make them progressively switch from non-malignant cells with a neutral or anti-tumorigenic role towards cancer-associated fibroblasts (CAF), tumour-associated macrophages (TAM) or vascular and perivascular cells with a pro-tumorigenic role (Fang and Declerck, 2013) (Figure 1-7). Therefore, signals originating from the tumour cells induce the pro-tumorigenic nature of the microenvironment, while the signals originated by these cells promote tumour development.

Tumour formation and maintenance, as organs, requires the self-renewal of cancer cells. This self-renewal property is provided by cancer stem cells (CSCs) (Wang et al., 2013). CSCs can develop from the normal tissue stem cells due to oncogenic mutation or can be normal somatic cells that acquire oncogenic mutations that give them the ability to self-renew and differentiate into specialised, mature cell types (Adjei and Blanka, 2015). The tumour stroma also recruits MSCs from the circulation and nearby tissues by secreting CXCL12 and CCL2. Recruited MSCs secrete CCL5, which induces migration, invasion and

metastasis of cancer cells due to the malignant transformation of CAFs and TAMs (Karnoub et al., 2007). Thus, the tumour microenvironment allows the recruitment and activation of CSCs through chemokines and those CSCs secrete more chemokines that enhance the pro-tumorigenic role of the tumour microenvironment. Nevertheless, it has also been shown that MSCs are able to inhibit Akt protein kinase activity and downregulate B cell lymphoma 2 (Bcl2) in cancer, inducing apoptosis (Qiao et al., 2008).

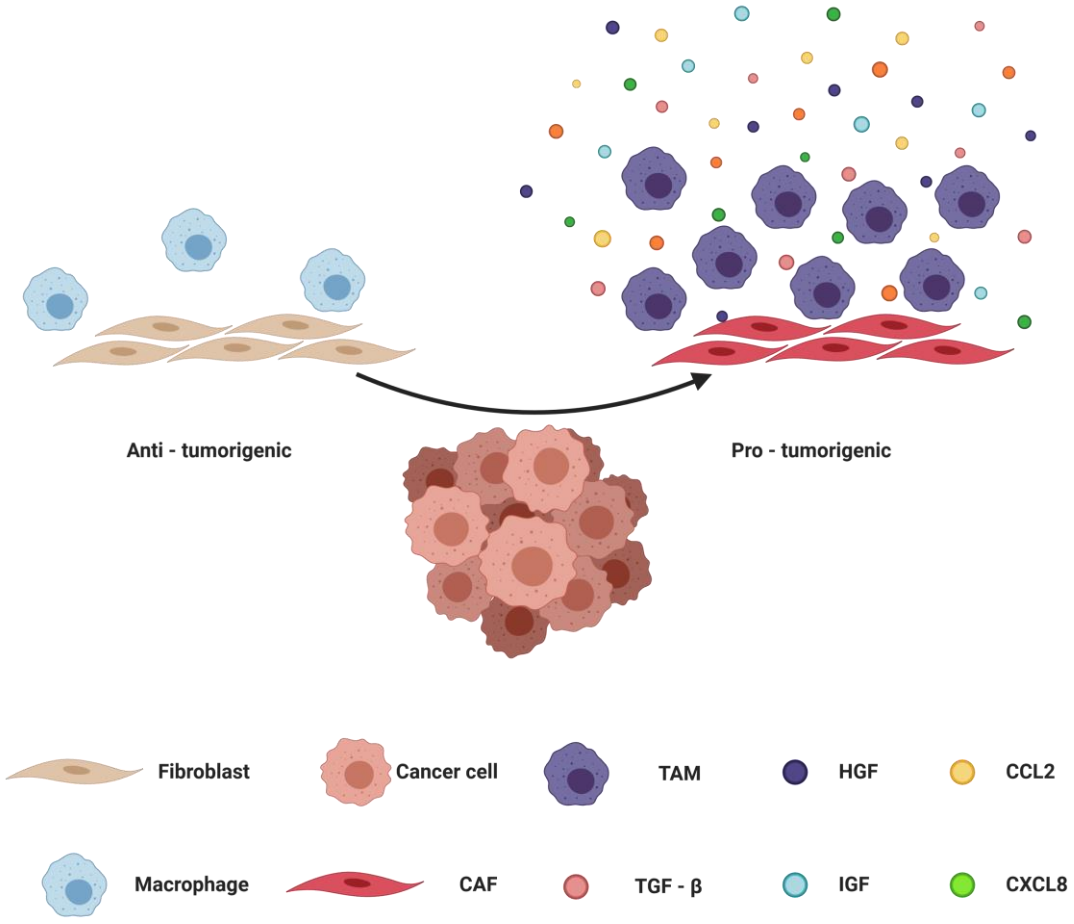


Figure 1-7. Schematic representation of the effect of cancer cells in tumour microenvironment.
Secretion of cytokines and growth factors, including IL-4, macrophage colony-stimulating factor and granulocyte-macrophage colony-stimulating factor by tumour cells promotes the generation of a tumour promoting environment where fibroblasts and macrophages switch from being anti-tumorigenic to have a pro-tumorigenic role. Cancer associated fibroblasts (CAFs) and tumour associated macrophages (TAMs) secrete pro-inflammatory molecules that recruit further TAMs as well as promoting angiogenesis, tumour growth and metastasis

CAFs secrete growth factors, transforming growth factor ß, hepatocyte growth factor, insulin like growth factor 1/ 2, and chemokines (CCL2 mainly) that enhance angiogenesis and therefore, proliferation and invasion of cancer cells due to the activation or transformation of both the microenvironment and the cancer cells (Adjei and Blanka, 2015). Many tumours secrete monocyte chemoattractant proteins, such as CCL2, producing an increase of TAMs, which mediate immunoinhibitory effects and facilitate tumour metastasis by generating changes in the tumour microenvironment. In fact, the blockade of CCL2-CCR2 signalling by monoclonal antibodies, such as CNTO 888, has been shown to augment $CD8^+$ T cell mediated responses and to inhibit metastatic seeding (Tsai et al., 2014).

CXCL8 and CXCL1 are usually upregulated in the tumour microenvironment, leading to an aberrant recruitment of neutrophils and angiogenesis (Sparmann and Bar-Sagi, 2004).

CCR7 and CXCR4 both have very important roles controlling the migration of immune cells to secondary lymphoid organs. CCR7 has been found to be upregulated in many cancers, including gallbladder cancer (Hong et al., 2016), non-small cell lung cancer (Sun et al., 2015) and oesophageal squamous cell carcinoma (Irino et al., 2014) among others, promoting metastasis. Moreover, CXCR4 and CCR7 enhance metastasis by inhibiting anoikis, a programmed cell death that takes place when anchorage-dependent cells detach from the extracellular matrix (Kochetkova et al., 2009). Lastly, signal transducers and activators of transcription (STATs), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and hypoxia inducible factor 1α (HIF1α) are also involved in inflammation and are deregulated in many cancers. HIF1*α* produces an increase of CXCR4, promoting metastasis by both metastatic migration towards CXCL12 and inhibition of anoikis.

1.3.8.4 Transplant rejection

Organ transplantation is one of the greatest achievements in medicine as it is the unique life-saving strategy for patients with irreversible failure of a wide range of organs, such as kidney, heart, lung, pancreas and liver (Salvadori and Bertoni, 2014). However, the main problem of this strategy is that the long-term

outcome can be jeopardised due to tissue or organ rejection. To avoid rejection, patients are supplied with immunosuppressants; however, these drugs further aggravate their quality of life as they are prone to infections, neoplasms, nephrotoxicity and diabetogenicity (Crescioli, 2016). Due to the role of chemokines in inflammatory and immune responses, it was not a surprise to find that they had a major role in transplant rejection and that they were the molecules involved in alloreactive cell recruitment (Heidt et al., 2011).

Chemokine ligands and their receptors are involved in transplant rejection due to their role driving and directing leukocyte migration, promoting an inflammatory environment surrounding the graft. Upon transplantation, inflammatory cytokines, such as IL-1, IFN-γ and TNF-α, are released at both local and systemic levels. This inflammatory environment promotes the release of pro-inflammatory chemokines, as CCL2, CXCL8, CXCL9, CXCL10 and CXCL11, by endothelial cells (Barker et al., 2014). The relevance of chemokines in graft rejection was shown when it was observed that neutralization of intra-graft CXCL10 allowed longer graft survival and therefore, a better outcome (Hancock et al., 2000).

1.4 The stromal compartment

The stromal compartment is the connective tissue of any organ and it includes a wide range of cells with a similar phenotype but different cellular functions. Among these cells, fibroblasts are the most common population, while mesenchymal stromal cells are present at a very low percentage. The stromal compartment is a major component of immune responses as it secretes a wide range of mediators to generate a pro-inflammatory or an anti-inflammatory environment according to the signals it receives.

1.4.1 Mesenchymal Stromal Cells

Mesenchymal stromal cells (MSCs) were first named in 1976 by Alexander Friedenstein as colony-forming unit fibroblasts (CFU-Fs). These cells were spindle shaped, clonogenic in monolayer cultures and could serve as feeders in

the bone marrow for haematopoietic stem cells (Friedenstein et al., 1976). The term mesenchymal stem cell was first used by Maureen Owen in 1985 because of their ability to self-renew and the potential to differentiate into discrete connective tissues cells. Although MSCs where first isolated from bone marrow, they can be isolated from a wide range of tissues around the body, including umbilical cord, cord blood, placenta, dental pulp, periodontal ligament, adipose tissue and from the islets within the pancreas (Lv et al., 2014).

Due to different culture conditions and the intrinsic variability among MSCs derived from different donors and strains, there is controversy regarding MSCs properties in the literature. For this reason, the International Society of Cellular Therapy (ISCT) proposed to call these cells mesenchymal stromal cells (Horwitz et al., 2005) and specified the criteria that human cells had to reach in order to be defined as MSCs. These criteria are plastic-adherence when maintained in standard culture conditions and specific surface antigen expression along with the potential to differentiate into osteoblasts, adipocytes and chondroblasts. MSCs must express CD105, CD73 and CD90 and, as additional criteria, they must lack the expression of haematopoietic antigens such as CD45, CD34, CD14 or CD11b, CD79α or CD19 and MHC Class II (Dominici et al., 2006). MSCs express human leukocyte antigen (HLA)-DR isotype upon stimulation with IFN-γ; in this case, cells are still termed MSCs but they have to be qualified with adjectives such as activated, licensed or stimulated MSCs to explain that the cells are not in a baseline state. MSCs not only express these markers, but also variable levels of other markers as CD29, CD44, CD166, CD146 and CD271, which can allow the isolation of tissue specific MSCs. CD271, for example, allows the identification of bone marrow MSCs (BM MSCs) (Álvarez-Viejo et al., 2015).

Ideally, these characteristics should allow the identification of MSCs; however, MSCs are similar to fibroblasts in that they have a similar morphology and are plastic-adherent (Haniffa et al., 2009). Moreover, human dermal fibroblasts have also been proven to have tripotency (Junker et al., 2010) and immunoregulatory functions similar to MSCs (Haniffa et al., 2007) and to express most MSC markers such as CD105, CD73 and CD90 (Schwab et al., 2008). Fibroblast contamination is a potential impediment for regenerative medicine due to their senescence and tumour transformation upon long-term expansion (Zhou et al., 2006); thus,

proper MSC isolation is essential. CD146 expression has been found to be weak for fibroblasts and high for MSCs (Lv et al., 2014) and CD166 was significantly higher in MSCs; therefore, these two markers could allow the discrimination between MSCs and fibroblasts. However, according to Halfon et al., markers discriminating MSCs from fibroblasts are downregulated in human MSCs of passage 6; murine MSCs are difficult to expand and therefore, are not well described (Halfon et al., 2011).

1.4.2 Tissue of origin of MSCs impacts their phenotype and function

MSCs can be isolated from a wide range of tissues around the body, including umbilical cord, cord blood, placenta, Wharton's jelly, dental pulp, periodontal ligament, adipose tissue and from the islets within the pancreas (Lv et al., 2014). BM MSCs are considered the gold standard and are the most used MSCs in the clinic; however, their isolation requires an invasive and painful procedure. As MSCs can be isolated from a large variety of tissues, alternative sources could be more suitable for clinical use. Adipose tissue, umbilical cord and islets are a potentially better source for MSC isolation as they are easily accessible and MSCs can be isolated in larger amounts; moreover, as these tissues are clinical waste there is no burden on the donor. As an example, adipose tissue yields a 500-fold higher frequency of colony forming units (CFU-F) than bone marrow and can be obtained very easily (Fraser et al., 2006); MSCs from the umbilical cord could also be a good choice as they are very abundant and they are ontogenically primitive (Choudhery et al., 2013).

Comparison of MSCs from different sources have shown that they are all phenotypically similar as they all have the spindle-like morphology and similar surface expression levels of the markers stated by the ISCT. However, they differ in origin, proliferative rate, differentiation potential, immunomodulatory capacity and cytokine secretion profiles (Hass et al., 2011, Wu et al., 2018). Adipose tissue derived MSCs (Ad MSCs) have a greater ability to differentiate into adipocytes compared to BM MSCs and umbilical cord derived MSCs (UC MSCs), while UC MSCs differentiate much more easily in osteoblasts than Ad and BM MSCs (Han et al., 2017). Few studies compared the immunomodulatory potential of MSCs among tissues; even if they all used a mixed lymphocyte reaction assay

or T cell proliferation measurement assays upon CD3/ CD28 stimulation the results are contradictory. On the one hand, Puissant et al. did not find statistical differences between BM MSCs and Ad MSCs in the inhibition of T cell proliferation (Han et al., 2017, Puissant et al., 2005). On the other hand, Ribeiro et al. determined that Ad MSCs have a stronger effect on T cell activation compared to BM MSCs and UC MSCs (Ribeiro et al., 2013), while Xishan et al. showed that BM MSCs had the highest immunosuppressive effect (Xishan et al., 2013). Little is known about the migratory abilities of MSCs according to their tissue of origin, which should be considered for clinical studies with these cells.

It is also very important to consider the species variation in the MSCs biology. Immunosuppressive effects of MSC have been reported for both mice and humans but there are important differences regarding the genomic stability, the spontaneous expression of neural markers, self-renewal and differentiation capabilities, as well as the immunoregulatory capacities (Scuteri et al., 2014, Hass et al., 2011). Under the same culture conditions, cytokine-mediated inflammatory stimulation of human and murine BM MSCs led to differential increase of the expression levels of IDO and inducible nitric oxide synthase (iNOS); human BM MSCs expressed high levels of IDO and little iNOS, and mouse MSCs expressed very low levels of IDO and high levels of iNOS. The immunosuppressive mechanism of MSCs is poorly understood but IDO and iNOS, respectively in humans and mice, have been shown to be involved, at least in part, in the suppressive effect of MSCs on cytotoxic cell proliferation, and thus, immunomodulation (Ren et al., 2009). For these reasons, the transfer of results obtained from animal MSCs must be carefully interpreted.

1.4.3 MSCs and the immune system

In 1997 the ability of MSCs to repair a craniofacial defect (Kadiyala et al., 1997) was demonstrated, while in 1998 it was observed that MSCs grown and expanded *ex vivo* in culture were able to give rise to long-lasting connective tissue cells after injection into irradiated isogenic mice (Pereira et al., 1998). In 2005, MSCs were found to be weakly immunogenic due to their lack of HLA antigens and co-stimulatory molecules as CD80 and CD86. This property, in combination with their ability to be immunosuppressive, immunomodulatory and regenerative in both human and animal models led to an increasing interest in the therapeutic

potential of MSCs (Keyser et al., 2007, Klyushnenkova et al., 2005). MSCs are found in perivascular locations *in vivo*, more precisely within epithelial niches close to the vessel walls, which enables blood vessel formation and the recruitment and interaction with immune cells (Liu et al., 2014). MSCs can interact with, and immunomodulate, not only the innate and the adaptive responses, but also the humoral immune system. Moreover, MSCs have been shown to recruit more leukocytes and to have stronger immunomodulatory and anti-inflammatory properties in the presence of inflammation; therefore, this is mimicked *in vitro* by stimulating MSCs with a cocktail of TNF-α, IFN-γ and/ or IL-1ß, also known as licensing of MSCs (Krampera et al., 2006, Krampera et al., 2007, Di Nicola et al., 2002, Beyth et al., 2005).

1.4.3.1 MSCs and the humoral immune system

As previously mentioned in Section 1.1.1.2, the humoral immune system is composed of macromolecules present in extracellular fluids such as complement proteins and secreted antibodies.

Interaction of MSCs with the complement system has been described, but it is not fully understood. Infusion of MSCs leads to complement activation after their contact with serum due to the expression of complement receptors C3aR and C5aR, allowing chemotaxis of MSCs towards the complement components C3 and C5 and leading to increased resistance to oxidative stress and prolonged survival by MSCs (Li and Lin, 2012, Schraufstatter et al., 2009). But at the same time, licensing of MSCs with TNF-α and IFN-γ leads to the secretion of complement factor H, which inhibits the formation of C5 and C3 convertases and the subsequent activation of the complement system and inflammation (Tu et al., 2010b). Moreover, BM MSCs can secrete C3 protein, inhibiting the immunoglobulin production by B cells without affecting their activation status (Lee et al., 2014). Antibody secretion leads to the activation of the classical pathway of the complement system as well as to opsonization upon antibody binding to a target. Thus, by dampening Ab secretion, MSCs avoid the secretion of C3a and C5a anaphylatoxins that would lead to further activation of T cells and macrophages, mast cell degranulation and chemotaxis of immune cells that would lead to increased inflammation.

1.4.3.2 MSCs and the innate immune system

As discussed in Section 1.1.1, the innate immune response is essential in the process of inflammation and is involved in transplant rejection and autoimmune diseases (Murphy et al., 2011, Prame Kumar et al., 2018). For this reason, it is essential to understand the interaction and the immunomodulatory and anti-inflammatory effect of MSCs on the cells of the innate immune system to understand the role of MSCs within a clinical setting.

1.4.3.2.1 MSCs and monocytes and macrophages

Macrophages are phagocytic immune cells, the everyday essential in host defence and tissue homeostasis and, monocytes can differentiate into pro-inflammatory or anti-inflammatory macrophages depending on microenvironmental signals. MSCs have been described to promote the differentiation of monocytes into M2 anti-inflammatory macrophages through a poorly understood mechanism involving the secretion of IDO, prostaglandin E2 (PGE2) and tumour necrosis factor-inducible gene 6 (TSG-6) (Wise et al., 2014, Choi et al., 2011, Németh et al., 2009). Moreover, differentiation of monocytes into M2 prolongs MSC survival (Freytes et al., 2013). Contradictorily, in a murine model of GVHD, Galleu et al. described how MSCs must be targeted by cytotoxic T cells to undergo apoptosis, as engulfment by macrophages leads to the production and secretion of IDO by macrophages, which are capable of mediating immunosuppression (Galleu et al., 2017).

1.4.3.2.2 MSCs and neutrophils

Neutrophils are fast responders to inflammatory signals and can exert their inflammatory role in three different manners: phagocytosis, degranulation and the formation of NETs. MSCs have been described to increase the phagocytic potential of neutrophils and at the same time, to reduce the respiratory burst of neutrophils by suppressing the production of nitric oxide and hydrogen peroxide by active neutrophils, diminishing in this way their inflammatory potential and NET formation (Joel et al., 2019, Jiang et al., 2016).

1.4.3.2.3 MSCs and DCs

MSCs are also able to supress the immune response by interfering with the maturation and activation process of DCs and the subsequent activation of T cells. MSCs secrete IL-6 and PGE2 suppressing the maturation of granulocyte macrophage colony stimulating factor (GMCSF) and IL-4 stimulated monocytes into DCs (Jiang et al., 2005, Spaggiari et al., 2009). Co-culture of MSCs and DCs promotes the secretion of the anti-inflammatory cytokine IL-10 by DCs while impairing their ability to endocytose, upregulate co-stimulatory molecules and secrete IL-12 and TNF-α (Aggarwal and Pittenger, 2005, Zhang et al., 2004).

1.4.3.2.4 MSCs and NK cells

As previously described in Section 1.1.1.1.8, NK cells recognise, and target, cells expressing low levels of MHC Class I, which makes MSCs a target for NK cells. However, co-culture of NK cells and MSCs under inflammatory conditions leads to the upregulation of MHC Class I in MSCs to avoid NK cell-mediated cytotoxicity, while downregulating the expression of NK cell activation receptors (Spaggiari et al., 2006, Poggi et al., 2005). Additionally, MSCs secrete TGF-ß1 and PGE2 which inhibits proliferation and IFN-γ production of IL-2 and IL-15 activated NK cells (Sotiropoulou et al., 2006). These findings suggest that under resting conditions NK cells would be able to recognise and kill MSCs, while MSC licensing increases the immunoregulatory potential of MSCs and enables the regulation of the activation state of NK cells.

1.4.3.3 MSCs and the adaptive immune system

The innate immune system is a collection of non-specific mechanisms to protect the host against pathogens, while the adaptive immune response generates immunological memory towards specific antigens. The adaptive immune system can also generate self-reactive antibodies which can give rise to autoimmune diseases and it is also essential in developing immunotolerance. Thus, it is essential to understand the interaction, and the immunomodulatory and anti-inflammatory effect of MSCs, on the adaptive immune system to understand the role of MSCs within a clinical setting.

1.4.3.3.1 MSCs and T cells

T cells are key mediators in inflammation and MSCs have been shown to suppress T cell proliferation both directly and indirectly (Di Nicola et al., 2002). BM MSCs interact with T cells via the engagement of programmed cell death protein 1 (PD1) *in vitro*, suppressing T cell proliferation (Augello et al., 2005). Engulfment of apoptotic MSCs by macrophages leads to IDO secretion, which has been described to promote T cell anergy, a tolerance mechanism in which the T cell remains alive but is inactivated following an antigen encounter (268). Furthermore, humans and murine MSCs secrete high levels of PGE2 *in vitro*, PGE2 inhibits TCR signalling, and inhibitors of PGE2 production decreased the immunosuppresive effect on T cells (Puccetti and Grohmann, 2007, Wiemer et al., 2011, Aggarwal and Pittenger, 2005). Another soluble factor that seems to be involved in the suppression of T cell proliferation is adenosine. Interaction between MSCs and T cells leads to increased production of adenosine by MSCs, which interacts with adenosine A2A receptor on the surface of T cells reducing their proliferation in both human and mice (Sattler et al., 2011, Haddad and Saldanha-Araujo, 2014). MSCs secrete hepatocyte growth factor (HGF) and IL-10, which in combination with IDO and PGE2 leads to T cell proliferation inhibition and differentiation into Tregs (Meisel et al., 2004). Moreover, secretion of IL-6, IL-10 and PGE2 by MSCs suppresses maturation and the antigen presentation potential of DCs, reducing $CD4^+$ T cell proliferation in an indirect manner (Aggarwal and Pittenger, 2005). MSCs can reduce IFN-γ secretion by Th1 cells while promoting an increased secretion of IL-4 by Th2 cells and increase the number of Tregs (Aggarwal and Pittenger, 2005). Therefore, MSCs can modulate T cell responses by inhibiting antigen specific T cell responses and promoting the differentiation of $CD4^+$ T cells into Tregs.

1.4.3.3.2 MSCs and B cells

MSCs inhibit B cell proliferation and activation in vitro, as well as differentiation via the programmed death 1 pathway (Augello et al., 2005). CCL2 and C3 secretion by MSCs results in the inhibition of immunoglobulin synthesis and secretion (Feng et al., 2014, Lee et al., 2014). As previously explained, by dampening Ig secretion, MSCs avoid the secretion of C3a and C5a anaphylatoxins that would lead to further activation of T cells and macrophages, mast cell degranulation and chemotaxis of immune cells that would lead to increased

inflammation. However, MSCs can also support the proliferation and differentiation of antibody-releasing B cells and this is inflammatory signal dependent, more precisely, IFN-γ dependent. Licensing of MSCs with IFN-γ increases the immunoregulatory potential of MSCs and enables the immunoregulation of B cells (Krampera et al., 2006, Saparov et al., 2016).

1.4.4 MSCs clinical use

In 1997, MSCs were shown to repair a craniofacial defect (Kadiyala et al., 1997), leading to an increasing interest in the therapeutic potential of MSCs due to their tissue regenerative capacity, which could be highly beneficial within the clinic for tissue degrading diseases such as rheumatoid arthritis and multiple sclerosis (Kemp et al., 2010, Sakaguchi et al., 2005). Moreover, their immunosuppressive and immunomodulatory properties led to the idea of MSCs being used in the treatment of autoimmune disorders and to suppress graft rejection and graft versus host disease (GVHD) (Farini et al., 2014). More importantly, the low expression of HLA molecules results in MSCs having poor immunogenicity *in vitro*, in pre-clinical and in human studies, which enables the use of allogeneic donors (Klyushnenkova et al., 2005, Koc et al., 2002). On top of this, MSCs can be isolated from a variety of tissues and can be easily expanded for mass production of a good manufacturing practice (GMP)-grade cell product. As a result, MSCs have huge potential as cellular therapeutics within different diseases including GVHD and type 1 diabetes mellitus (T1DM) among others.

1.4.4.1 MSCs migratory capacity

For MSCs to be effective within a clinical setting, MSCs must migrate to sites of tissue injury. MSCs have been shown to migrate to sites of injury. As examples, after cerebral ischemia MSCs have been shown to migrate into sites of brain injury (Mahmood et al., 2003, Chen et al., 2001) and in a model of allograft rejection, MSCs were able to migrate into the infarcted myocardium (Barbash et al., 2003). However, an engraftment study using three different administration methods in cardiac disease proved that only 1 to 5% of delivered cells engraft within the target site regardless of the delivery route (Freyman et al., 2006). Intravenous (IV) infusion of MSCs leads to engraftment of MSCs mostly in the lungs, which is likely generated by the small capillary size, the large size of the

cells and their strong adhesive properties. However, MSCs can also be found in the spleen, liver, bone marrow, thymus, kidney and skin seconds or minutes after IV injection, but, it is still unknown if MSCs migrate specifically to these organs or they just get trapped (Devine et al., 2003, Fischer et al., 2009, Freyman et al., 2006, Schrepfer et al., 2007).

The mechanisms used by MSCs to migrate from periphery into tissue injury remain unknown, but it is likely that they comprise a combination of adhesion molecules and chemokines and their receptors. To support this hypothesis, there is a large body of literature describing the constitutive expression of chemokine receptors on MSCs. CCR1, CCR7, CXCR2, CXCR6, CX3CR1 and, more importantly, CXCR4 are decisive receptors in the context of homing. However, the expression of these markers varies among reports in the literature due to tissue source of isolation, cell culture methods and passage number of the cells. CXCR4 has been described as a major player in directing MSC homing to sites of injury but some groups have described no expression of this receptor on MSCs (Von Luttichau et al., 2005), some have found a small expression (Wynn et al., 2004) whilst others have shown functional CXCR4 in MSCs (Kortesidis et al., 2005). Murine BM MSCs have been described to express CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR9, CCR10, CXCR3, CXCR4 and CXCR7 and to have functional CCR3, CCR4, CCR5, CCR7, CCR10 and CXCR4 (Alexeev et al., 2013). Ad MSCs have higher CXCR4 expression and migration capacity than BM MSCs and more interestingly, the chemokine receptor profile is sensitive to time in culture (Heirani-Tabasi et al., 2017). In addition, expression of chemokine receptors and ligands can be upregulated by cytokine-mediated stimulation and hypoxia (Croitoru-Lamoury et al., 2007, Jin et al., 2018). All this together suggests that MSCs isolated from different sources exhibit differential chemokine receptor expression and therefore, differential homing potential to sites of inflammation, which could be of high relevance to enhance migration when using MSCs as cellular therapeutics.

1.4.4.2 MSCs in Graft Versus Host Disease and Solid Organ Transplantation

Some haematological malignancies and blood cells disorders, such as sickle-cell anaemia, can be treated with allogeneic haematopoietic stem cell transplant (HSCT). These allografts contain mature T cells that can target and eradicate

malignant cells in the recipient; however, these cells can also target the recipient as nonself leading to GVHD (Korngold and Sprent, 1978).

To better understand GVHD, mouse models were created using radiation or chemotherapy to deplete haematopoietic cells and reconstituting the immune system with allogeneic bone marrow cells. Due to the immunomodulatory and anti-inflammatory properties of MSCs, it was hypothesised that MSCs could improve the engraftment of HSCs and increase the longevity of the graft by diminishing GVHD. Infusion of BM MSCs in an MHC mismatched mouse model of HSCT showed that BM MSCs decreased the severity of GVHD while promoting graft survival. These results were coupled with reduced infiltration of T cells and inhibition of the co-stimulatory molecules CD80 and CD86 on host DCs (Wen et al., 2015).

In humans, GVHD is treated with high-dose corticosteroids but 40 to 60 % of patients develop steroid resistance, which has a very poor prognosis. For these patients, a promising alternative to immunosuppressants is MSC infusion. Between 2001 and 2007, 55 patients who had developed steroid resistance were infused with 1 to 5 doses of MSCs obtained from matched sibling donors, haploidentical donors and third-party HLA-mismatched donors. 70% of the patients showed clinical improvement and 54% of the patients had complete responses to the treatment regardless of donor major histocompatibility matching (Le Blanc et al., 2008). The first industry-sponsored phase III trial of MSCs for treatment of steroid-refractory GVHD was completed in 2009 and was deemed a failure as complete remission at day 28 after infusion of MSCs was not increased compared to placebo. This study included both children and adults with any grade of steroid-resistant GVHD. However, children were described to respond better to allogeneic MSC treatment and gut and liver GVHD were observed to be more responsive than skin GVHD. Taking this into account, a new study to treat paediatric GVHD was performed and in 2018 it was shown to be successful as MSC infusion significantly improved day 28 overall response in steroid-refractory GVHD paediatric subjects (Galipeau and Sensébé, 2018). For this reason, MSCs are being studied as a promising strategy to regulate anti-donor immune responses in solid organ transplantation (SOT) to achieve long-term stable graft function without the need for immunosuppression. Phase 1

clinical trials have been carried out in kidney, liver and lung transplantation infusing allogeneic BM MSCs or UC MSCs and in all the cases MSC-treated patients showed good graft function at one year (Soeder et al., 2015, Sun et al., 2018, Keller et al., 2018). More importantly, time of MSC infusion has been shown to be critical; MSC infusion prior to transplantation leads to migration of MSCs to lymphoid organs where they promote the expansion of Tregs. In contrast, MSC infusion after kidney transplantation resulted in premature graft dysfunction coupled with neutrophils and complement deposition (Casiraghi et al., 2012). This is likely explained by the lack of MSC licensing; infusion of MSCs post-transplantation does not enable interaction of the MSCs with the inflammatory environment for long enough to exert their anti-inflammatory and immunomodulatory properties (Krampera, 2011).

1.4.4.3 MSCs in Diabetes Type 1

Type 1 Diabetes mellitus is a chronic autoimmune disorder resulting in the destruction of the insulin producing beta cells in the islets of Langerhans as a result of targeting islet cell autoantigens. Autoreactive T cells are considered the main effectors of beta cell destruction but macrophages, DCs and B cells are also involved (Yoon and Jun, 2005). The most comprehensive analysis to date concludes that there are 350,000 people in the UK living with T1DM, costing the NHS £1 billion per year (Stedman et al., 2020).

Daily insulin injections with the aim of restoring blood glucose levels is the most common method of treatment for individuals with T1DM. T1DM can be fatal if untreated and in some cases the disease cannot be well controlled with insulin, leading to hypoglycaemic unawareness. For these patients, the Scottish National Blood Transfusion Service (SNBTS) offers an islet transplant service to re-establish glycaemic awareness and have a better control of the disease. This procedure involves the isolation of islets of Langerhans from the pancreas of a donor to infuse them into the diabetic patient. The pancreas of diabetic patients is the site of autoimmune destruction and surgical intervention in this organ is highly complicated. Due to the high irrigation and the unique blood supply that promotes a tolerogenic state, the liver is the recipient of these cells. The success of the procedure avoids the patients having to use insulin. However, the success rate, defined as absence of insulin requirement 3 years after the

intervention, is just 44% due to long term deterioration and rejection (Bruni et al., 2014). For this reason, there is undergoing research on protecting the islets from recipient immune attack; current proposals include physically encapsulating the islets within a semi-permeable membrane consisting of polymer (Sakata et al., 2012) and MSCs co-transplantation to immunoregulate and dampen inflammation improving graft survival (Figliuzzi et al., 2014).

Two main models have been used to study the effect of MSC administration in T1DM, the non-obese diabetic (NOD) mouse model and the administration of streptozotocin (STZ). NOD mice have a defective cytotoxic T lymphocyte-associated protein 4 (*CTLA4*) gene, which is essential in the suppression of T cells. In this way, spontaneous autoimmune diseases are generated, including T1DM-like ß cell destruction (Kikutani and Makino, 1992). The administration of STZ damages ß cells resulting in the accumulation of immune cells and immune mediated destruction of ß cells. Both of these models have shown that MSC administration can protect against T1DM when MSCs are administered before onset and that they can reverse the disease when MSCs are administered after onset as they can revert hyperglycaemic animals to normal blood glucose levels (Madec et al., 2009). Co-transplantation of insulin producing islets of Langerhans and MSCs has shown that MSCs can significantly delay graft rejection (Forbes et al., 2020), improve insulin secretion (Kerby et al., 2013) and delay allograft rejection (Ben Nasr et al., 2015). These observations have been paired with a decrease of $CD4^+$ T cells, an increase of Th2 response mediated by a switch in cytokine secretion, an induction of T regulatory cells and an increase in vascularization (Madec et al., 2009, Forbes et al., 2020).

1.5 Thesis aims

This introduction has highlighted the anti-inflammatory and immunomodulatory potential of MSCs for use as cellular therapeutics in several diseases, including autoimmune diseases, cancer and transplantation. As SNBTS provides an islet transplant service, the particular interest of this thesis is focused on the co-infusion of MSCs with islets of Langerhans to increase engraftment and survival of the islets. Nonetheless, our interest is not limited to this and the knowledge

generated in this thesis extends understanding of the immunomodulatory potential of MSCs within any clinical setting. It is important to bear in mind that most of the literature regarding phenotype, function and immunomodulatory properties is focused on human BM MSCs, while there is a lack of understanding of MSCs isolated from other tissues or other species. Murine MSCs have been described to be more difficult to expand in culture than human or rat MSCs, which results in a smaller body of literature on murine MSCs (Caroti et al., 2017). However, mouse models are essential for performing mechanistic studies and preclinical testing of new therapeutics, which makes it essential to understand the phenotype, function and immunomodulatory properties of murine MSCs.

A PhD study comparing human MSCs from tissues considered medical waste- islets and adipose tissue- to BM MSCs has already been performed (Thirlwell, 2018), but this study could not take into account the xeno-challenge of using human MSC in the mouse models. Thus, the use of autologous murine MSCs to study and compare the immune reactivity and immunomodulatory potential of MSCs from different sources is essential.

Chemokines are master regulators of immune cell trafficking under resting and inflammatory conditions; for this reason, this study set out to understand the chemokine receptor expression of murine MSC types. This would let us understand whether tissue of origin could affect the migration to specific target tissues if infused systemically. For local infusion, low expression of chemokine receptors could be preferred to avoid MSCs from migrating away from the graft site.

Moreover, it was essential to understand how tissue of origin could influence the interaction of MSCs with their surrounding environment after infusion into a patient. Understanding the recruitment potential and the interaction between immune cells and MSCs isolated from different sources would provide insight of MSCs anti-inflammatory, immunomodulatory and pro-regenerative capacity *in vivo*. To study this, we analysed the chemokine and immunomodulatory protein secretion profiles of MSCs isolated from various sources and their interaction with immune cells in a mouse air pouch model. Altogether, the layout of this study and overall aims of each chapter are outlined below:

1. The aim of Chapter 3 was to fully phenotype BM, Is and Ad derived MSCs at rest and under inflammatory stimulation (MSC licensing).

2. The aim of Chapter 4 was to assess and compare the mRNA expression of the most relevant chemokines and their receptors by MSCs at rest and under inflammatory stimulation. Moreover, we aimed to determine if the expression patterns of chemokines would persist at protein level at rest and under inflammatory stimulation.

3. The aim of Chapter 5 was to determine the immune cell attraction profile of MSCs at rest and under inflammatory stimulation *in vivo*, assessing in this manner the functionality of the secreted chemokines.

4. The aim of Chapter 6 was to assess and compare the mRNA expression of toll-like receptors, the complement system family and other immunoregulatory and anti-inflammatory molecules by MSCs at rest and under inflammatory stimulation. Moreover, we aimed to determine if the expression patterns would persist at protein level at rest and under inflammatory stimulation to better understand the immunomodulatory potential of MSCs isolated from different sources.

In this manner, by looking at the chemokine and chemokine receptor expression, the immune cell attraction profile and the expression of immunoregulatory, anti-inflammatory and angiogenic molecules by MSCs isolated from different sources we aimed to understand MSC properties and functions *in vitro* and *in vivo*. This knowledge would enable the prediction of roles for these cells and preferential MSC tissue of isolation to be administered as cellular therapeutics within specific clinical settings.

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Cell culture methods

2.1.1 Growing and harvesting cells

Manipulation of cells was done in all cases in a sterile environment using a laminar flow hood with HEPA filtration. Mesenchymal stromal cells were grown in Dulbecco's Modified Eagle Medium *(*DMEM) with high glucose and sodium pyruvate (Invitrogen) supplemented with 20% (v/v) heat-inactivated foetal calf serum (FCS) (Invitrogen) and 2 mM glutamine (Sigma), which is referred as complete medium. 3T3 Mouse Embryonic Fibroblasts (MEF) cell line (ATCC CRL-2752) was grown in DMEM with high glucose and sodium pyruvate supplemented with 10% (v/v) FCS. Cells were maintained and grown in a humidified incubator at 37 ºC and 5% CO_2.

Cultures were examined daily for growth using a Zeiss optical microscope and when cultures achieved a cell density of greater than 80% confluence, cells were sub-cultured. Briefly, the medium was removed, and the cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS) (Sigma). To detach the cells, cells were incubated with TrypLE™ Express Enzyme (Thermo). TrypLE was then inactivated in the flask with medium. Cells were then placed into a 15 ml falcon tube and spun down at 400 x g for 5 minutes, the supernatant was then removed, and cells were resuspended into an appropriate volume of culture medium (0.26 ml/ cm^2). Cells were then distributed into new flasks at 3500 cells/ cm^2 for sub-culturing or samples were taken. A passage here describes detaching the cells from the flask and re-seeding them into a new one.

2.1.1.1 Cell counting

Cells were counted using a Neubauer Haemocytometer (Hawksley). Dead cells were excluded using Trypan blue (Sigma). Trypan blue was diluted 1:10 in phosphate-buffered saline (PBS). Cell were incubated for 5 minutes at room temperature with a 1:1 dilution of Trypan blue and loaded into the haemocytometer chamber. Live cells in the 4 large outer squares were counted, divided by 4 to obtain the average cell number, multiplied by 2 to account for

the trypan blue dilution and then by 10^4 to get the number of cells per 1 ml of the cell suspension.

2.1.1.2 Freezing cells

Cells were washed in PBS, detached with TrypLE as described above, spun down and resuspended at a density of approximately $1x10^6$ cells/ mL in Cellbanker cell freezing media (Amsbio). 1 mL aliquots were transferred to 2 mL cryo-vial (Thermo-scientific) and stored in a freezing vessel (Nalgene, Hereford), containing isopropanol. This was then placed at -80 ^{0}C (cooling 1 ^{0}C per minute) and transferred to liquid nitrogen tanks within two days.

2.1.1.3 Thawing cells

Cells were recovered from liquid nitrogen and rapidly thawed in a 37 ^{0}C water bath. The cells were then transferred from a cryo-vial to a 15 mL falcon tube. Warmed culture medium was slowly added drop by drop until cells were suspended in 7 mL of culture medium. The cells were then spun down at 300 x g for 5 minutes and supernatant was discarded. The remaining pellet was then resuspended in the appropriate amount of culture medium and transferred to tissue culture flask(s).

2.1.2 MSCs isolation

To isolate MSCs, female mice aged 7 to 8 weeks were used in all the cases. Details on how MSCs were isolated are described below. All tissues were processed within 30 minutes following animal death to ensure high cell viability.

2.1.2.1 Isolation of Bone Marrow derived mesenchymal stromal cells (BM MSCs)

Mice were killed with CO_2 or cervical dislocation and the cadaver was laid with the abdominal side facing up, the limbs were stretched and fixed with pins and then the skin was sterilised using 70% ethanol. The skin from the hind limbs was removed by pulling toward the foot, which is cut at the anklebone. This eliminates further contact of the hind limb with the animal's fur, which is a source of contaminating bacteria. Muscles, ligaments and tendons were dissociated from tibias and femurs using micro dissecting scissors and surgical

scalpel. Then, hind limbs were dissected from the trunk of the body by cutting along the spinal cord with care not to damage the femur. Hind limbs were stored on ice in Hanks' Balanced Salt Solution (HBSS) (Sigma) while awaiting further dissection or digestion.

Further dissection of the hind limbs was performed under the hood. Muscle and connective tissue from both the tibia and the femur were further removed and each hind limb was dissected by cutting through the knee joint. The ends of the tibia and femur were cut just below the end of the marrow cavity. A 27 G needle attached to a 10 mL syringe with complete medium was inserted into the spongy bone, exposed by removal of the growth plate, and was used to flush the bone marrow out, which was collected in a 100 mm sterile Petri dishes (Fisher).

Plates were incubated at 37 ^{0}C with 5% CO_2 in a humidified chamber leaving the solid mass in the medium, without disturbing them. On Day 5 cells were washed with DPBS twice and trypsinised for 2 minutes at 37 ^{0}C, then the trypsin was neutralised using complete medium. The trypsinization lasted less than 2 minutes as longer digestion is harmful for MSCs and could lift non MSCs from the dish. Cells were then placed into a 15 mL falcon tube and spun down at 400 x g for 5 minutes, the supernatant was then removed, and cells were resuspended in an appropriate volume of culture medium (0.26mL/cm^2). Cells were then distributed into new flasks at 3500 cells/cm^2. From then on, cells were checked daily for growth and medium was changed every 2-3 days to remove dead cells. When cells achieve a confluency of 80%, MSCs were passaged as described in Section 2.1.1.

2.1.2.2 Isolation of Pancreatic Islet derived mesenchymal stromal cells (Is MSCs)

To ensure the viability of the islets mice were killed by cervical dislocation and immediately laid with the abdominal side facing up. A midline incision was made around the abdomen and the skin was retracted using straight tweezers. The thoracic cavity was opened and heart and ribs were removed to provide a better access to the liver. The liver was then pushed up to the thoracic cavity and the intestines and stomach were pushed to the left. The rectum was cut and the intestines were taken out of the abdomen by pulling out from the rectum to

increase the visibility of the pancreas. The mesenteric fat attached to the intestines was removed to avoid confusion with the pancreas. Once the pancreas was visible, the spleen was identified and lifted to facilitate the access to the tail of the pancreas, which has been described to have a higher and more compact number of islets compared to the head of the pancreas (Elayat et al., 1995). The pancreas was then micro-perfused with 4 mL of 0.6 mg/mL cold collagenase P in HBSS using a 30 G needle with a 5 mL syringe.

Pancreas was then removed from the abdomen and put into 1 mL of 0.6 mg/mL collagenase P in HBSS on ice. Next, 10 mL of warm HBSS was added to the tissue and the collagenase was activated by placing the tissue at 37 ºC for 19 minutes. After digestion, collagenase was inactivated by adding 10 mL of cold HBSS. Tissues were then agitated twice per second for a minute. 40 mL of room temperature HBSS was then added to the tissue, which was followed by centrifugation at 1200 x g for 2 minutes. The supernatant was discarded and the pellet was resuspended in 10 mL of HBSS. The sample was then filtered through a 400 µm cell strainer. An extra 10 mL of HBSS was added to the tube containing the islets to ensure all the islets went through the filter. Samples were centrifuged at 1200 x g for another 2 minutes; the supernatant was discarded and the pellet was dried. The pellet was then resuspended into Histopaque 1077 (Sigma) and HBSS was carefully added without resuspending it with the Histopaque 1077. Centrifugation of the sample at 1200 x g for 20 minutes with the brake off leads to the generation of a density gradient, also known as Ficoll gradient centrifugation, that allows the isolation of the islets from the remaining acinar tissue (Orloff et al., 1987). Islets were carefully removed with a Pasteur and where counted under the microscope.

Islets were centrifuged and plated out on 100-mm sterile Petri dishes (Fisher) at a concentration of 10 islets/ cm^2 and were cultured at 37 ºC in 5% CO_2 in MSC culture medium. At day 7, MSC cells had already started the migration from the islets to the plate and therefore, media was carefully replaced and then changed every 3-4 days. Once the cells had reached 80% confluency the adherent MSCs were passaged as described in Section 2.1.1 with an exception; to remove islets and cell debris cells were passed through a 100 µM cell strainer. The cells were counted using a haemocytometer and classified as passage 2 (P2).

2.1.2.3 Isolation of Adipose Tissue derived mesenchymal stromal cells (Ad MSCs)

Animals were sacrificed as above. A midline incision was made around the abdomen and the skin was retracted using straight tweezers. The muscular wall was then opened to expose the liver and intestines. Perigonadal adipose tissue was harvested. Adipose tissue was digested using 0.2mg/mL Collagenase P (Roche) and 0.1mg/mL DNAse (Roche) in HBSS for 40 minutes at 37 ^{0}C. After incubation, collagenase was inactivated using medium and the soft tissues were plated into 100 mm sterile Petri dishes (Fisher).

Plates were incubated at 37 ^{0}C with 5% CO_2 in a humidified chamber leaving the solid mass in the medium, without disturbing it. On Day 5 cells were washed with DPBS twice and trypsinised for 2 minutes at 37 ^{0}C, then the trypsin was neutralised using complete medium. Cells were then placed into a 15 mL falcon tube and spun down at 400 x g for 5 minutes, the supernatant was then removed and cells were resuspended in an appropriate volume of culture medium (0.26mL/cm^2). Cells were then distributed into new flasks at 3500 cells/cm^2. From then on, cells were checked daily for growth and medium was changed every 2-3 days to remove dead cells. When cells achieve a confluency of 80%, MSCs were passaged as described in Section 2.1.1.

2.1.3 MSC licensing with stimulatory molecules

When cultures achieved a cell density >80% confluence, the medium was discarded and the cells were washed twice with DPBS. MSC culture medium was replaced with medium supplemented with stimulatory molecules as detailed in Table 2-1.

Table 2-1. List of inflammatory reagents used for MSC licensing.

Name given in figures	Reagent	Company	Final concentration
Cyt	Interferon-γ	Peprotech	40 ng/mL
	Tumour Necrosis Factor-α	Peprotech	40 ng/mL
	Interlukin-1β	Peprotech	40 ng/mL
LPS	Lipopolysaccharides from Escherichia coli O127:B8	Sigma Aldrich	100 ng/mL
LTA	Lipoteichoic acid from Staphylococcus aureus	Sigma Aldrich	100 ng/mL
Poly I:C	Polyinosinic–polycytidylic acid sodium salt	Sigma Aldrich	4 mg/mL

Three different licensing conditions were tested.

In the first one, cells were stimulated for 48 hours, after which they were washed twice with PBS and fresh culture medium was added; cells were harvested 24 hours later.

In the second condition, cells were washed twice with PBS, the culture medium was replaced with fresh medium and the cells were left growing for 48 hours. Cells were then washed twice with PBS, the culture medium was replaced with supplemented medium and the cells were harvested 24 hours later.

In the last condition, cells were stimulated for 48 hours, after which cells were washed twice with PBS and were stimulated again for another 24 hours. Figure 2-1 illustrates the time points at which supplemented medium was added. Culture medium, collected at the same time as the cells were harvested, was kept for experimental procedures.

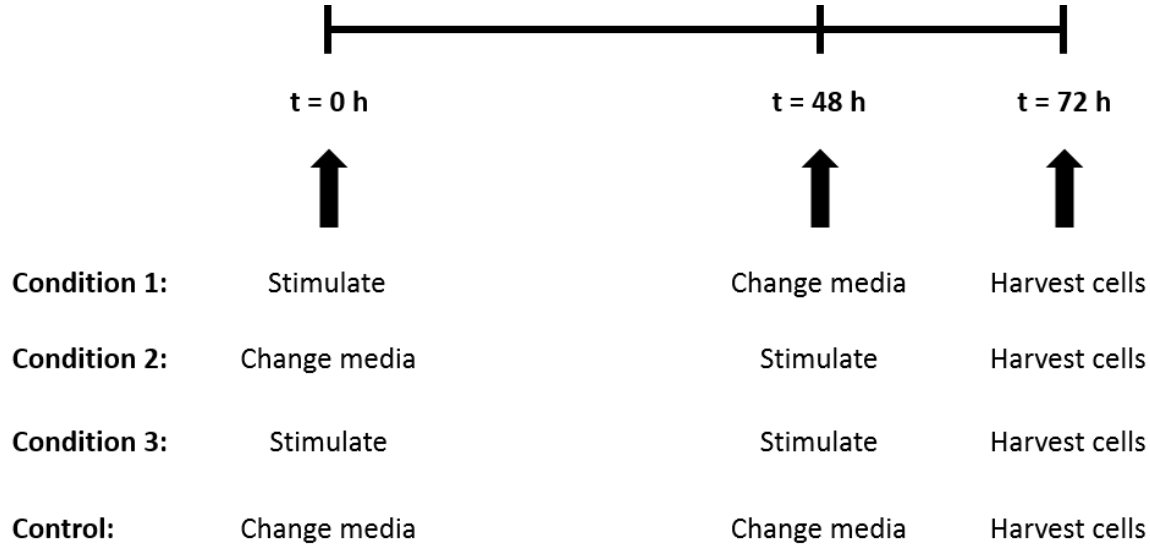


Figure 2-1. Diagrammatic illustration of the time course of the MSC licensing.

2.1.4 Differentiation assays

MSCs were differentiated into adipocytes, chondrocytes and osteoclasts using the Mouse Mesenchymal Stem Cell Functional Identification Kit (R&D Systems) following the manufacturer's instructions. All reagents and materials are listed in the manufacturer's handbook.

2.1.4.1 Adipogenesis and osteogenesis

MSCs from different sources were plated at $2.1x10^4$ (adipogenesis) or $4.2x10^3$ (osteogenesis) confluency in 4-well Nunc™ Lab-Tek™ Chamber Slides (Thermo Fisher Scientific). Cells were cultured in 0.5mL/well 90% α-MEM, 10% (v/v) FCS and 1% (v/v) 100X Penicillin-Streptomycin-Glutamine. For adipogenesis, when MSCs reached 100% confluency, culture medium was replaced by 0.5mL/well of adipogenic differentiation medium (10µl/mL of Adipogenic Supplement added to α-MEM culture medium). For osteogenic differentiation, once cells were 50-70% confluent, culture medium was replaced with 50 µl/mL of osteogenic supplement added to α-MEM culture medium. From then on, in both cases, freshly prepared supplemented culture medium was replaced every 3-4 days for 14-21 days. Cells were then fixed and stained as stated in Section 2.3.2.1.

2.1.4.2 Chondrogenesis

For the differentiation of MSCs into chondrocytes, $2.5x10^5$ MSCs were transferred into a 15mL conical tube in chondrogenic culture medium (99% DMEM/ F12, 1% Insulin-Transferrin-Selenium supplement and 1% Penicillin-Streptomycin-Glutamine) and spun down at 200 x g for 5 mins at room temperature. Supernatant was poured off and MSCs were resuspended in chondrogenic culture medium and spun again at 200 x g for 5 mins. MSCs were left in the chondrogenic culture medium as a pellet and placed into the incubator with the 15 mL conical tube lids slightly loosened for gas exchange. Chondrogenic culture medium was replaced every 2-3 days with caution so as not to disturb the pellet and cultured for 21 days. After 21 days, the spherical mass of cells was removed and prepared for cryosectioning and further staining as explained in Section 2.3.2.

2.1.5 Depletion of CD45 positive population

Flow cytometry-based analysis of Islet derived MSCs showed a CD45 positive population. To further examine this population, CD45 MicroBeads (Miltenyi Biotec) were used to isolate the CD45 positive cells. Cell separation buffer was prepared by diluting MACS BSA Stock Solution (Miltenyi Biotec) 1:20 in autoMACS Rinsing Solution (Miltenyi Biotec).

Cells were harvested as explained in Section 2.1.1. Cells were then counted and centrifuged at 300 x g for 10 minutes and the supernatant was pipetted off. The cell pellet was then resuspended in 90 µL of degassed buffer per 10^7 cells. Next, 10 µL of CD45 MicroBeads per 10^7 cells were added and the mix was incubated for 15 minutes on ice. Cells were then washed by adding 2 mL of buffer per 10^7 cells and centrifuged at 300 x g for 10 minutes. The supernatant was removed and cells were resuspended in 500 µL of buffer. Cells were then ready for magnetic separation. The CD45 depletion was done using LS Columns (Miltenyi Biotec) and the MACS MultiStand (Miltenyi Biotec) separator. The LS columns were placed in the magnetic field of the separator and columns were prepared by rinsing with 3 mL of the buffer. The cell suspension was then applied onto the column and the unlabelled cells, which passed through the column, were collected. The column was washed three times with 3 mL of buffer. The total effluent was collected and labelled as $CD45^-$ cells. To harvest the magnetically

labelled cells, columns were removed from the separator and placed on a suitable collection tube. 5 mL of buffer was pipetted onto the column and the cells were flushed out by firmly applying the plunge supplied with the column. This fraction was labelled as $CD45^+$ cells. Cells were then distributed into new flasks at 3500 cells/ $square^2$ for sub-culturing or samples were taken.

2.2 Molecular biology

2.2.1 Ribonucleic acid (RNA) extraction from cells

Pipettes, bench surfaces, tubes and labware were cleaned before use with RNase AWAY® and DNA AWAY™ Surface Decontaminants (Molecular BioProducts) to reduce the degradation of RNA from environmental RNases. A pellet of a maximum of 5×10^6 cells was disrupted and homogenised using the QIAshredder system (Qiagen) and total RNA was then extracted with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, which included the optional DNase digestion step using the RNase Free DNase Set (Quiagen). RNA was eluted by the addition of 50 µL of RNase free water. The concentration and quality of the RNA samples was tested by ultraviolet absorption at 260/280 nm in a ThermoFisher Nanodrop 1000 spectrophotometer. The quality of the RNA was tested in a 1% agarose gel. RNA was immediately used or stored at -80 ^{0}C in an eppendorf tube until needed; RNA was quantified after each defrost.

2.2.2 RNA reverse transcription

RNA was reverse-transcribed by QuantiTect Reverse Transcription Kit (Qiagen), which includes a genomic DNA elimination step. Genomic DNA elimination of 1µg of RNA was performed using the reaction mixture explained on Table 2-2 and incubating the mixture for 2 minutes at 42 ^{0}C and then placing it immediately on ice. Reverse transcription was accomplished using the reaction mixture detailed in Table 2-3 and applying an initial step at 42 ^{0}C for 15 minutes followed by a denaturation step at 95 ^{0}C for 3 minutes. cDNA was stored at -20 ^{0}C until it was required for gene expression studies. Reverse transcription controls were used to ensure the efficacy of this project; these controls were made by replacing the enzyme with RNase free water.

Table 2-2. Reaction mixture for genomic DNA elimination using the QuantiTect Reverse Transcription Kit (Qiagen).

Reagent	Volume (µl)	Final concentration
gDNA Wipeout Buffer, 7x	2	1x
Template RNA, 1 µg	Variable	1x
RNase free water	Up to 14	NA

Table 2-3. Reaction mixture for RNA reverse transcription using the QuantiTect Reverse Transcription Kit (Qiagen).

Reagent	Volume (µl)	Final concentration
Quantiscript Reverse Transcriptase	1	1x
Quantiscipt RT Buffer, 5x	4	1x
RT Primer Mix	1	1x
Entire genomic DNA elimination reaction	14	NA

2.2.3 Primer design

Pairs of primers were designed to relatively quantify the amount of specific cDNA in a sample by SYBR Green. The cDNA sequences used to design the primers were obtained from the NCBI Nucleotide database, which is available online at: https://www.ncbi.nlm.nih.gov/nucleotide/. Primers were designed using the Primer 3 Plus software, which is available online at: http://primer3plus.com/cgi-bin/dev/primer3plus.cgi. To ensure an accurate amplification and a high efficiency, primers were designed using strict criteria. Primers had to be between 18 and 24 base pairs (bp) in length, with 20 bp as the optimal length. The GC content had to be between 40% and 60%, with 50% the ideal percentage, avoiding stretches of more than 4 G or C bases and avoiding more than two G or C bases in the last 5 bases situated at the 3' end of each primer. The melting temperature (T_m) should be as close to 60 ºC as possible and the amplicon size should be less than 150 bp. However, the most important was that the 3' self-complementary should not be higher than 1 while the self-complementary should not be higher than 2. In the cases in which all these criteria could not be matched, all the conditions but the 3' self-complementary were relaxed.

To ensure the specificity of the primers for the gene of interest the BLAST analysis online tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used. For SYBR Green assay custom made primers were obtained from Integrated DNA

Technologies (Table 2-5). To further confirm the specificity of the primers, the primers were first used to run a PCR reaction with any cDNA containing the gene of interest. The reaction mixture was set up as outlined in Table 2-4 and the cDNA was amplified as follows: an initial step at 98 ^{0}C for 3 minutes, 35 cycles of denaturation at 98 ^{0}C for 10 seconds, followed by the annealing at the T_m of each pair of primers for 30 seconds and elongation at 72 ^{0}C for 20 seconds. The 35 cycles were followed by a final elongation step at 72 ^{0}C for 10 minutes. The PCR product was then run at 75 V in a 1% agarose gel and specificity was confirmed if a single amplification product with the expected molecular weight was observed.

Table 2-4. Reaction set up for PCR indicating the reagents and their concentrations.

Reagent	Volume (µl)	Final concentration
Phusion HF Buffer x5	5	1x
dNTPs (10mM)	0.5	200 µM each dNTP
Forward primer (10µM)	1.25	0.5 µM
Reverse primer (10µM)	1.25	0.5 µM
DMSO	0.75	3%
Phusion DNA Polymerase	0.25	0.5 U
cDNA (1:5)	1	NA
ddH_2O	Up to 25	NA

Table 2-5. List of forward and reverse primers used during this study.

Gene	Forward primer	Reverse primer
CD45	TGGTGTGCAGCTATGAGCAA	GTCCATTCTGGGCGGGATAG
CD45	GACAACCTTCGTGCCCAAAC	TGACGAGTTTTACACCGCGA
B2M	GGTGACCCTGGTCTTTCTGG	TGTTCGGCTTCCCATTCTCC
CCL2	AGCCAACTCTCACTGAAGCC	GCGTTAACTGCATCTGGCTG
CCL3	CAGCCAGGTGTCATTTTCCT	CAGGCATTCAGTTCCAGGTC
CCL4	TGACCAAAAGAGGCAGACAGAT	GCTGTGCCACATCTCTTGGT
CCL5	CTGCTGCTTTGCCTACCTCT	ACACACTTGGCGGTTCCTT
CCL7	TGAAAACCCCAACTCCAAAG	TTAGGCGTGACCATTTCACA
CCL9	CTCACAACCACGGACCTACA	CACTGGGGAAGACCAAAGAA
CCL11	GCACCCTGAAAGCCATAGTCT	TGGGGTCAGCACAGATCTCT
CCL19	GTGCCTGCTGTTGTGTTCAC	CAAGACACAGGGCTCCTTCTG
CCL20	CGACTGTTGCCTCTCGTACA	CTTCATCGGCCATCTGTCTT
CXCL1	CCGAAGTCATAGCCACACTCA	AGGTGCCATCAGAGCAGTCT
CXCL2	CCTCAACGGAAGAACCAAAG	AGGCACATCAGGTACGATCC
CXCL5	GCCCTACGGTGGAAGTCATA	GTGCATTCCGCTTAGCTTTC
CXCL10	GCTCAAGTGGCTGGGATG	GAGGACAAGGAGGGTGTGG
CXCL12	CCTCAACCCACCATGCTCAT	GAGACAGTCTTGCGGACACA

Gene	Forward primer	Reverse primer
CXCL13	CATACCCAACCCACATCCTT	GCCTGTTCTCAAATAGCCTTTC
CXCL16	TGCTGACCCTTTGCCTCTAC	GGCTGGCTTGGACTAAATAACA
CCR1	GCCCTCATTTCCCCTACAA	CGGCTTTGACCTTCTTCTCA
CCR2	TGTGGGACAGAGGAAGTGG	GGAGGCAGAAAATAGCAGCA
CCR3	ACCTTCGGCTCTTTTTCCAC	TGTTCTTTCCATTTTCTCACCA
CCR4	GCACCAAGGAAGGTATCAAGG	TGAACAGGACCAGAACCACA
CCR5	GGATTTTCAAGGGTCAGTTCC	GAAGACCATCATGTTACCCACA
CCR6	TCTTCACCCCTTTGCTGTTT	GCTCTGTGCCTCTTGGAGTT
CCR7	ATTGCTGCTGAGGGAAGAG	ACTTTTGGCTGTCGTTTTGG
CCR8	CCCTTTGCCATCCTCCTGTT	ATGGCTCTGGTCCTGTTGTG
CCR9	GAGTCTTGCTCCCAATCCAC	TAGGTTCCCACCATCCAAAC
CCR10	CCTGCTCTGCTCCTACTGAGA	CCTGGGATTGTTTCTTTAGCC
CXCR1	TGTCCCTTCTGAGCTTGCTG	CCAAGAAGGGCAGGGTCAAT
CXCR2	TGTCTGCTCCCTTCCATCTT	CCATTTCCTCTCCTCCACCT
CXCR3	AGTGCTTGTCCTCCTTGTAGTTG	GGTGTTGTCCTTGTTGCTGA
CXCR4	CTACAGCAGCGTTCT CAT CCT	CTTTTCAGCCAGCAGTTTCCT
CXCR5	ACTGTGATCGCTCTG CAC AA	GTGCAGGTGATGTGGATGGA
CXCR6	GACTCTGGGGTTCTT CCT GC	CAGCAGGAACACAGCCACTA
CX3CL1	CAACTTCCGAGGCACAGGAT	AGATGTCAGCCGCCTCAAAA
ACKR1	CCCTATGCAACCTGGGCTAC	TGGGGTTCAGGCAAGCATAG
ACKR2	TTCTCCCACTGCTGCTTCAC	TGCCATCTCAACATCACAGA
ACKR3	TGTCCCTGCCTGATACCTACT	GGACAGCAAAGCCCAAGATG
ACKR4	CCGAGACCCAACCATCAACA	TCCACACTTTGCCCACTTGT
CX3CL1	CAACTTCCGAGGCACAGGAT	AGATGTCAGCCGCCTCAAAA
CX3CR1	ACCCCTTTATCTACGCCTTTG	CTGTCCTGCCTGCTCCTCT
XCR1	GGGATCAAGTTCCGCAGACA	ATGTGCCCATCCTCTCCTCT
IL1R1	CCGAACCGTGAACAACACAA	TCAATCTCCAGCGACAGCAG
IL1R2	GATGTCTGGGCATCTGCTTTC	CCCTTGGAGCCCAATGCTAT
TNFRSF1a	GCTGTTGCCCCTGGTTATCT	ATGGAGTAGACTTCGGGCCT
TNFRSF1b	CAGGACCCTTGGCGTTACAT	TTGGCAAGGTGGTTGTCAGT
IFNGR2	CCAGCAATGACCCAAGACCA	TTCGGCTCCAGCAACCTATG
IFNGR1	ACGGTGATCTGTGAAGAGCC	TGCGTCTTTGTGTCGGAGTT
TLR1	GGCACGTTAGCACTGAGACT	GCTGACGGACACATCCAGAA
TLR2	CGTTGTTCCCTGTGTTGCTG	GGATAGGAGTTCGCAGGAGC
TLR3	GAACAACGCCCAACTGAACC	GAGAAAGTGCTCTCGCTGGT
TLR4	GCATGGCTTACACCACCTCT	TTTGTCTCCACAGCCACCAG
TLR5	TCTCCAGACGCCTCATCTCA	GTTCCAAGCGTAGGTGCTCT
TLR6	ACGAAGCTGACTTTCCTGGG	GTGAGCAACTGGGAGCAGAT
TLR7	ACCCATACTTCTGGCAGTGC	CCAAGGCATGTCCTAGGTGG
TLR8	TGCACATTCCCTGGAGACAC	AGAGGAAGCCAGAGGGTAGG
TLR9	AATGGCTCTCAGTTCCTGCC	CCTGCAACTGTGGTAGCTCA

Gene	Forward primer	Reverse primer
TLR11	CCTTACCTTGACTGGCTGGG	GCAAGATGCCAAAAGGTGCA
TLR12	ATTCCAGGAGCTCCAGCAAC	CTTACCCAGGTGAAGCAGGG
TLR13	CAAGTGCCAGCTCTCCTTCA	TGGCTCAGATCTAGGCTGGT
C1QA	AAGGGTCGCATTTACCAGGG	GCCGAGGGGAAAATGAGGAA
C1QB	TCTTCCTGCCTCTAGGGACC	CCTGCTGCTGTCCTCAGAAA
C1QC	CCAACAGCGTCTTCTCTGGT	CAGGAACCAGGGTGGACTTC
C1R	AGCAGCAATGCAGTGGATCT	TGGTGTAGTGCAGCTTCCAG
C1Sa	TAGAGCCGTCAGAGAGCTGT	TTCCTCGATGCCTCCTGAGA
C2	ATCACCTTTGCCTCTCAGCC	TCCGTCACATCCTGGGATCT
C3	TGCTGGCCTCTGGAGTAGAT	AGGCAGTCTTCTTCGGTGTG
C3AR1	CCCCAAGACATTGCCTCCAT	GACTGTGTTCACGGTCGTCT
C4	TCGCAGACATCACCCTCCTA	GCCCGTCAGTCTCAAAGTGA
C5	CCTGCTGAAGCCCAAGAGAA	GCAGGGTGTTTTCAAGCAGG
C5AR1	GCCTAGCTGCTCCTTTTCCA	TCATGAGGATCCTGGGCTCA
C6	AGGAGAGCCCAGAGGAGAAG	GGCTCGACTGGTCTTGACAA
C7	CGTGTGAGCAAGGAGTCCTT	CAACCTCCATCAACCCCTCC
C8A	AACTGCCGGTGTCAGTGTAG	CTGACCAGGAACTCCAGCAG
C8B	GGGGCATCTACGAGTACACG	CTCCAGCACAGGCAGTAACA
C8G	ACCTACTGTCCGTAGCAGGT	ATAGTGCTGATGGGGGAGGT
CD46	GGAGCTCTTATCCCCATGCC	GACTGAGTGTGGAAGGCACA
CD59	TGGTAGCCCAGCACAATGAG	TGTGAGGCTAACAGCTGTGG
CFB	GTCAGGCCCTGGAGTACCTA	TCTTTTGGTCTCGGGTCTGC
CFD	CAATCTGCGCACGTACCATG	CCACGTAACCACACCTTCGA
CFH	ACAACGGGTTTTCACCACCT	GTGCAACGAAGGTAGTCCCA
CFHR1	TGGTCACCAACTCCGAAGTG	AGCCTTGATTGCAGACCACT
CFI	GCGGGGGTAGTGTGTTACAA	TCGCTTTGGTCTCCACAGTC
CFP	GGCCCTGCTCAGTTACATGT	AGCTGCCACTCAAGAGTTCC
CR1L	GGATTCCAGAAGGGGTTGGG	TTCCAGCTGCCATCAGACTG
CR2	GCCCCGATCCAGAAGTCAAA	TGCCGTTCATGATGAAGCCT
ITGAX	CAAGATGCCACCAAGGTCCT	CGAATGATGCTTGCAGCCTC
ITGB2	GCAGAAGGACGGAAGGAACA	CCAGATGACCAGGAGGAGGA
CD142	TGCTTCTCGACCACAGACAC	ATAGGCCCAGGTCACATCCT
CD274	CAGCAACTTCAGGGGGAGAG	CTGTGATCTGAAGGGCAGCA
COX2	CATCCCCTTCCTGCGAAGTT	GGCCCTGGTGTAGTAGGAGA
GMCSF	AGGCTAAGGTCCTGAGGAGG	GGGCTTCTTTGATGGCCTCT
HGF	TGAGTTATGTGCTGGGGCTG	CACATCCACGACCAGGAACA
IDO 1	TGGTGGAAATCGCAGCTTCT	TTGACGCTCTACTGCACTGG
IDO 2	ACCTCCCTCGTCCCTTAGTC	AGAGAGTAAGCAGGGGAGGG
IFNβ	CACAGCCCTCTCCATCAACT	GCATCTTCTCCGTCATCTCC
IL-10	CAGAGAAGCATGGCCCAGAA	GCTCCACTGCCTTGCTCTTA
IL-1B	CGCTCAGGGTCACAAGAAAC	GAGGCAAGGAGGAAAACACA

Gene	Forward primer	Reverse primer
IL-6	TTCCATCCAGTTGCCTTCTT	ATTTCCACGATTTCCCAGAG
iNOS	GAGCCACAGTCCTCTTTGCT	CAACCTTGGTGTTGAAGG G
MMP9	AAACCCTGTGTGTTCCCGTT	CCTTTAGTGGTGCAGGCAGA
TGF- β1	CTTTGTACAACAGCACCCGC	CATAGATGGCGTTGTTGCGG
TNF-α	CACCACCATCAAGGACTCAA	GAGGCAACCTGACCACTCTC
TSG-6	CGGATACCCCATTGTGAAAC	TCCTTTGCATGTGGGTTGTA
VEGF a	AACGATGAAGCCCTGGAGTG	GCTGGCTTTGGTGAGGTTTG
VEGF b	AGAGTGCTGTGAAGCCAGAC	GATGATGTCAGCTGGGGAGG
VEGF c	AACCTCCATGTGTGTCCGTC	TGCTGAGGTAACCTGTGCTG
VEGF d	TTCAGGAGCGAACATGGACC	CCACAGCTTCCAGTCCTCAG

2.2.3.1 Buffers and reagents for agarose gel electrophoresis

Table 2-6. List of buffers and reagents for agarose gel electrophoresis and their composition

Solution	Composition	Concentration
10% TAE	TRIS Glacial acetic acid EDTA	0.4 M 17.4 M 0.5 M
1% agarose gel	Agarose SYBR Safe DNA gel stain TAE	1% (w/v) 7% (v/v) 1x

Length of DNA was assessed with the use of GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific) and GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific).

2.2.4 Quantitative reverse transcription PCR (qRT-PCR)

To quantitatively study gene expression a SYBR Green quantitative reverse transcription PCR assay was performed using the Applied Biosystems 7900HC Fast Real-Time PCR Systems detection system (Applied Biosystems).

Reactions were set up in 96 well plates as outlined in Table 2-7 and cDNA was amplified as follows: denaturation and polymerase activation at 95 ^{0}C for 3 minutes, amplification during 40 cycles at 95 ^{0}C for 3 s followed by 60 ^{0}C for 30 s. The direct detection of the PCR products was followed by the measurement of the fluorescence released from the SYBR Green I Dye when it binds to the double-stranded product after each cycle.

Table 2-7. Reaction set up for real time quantitative PCR analysis indicating the reagents, and their concentrations.

Reagent	Volume (µl)	Final concentration
PerfeCTa SYBR Green FastMix with ROX dye (VWR) x 2	20	1x
Forward primer (100 µM)	0.3	0.75 µM
Reverse primer (100 µM)	0.3	0.75 µM
cDNA (1:5)	4	NA
ddH_2O	Up to 40	NA

All samples were processed in triplicate. In all cases, samples were tested using the *beta-2 microglobulin (B2M*) gene as reference for data normalization. The difference between the C_t value and the housekeeping gene (ΔC_t) was calculated for each point of the triplicated sample:

$$\Delta C_t = C_t\ (target\ gene) - \ C_t(Housekeeping\ gene)$$

As this study did not have a reference sample, data were represented as $2^{(-\Delta CT)}$, which enables the visualization of expression levels of specific genes normalised to B2M for each sample. Due to the nature of normalisation, genes that generated a CT of 35 or above resulted in $2^{(-\Delta CT)}$ less than, or equivalent to, ~0.0001. Genes with $2^{(-\Delta CT)}$ values similar to ~0.0001 are marked with a red box on the following graphs and are likely not transcribed at meaningful levels by MSCs.

$$\boldsymbol{Gene\ expression} = \ \boldsymbol{2^{-\ \Delta C_t}}$$

As triplicates had been done, the median of the RQ values of each sample was used for statistical analysis. All the experiments were done at least three times and the average and standard deviations were calculated. D'Agostino-Pearson omnibus normality test was used in order to determine if the values come from a Gaussian distribution. Due to the low sample size, data was considered to be non-parametric and differences between the samples were analysed using the appropriate statistical tests, which are indicated in the figure legends. Differences were considered to be statistically significant at $p < 0.05$.

2.3 Protein analysis

2.3.1 Flow cytometry

2.3.1.1 Flow cytometry staining

When cultures achieved a cell density of greater than 80% confluence, the medium was removed and the cells were washed twice with DPBS. To detach the cells, cells were incubated with TrypLE™ Express Enzyme at 37 ºC for 10 minutes and the enzyme was inactivated with medium. Cells were then distributed for both sub-culturing and flow cytometry experiments. Cells were centrifuged at 400 x g for 5 minutes following washing, detachment and enzyme inactivation.

For fixable cell viability staining, after the detachment and centrifugation of cells the supernatant was discarded and cell pellets were resuspended in PBS and centrifuged again at 400 x g for 5 minutes. The supernatant was removed and cell pellets were resuspended in an appropriate volume of PBS buffer. The staining was done using the appropriate amount of the fixable viability dye eFluor506. Cells were then ready for cell surface staining.

For cell surface immunofluorescence staining, cells were centrifuged and cell pellets were resuspended in fluorescence-activated cell sorting (FACS) buffer (2% FCS, 2 mM EDTA in DPBS) and were centrifuged again at 400 x g for 5 minutes. The supernatant was removed and cell pellets were resuspended in an appropriate volume of FACS buffer. Cells were distributed between FACS tubes with 1 million of cells per tube. The stains were carried out using the appropriate amount of each antibody followed by vortex and incubation at 4 ºC for 30 minutes in the dark. Upon incubation, cells were washed with FACS buffer and centrifuged at 400 x g for 5 minutes. The supernatant was discarded and cell pellets were resuspended in 300 µL FACS buffer and kept on ice until needed for flow cytometry.

For nuclear protein staining, Fixation/ Permeabilization Solution Kit (BioSciences) was used. Following cell surface protein staining, cells were washed and thoroughly resuspended in 100 µL of BD Cytofix/ Cytoperm solution and incubated at 4 ºC for 20 minutes. Cells were then washed twice in 1x Perm/ Wash solution. Fixed and permeabilised cells were then resuspended in 50 µL of

Perm/ Wash solution containing antibody and were left incubating at 4 ^{0}C for 30 minutes in the dark. Upon incubation, cells were washed with FACS buffer and were resuspended in 300 µL FACS buffer and kept on ice until needed for flow cytometry.

To calibrate the flow cytometer, single stains were done using UltraComp eBeads (Thermo); fluorescence minus one (FMO) controls were used to accurately gate positive staining due to the fluorescence spread of the lasers in the different channels. Data were acquired using the Fortessa (BD BioSciences) or the MACSQuant (Miltenyi Biotec) flow cytometers and analysed using the FlowJo version 10 software.

2.3.1.2 Surface molecule phenotype of MSCs

At P3, MSCs were left to grow until they were 80% confluent, detached from the flasks as described in Section 2.1.1 and prepared for flow cytometry as previously detailed in Section 2.3.1.1 using the “MSC phenotyping” panel of antibodies listed in Table 2-8 and run on Fortessa. Cells isolated from the islets required further characterisation using the “Islet characterisation” panel of antibodies listed in Table 2-8.

2.3.1.3 Surface molecule phenotype of MScs in homeostatic vs inflammatory conditions

At P3, MSCs were left to grow until they were 80% confluent, the medium was discarded and the cells were washed twice with DPBS. MSC culture medium was replaced with medium supplemented with stimulatory molecules as outlined in Section 2.1.3. After 72 hours of the first media change, cells were detached from the flasks as described in Section 2.1.1 and prepared for flow cytometry as previously detailed in Section 2.3.1.1 using the “MSC phenotyping in inflammation” panel of antibodies listed in Table 2-8 and run on the MACSQuant.

2.3.1.4 Analysis of epithelial origin of Islet derived MSCs

To analyse the cell types enclosed within the islets of Langerhans, islets were harvested as described in Section 2.1.2.2 and were purified by hand–picking the islets. Islets were then incubated with 2.5 mL of trypsin-

ethylenediaminetetraacetic acid (EDTA) for 15 minutes at 37 ºC with shaking. Trypsin was inactivated with culture medium and cells were washed twice prior to staining the cells as stated in Section 2.3.1.1 using the "Islet characterisation" panel of antibodies listed in Table 2-8. Samples were run on the MACSQuant.

2.3.1.5 Proliferation analysis

To analyse the percentage of proliferative cells and its relationship with the CD45 status, cells were harvested as outlined in Section 2.1.1 and were stained as follows. First, the extracellular staining was performed as explained in Section 2.3.1.1 with the antibody panel listed in Table 2-8. To determine the percentage of proliferative cells, the nuclear protein Ki67 was used. Expression of Ki67 occurs during G1, S, G2, and M phase, while in G0 phase the Ki67 protein is not detectable. Intranuclear staining was done according to the manufacturer's instructions. Shortly, after the extracellular staining, the cell pellet was loosened by vortexing. 3 mL of precooled 70% ethanol was added to the cell pellet while vortexing, followed by 30 seconds of extra vortexing and incubation at -20 ºC for 1 hour. After incubation, cells were washed with FACS buffer three times and resuspended in 100 µL. Ki67 antibody or the appropriate isotype was then added, and cells were left incubating at room temperature in the dark for 30 minutes. Cells were then washed twice with FACS buffer. All the antibodies used for proliferation analysis are listed in Table 2-8.

2.3.1.6 Defining the immune cell attraction profile of MSCs *in vivo*

The immune cell attraction profile of MSCs was tested *in vivo* by introducing MSCs into air pouch created in the dorsal skin of mice (described in Section 2.4.2). Cell surface immunostaining was performed as detailed in Section 2.3.1.1 using the "innate immune response" and "adaptive immune response" panel of antibodies listed in Table 2-8.

Table 2-8. List of antibodies use for flow cytometry during this study.

Antibody panel	Antigen	Fluorophore	Clone	Dilution	Supplier
Phenotyping MSCs	CD73	BV 421	TY/11.8	1:200	BioLegend
	CD19	BV 510	6D5	1:200	BioLegend
	CD11b	BV 650	M1/70	1:200	BioLegend
	MHC Class II	BV 785	M5/114.15.2	1:200	BioLegend
	CD90	FITC	30-H12	1:200	Miltenyi
	CD34	PE	MEC14.7	1:200	BioLegend
	CD45	PE/Cy7	30-F11	1:200	BioLegend
	CD105	APC	MJ7/18	1:200	BioLegend
	CD64	FITC	X54-5/7.1	1:200	BioLegend
	Viability dye	Draq7		1:200	BioStatus
MSC phenotyping in Inflammation	CD73	BV 421	TY/11.8	1:200	BioLegend
	Fixable viability dye	eFluor 506		1:1000	eBioscience
	CD166	FITC	eBioALC48	1:200	eBioscience
	MHC Class I	PE	M1/42	1:200	BioLegend
	CD146	PE/Cy7	ME-9F1	1:200	BioLegend
	CD 271	APC	REA648	1:200	Miltenyi
	MHC Class II	APC/Cy7	M5/114.15.2	1:200	BioLegend
Islet characterisation	Fixable viability dye	eFluor 506		1:1000	eBioscience
	Vimentin	FITC	RV202	1:200	Abcam
	CD45	PE/Cy7	K041ES	1:200	Biolegend
	CD45	PE/Cy7	I3/2.3	1:200	Biolegend
	CD326 (EpCAM)	APC	G8.8	1:200	eBioscience
Proliferation analysis	Fixable viability dye	eFluor 506		1:1000	eBioscience
	ki67	FITC	16A8	1:200	Biolegend
	IgG2a isotype control	FITC	RTK2758	1:200	Biolegend
	CD45	PE/Cy7	K041ES	1:200	Biolegend

Antibody panel	Antigen	Fluorophore	Clone	Dilution	Supplier
Innate immune response	CD45	BV421	30-F11	1:200	Biolegend
	Fixable Viability dye	eFluor 506		1:1000	eBioscience
	Siglec F	FITC	S17007L	1:200	Biolegend
	F4/80	PE	BM8	1:200	Biolegend
	CD11c	PerCP/Cy5.5	N418	1:200	Biolegend
	CD11b	PE/Cy7	M1/70	1:200	Biolegend
	Ly6g	APC	1A8	1:200	Biolegend
	Ly6c	APC/Cy7	HK1.4	1:200	Biolegend
Adaptive immune response	CD45	BV421	30-F11	1:200	Biolegend
	Fixable Viability dye	eFluor 506		1:1000	eBioscience
	CD8 alpha	PE	53-6.7	1:200	Biolegend
	CD73	PerCP/Cy5.5	TY/11.8	1:200	Biolegend
	CD4	PE/Cy7	RM4-5	1:200	Biolegend
	NK1.1	APC	PK136	1:200	Biolegend
	B220	APC/Cy7	RA3-6B2	1:200	Biolegend

2.3.1.7 Flow cytometry analysis

Prior to any analysis of flow cytometry experiments the following strategy was used to identify the live cells within the sample. As shown in Figure 2-2, this strategy started by gating the cells of interest using FSC and SSC. As the flow cytometer registers signals caused by debris and micro-particles present in the sample, a threshold of 50.000 was applied in the FSC, which allowed a better identification of the population. Doublets where excluded using SSC-H and SSC-A while live cells were gated based on exclusion of a live/dead cell marker. The exclusion of dead cells is essential as dead cells produce false positives as they have increased autofluorescence levels and higher non–specific binding of antibodies. Analysis of the data was performed using version 10 of the FlowJo software.

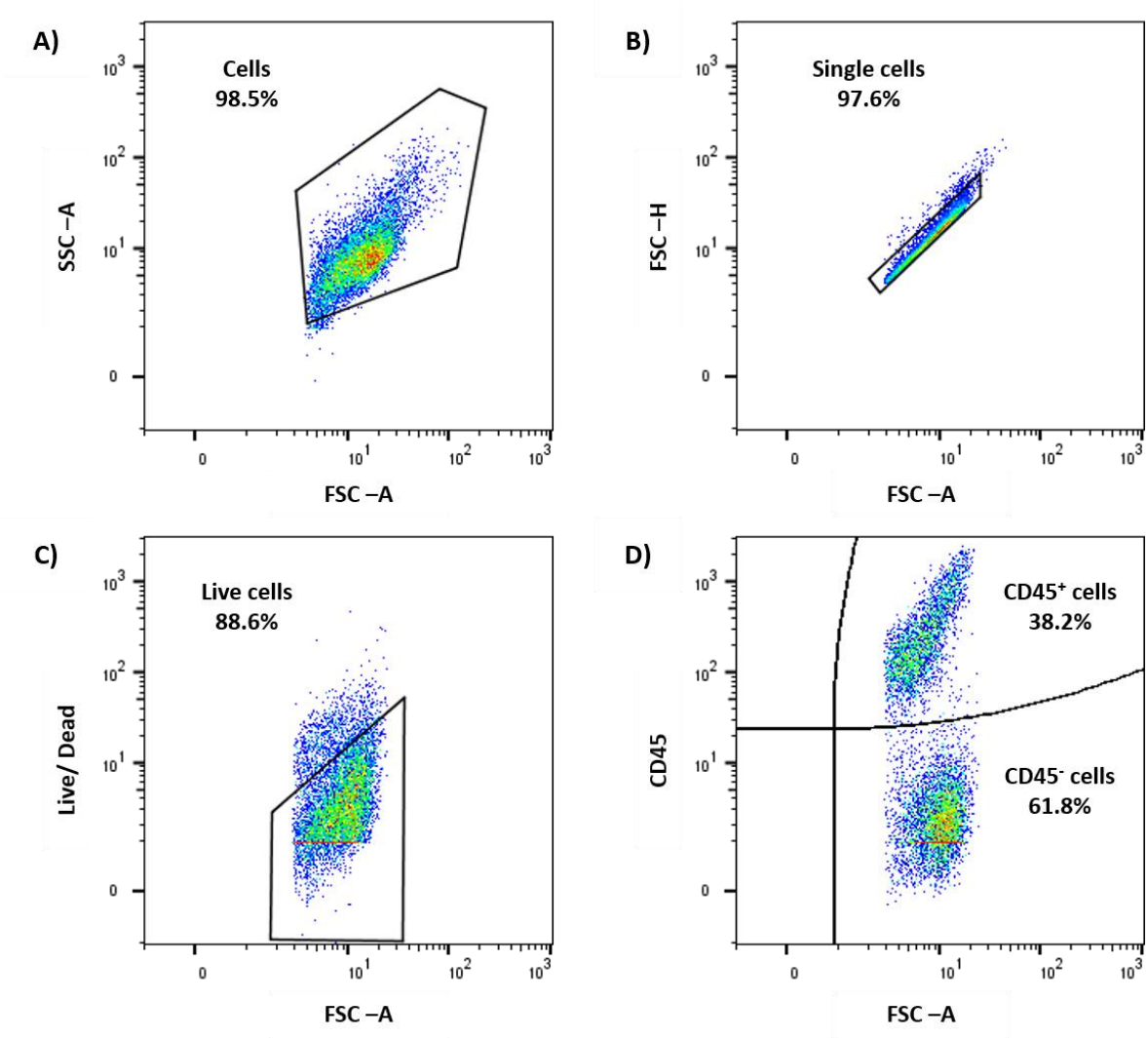


Figure 2-2. Flow cytometry basic cell gating strategy.
All the flow cytometry experiments were analysed following this initial gating strategy to identify single live cells. **A)** The first step was focused on identifying cells with the appropriate size and granularity, **B)** followed by single cells gating to avoid clumps of cells. **C)** Due to the autofluorescence of death cells, a live dead marker was used to identify non-stained, and therefore, live cells. **D)** Cells were then gated for further analysis. In this specific example, life cells were classified into CD45 positive and negative.

Multicolour flow cytometry panels can lead to fluorescence spread from the lasers into different channels. In order to identify a positive from a negative population we used a fluorescence minus one controls, which are samples that contain all the antibodies present in the panel minus one. For this reason, FMO controls were prepared for each fluorophore present in the panel. In this way, FMO controls behave as negative controls, letting the user know how the other fluorophores in the panel affect each channel, allowing a clear distinction between negative and positive staining as shown in Figure 2-3.

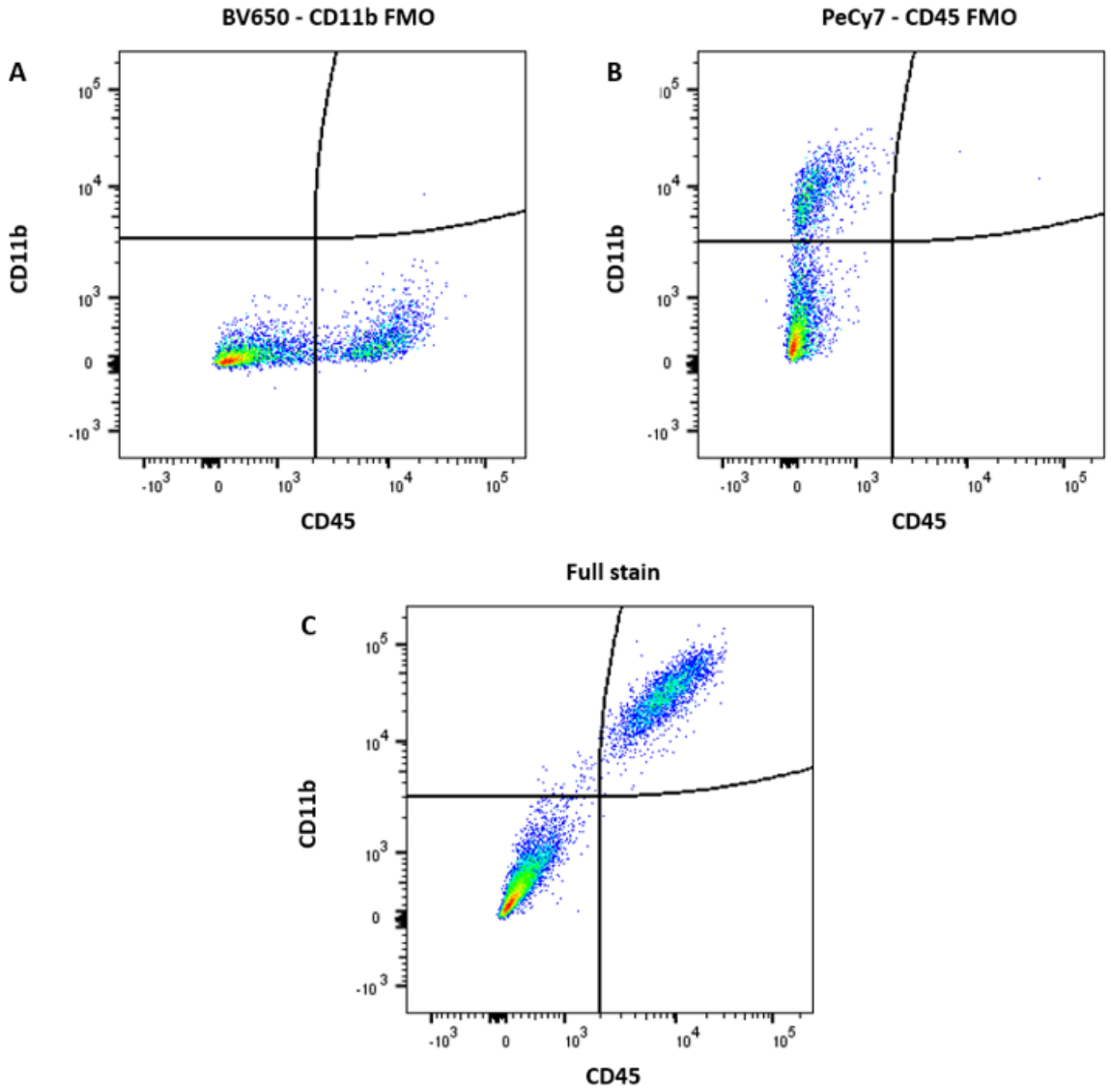


Figure 2-3. Use of Fluorescence Minus One (FMO) controls to accurately identify positive and negative populations.
A) BV650-CD11b FMO contains all the fluorochromes in the panel but BV650, the one corresponding to CD11b, to allow the identification of the fluorescence spread from the different lasers into BV650 channel. **B)** PeCy7-CD45 FMO contains all the fluorochromes in the panel but PeCy7, the one corresponding to CD45, to allow the identification of the fluorescence spread from the different lasers into PeCy7 channel. **C)** Use of FMOs allowed the accurate identification of positive and negative populations in full stained samples.

2.3.2 Immunocytochemistry

2.3.2.1 Immunocytochemistry for adipocytes and osteocytes

MSCs that had undergone adipogenic and osteogenic differentiation were grown and treated as described in Section 2.1.4.1. MSC differentiation medium was aspirated and cells were washed twice with 1 mL of PBS and then fixed with 0.5 mL of 4% paraformaldehyde in PBS for 20 mins at RT. Cells were washed three

times with 0.5 mL of 1% bovine serum albumin (BSA) (Thermo Fisher Scientific) in PBS for roughly 5 mins each wash. The cells were permeabilised and blocked with 0.5 mL of 0.3% Triton X-100 (Thermo Fisher Scientific), 1% BSA and 10% normal donkey serum (Sigma) in PBS for 45 mins. After blocking, cells were incubated with 300 µL/ well of the appropriate primary antibody (Table 2-9). Cells were left at 4 ºC overnight. A negative control was run using PBS containing 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum with no primary antibody and another negative control was run using PBS containing 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum with an isotype. After the incubation, cells were washed three times with 0.5 mL of 1% BSA in PBS for 5 mins each wash. Cells were then incubated with a diluted appropriate secondary antibody (Table 2-9) for 60 mins, in the dark, at room temperature. Cells were washed three times with 0.5 mL of 1% BSA in PBS for 5 mins each wash.

For nuclear visualization during fluorescent imaging PBS was removed, chambers were removed from the slide and one drop of ProLong Gold Antifade Mounting solution with DAPI (Thermo Fisher Scientific) was added to each well. Slides were sealed using DPX Mounting Media & Section Adhesive and slides were covered with 22 x 50 mm cover slips (Academy). All slides were imaged with a Zeiss epifluorescence microscope using the appropriate fluorescence channels and magnifications. Images were prepared using Zen software.

2.3.2.2 Immunocytochemistry for chondrocytes

The pellet of cells was washed twice with 1 mL of PBS, then fixed with 0.5 mL of Zinc formalin solution overnight at 4 ºC. The pellet was then washed twice with 1 mL PBS for 5 mins. The pellet was carefully removed and placed into a cryomould. Cryosectioning was carried out as detailed in Section 2.3.2.2.1. Using a liquid barrier pen, a hydrophobic barrier was drawn around each section and cells were then blocked and permeabilised with 0.15 mL of 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum in PBS at room temperature for 45 minutes. After blocking, sections were incubated with the appropriate primary antibody (Table 2-9) working solution overnight at 4 ºC. A negative control was run using PBS containing 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum with no primary antibody and another negative control was run using PBS containing 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum with an isotype control

antibody. Sections were washed three times with PBS containing 1% BSA for 5 mins. Sections were then incubated in the dark for an hour with an appropriate secondary antibody (Table 2-9). Sections were washed three times with PBS containing 1% BSA for 5 mins. Sections were then washed once with distilled water and excess water was removed.

For nuclear visualization during fluorescent imaging PBS was removed, chambers were removed from the slide and one drop of ProLong Gold Antifade Mounting solution with DAPI was added to each well. Slides were sealed using DPX Mounting Media & Section Adhesive and slides were covered with 22 x 50 mm cover slips. All slides were imaged with a Zeiss epifluorescence microscope using the appropriate fluorescence channels and magnifications. Images were prepared using Zen software.

2.3.2.2.1 Cryosectioning

Cells were placed in a small cryomould, OCT compound (Tissue-Tek) was gently poured on top, avoiding any air bubbles, samples were then snap frozen in liquid nitrogen and placed in the -80 ^{0}C freezer until sectioning. Frozen moulds were placed into the cryostat (Bright Instruments) at -25 ^{0}C and sectioned 8 µm thick onto superfrost glass slides.

2.3.2.3 Immunocytochemistry for islets and islet derived MSCs

10-20 islets per chamber or 3500 cells/cm^{2}were seeded in a 4-well Nunc™ Lab-Tek™ Chamber Slide™ (Thermo Fisher Scientific) and cells were allowed to grow for 7-10 days. Wells were then washed three times with 0.5 mL of room temperature PBS. 0.5 mL of 4% paraformaldehyde in PBS was added to each well and was left for incubation for 20 minutes at room temperature. 4% paraformaldehyde was then removed, and wells were washed three times with 0.5 mL of PBS for 5 minutes with agitation. 0.5 mL of permeabilization and blocking buffer (1% BSA, 10% goat serum and 10% Triton x100 in DPBS) were added per well to block non-specific antibody binding and was incubated for 1 hour at room temperature with shaking.

Primary antibody was diluted into the permeabilization and blocking buffer according to the manufacturer's instructions; 1:100 for EpCAM monoclonal

antibody and 1:200 for Vimentin monoclonal antibody. The blocking buffer was removed from the 24 well plate and 200 µL of the diluted antibodies were added into each well and left incubating at 4 ^{0}C overnight or for 1 hour at room temperature. After the incubation, wells were washed three times with 0.5 mL of room temperature PBS for 5 minutes with shaking. Secondary antibody was diluted into the permeabilization and blocking buffer according to the manufacturer's instructions (1:400). 200 µL of the diluted secondary antibodies were added into each well and was left incubating for 1 hour at room temperature protecting the plate from the light. After the incubation, wells were washed three times with 0.5 mL of room temperature PBS for 5 minutes with shaking while protecting the plate from the light. All the antibodies used for immunocytochemistry are listed in Table 2-9.

For nuclear visualization during fluorescent imaging, PBS was removed, chambers were removed from the slide and one drop of ProLong Gold Antifade Mounting solution with DAPI was added to each well. Slides were sealed using DPX Mounting Media & Section Adhesive and slides were covered with 22 x 50 mm cover slips. All slides were imaged with a Zeiss epifluorescence microscope using the appropriate fluorescence channels and magnifications. Images were prepared using Zen software.

Table 2-9. List of antibodies used for immunocytochemistry in this study.

Antigen	Host/ Isotype	Fluorophore	Clone	Supplier
EpCAM	Mouse/ IgG1	Unconjugated	323/A3	Invitrogen
Vimentin	Mouse/ IgM	Unconjugated	J144	Invitrogen
Mouse IgG1	Mouse/ IgG1	Unconjugated	P3.6.2.8.1	Invitrogen
Mouse IgM	Mouse/ IgM	Unconjugated	11E10	Invitrogen
Mouse IgG1	Goat / IgG	AF 488	Polyclonal	Invitrogen
Mouse IgM	Goat / IgG	AF 568	Polyclonal	Invitrogen
FABP4	Goat/ IgG	Unconjugated	Polyclonal	R&D Systems
Collagen II	Sheep/ IgG	Unconjugated	Polyclonal	R&D Systems
Osteopontin	Goat/ IgG	Unconjugated	Polyclonal	R&D Systems
Goat IgG	Goat/ IgG	Unconjugated	Polyclonal	Invitrogen
Sheep IgG	Sheep/ IgG	Unconjugated	Polyclonal	Invitrogen
Goat IgG	Donkey/ IgG	NL557	Polyclonal	R&D Systems
Sheep IgG	Donkey/ IgG	NL558	Polyclonal	R&D Systems

2.3.3 Western Blotting

2.3.3.1 Buffers and reagents for Western Blotting

Table 2-10. List of buffers and reagents for Western Blotting and their composition

Solution	Composition	Concentration
RIPA buffer	Tris-HCl 1M pH 7.4 NP-40 Sodium deoxycholate SDS NaCl EDTA Sodium fluoride	50 mM 1% (v/v) 0.5% (w/v) 0.1% (v/v) 150 mM 2 mM 50 mM
10x SDS running buffer	TRISBase Glycine SDS	0.25 M 2 M 0.035 M
Blocking solution	Milk Powder Tween 20 in PBS	10% (w/v) 0.1% (v/v)
10x TG	TRIS Glycine	0.48 M 0.39 M
Transfer buffer	10x TG MeOH SDS 20%	10% (v/v) 20% (v/v) 0.19% (v/v)

Molecular weight of proteins was assessed with the use of PageRuler™ Prestained Protein Ladder, 10 to 180 kDa (Thermo Fisher Scientific).

2.3.3.2 Sample preparation

To isolate splenocytes, mice aged 10 weeks were killed with CO_2 or cervical dislocation and the cadaver was laid with the abdominal side facing up, the limbs were stretched and fixed with pins and then the skin was sterilised using 70% ethanol. A midline incision was made around the abdomen and the skin was retracted using straight tweezers. The muscular wall was then opened to expose the liver and intestines. The liver was then pushed up to the thoracic cavity and the intestines and stomach were pushed to the left, which enables access to the spleen. Spleen was then harvested and placed in 1% BSA RPMI 1640 medium (Thermo Fisher Scientific). To isolate splenocytes, the spleen was transferred into a 70 μm cell strainer and was mashed with the plunger from a 2 mL syringe. Grinding circular movements were used to homogenise the tissue. 20 mL of PBS were used to wash out the cells from within the strainer. The homogenised cell

suspension was then centrifuged at 300 x g for 10 minutes at room temperature and the supernatant was discarded. Cells were then resuspended into 10 mL PBS and passed through a 40 µm cell strainer and centrifuged at 300 x g for 10 minutes at room temperature. 1mL per sample of ACK lysing buffer (Thermo Fisher Scientific) was used to remove the red blood cells from the spleen. Samples were incubated at room temperature for 1 minute and were then diluted in 20 mL of PBS followed by centrifugation at 300 x g for 10 minutes at room temperature. The supernatant was removed and discarded and the cell pellet was resuspended in 1 mL of PBS and the suspensions were transferred into microcentrifuge tubes and centrifuged for 2 minutes at 1000 rpm. The supernatant was removed and discarded.

MSCs and 3T3 MEF cells were harvested as detailed in Section 2.1.1.

2.3.3.3 Sample lysis

Cells were lysed using radioimmunoprecipitation assay (RIPA) lysis buffer containing leupeptin (2 µg/mL), aprotinin (2 µg/mL), Na_3VO_4 (1mM) and pefabloc (0.5 mM) leaving them on ice for 30 minutes and centrifuged for 15 minutes at 13,000 g at 4 ^{0}C. The supernatant was then recovered and protein concentrations were determined using the Thermo Scientific Pierce ™ BCA Protein Assay Kit (Thermo Fisher Scientific). SDS to 1x was added and samples were boiled at 95 ^{0}C for 5 minutes.

2.3.3.4 Western Blot

Samples were boiled in loading buffer and separated on a 10% SDS-PAGE at 80V until the samples were inside the gel and then the intensity was increased to 120V till the blue front of the gel disappeared. 2.5 µL of protein ladder was loaded as a molecular weight reference. Proteins were transferred to a Polyvinylidene Difluoride (PVDF) membrane (Amersham Pharmacia Biotech) using a wet blotter at 25 V. Membranes were then blocked for 1 hour with blocking solution shaking at room temperature, followed by incubation on a rocking platform at 4 ^{0}C with primary antibodies overnight. All the antibodies used in this study are listed in Table 2-11. After 3 washes with TBST 1x, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for

1 h. Antigen-antibody complexes were detected by using Clarity Western ECL Substrate solution (BioRad) and images acquired using darkroom development techniques for chemiluminescence.

Table 2-11. List of antibodies used for Western Blot in this study.

Antibody	Host	Dilution in 5% milk TBST	Product reference
Anti-CD45	Rat	1:1000	BioLegend (103101)
Anti-β-actin	Mouse	1:5000	Sigma Aldrich **(**SAB1305567)
Anti-mouse	Mouse	1:5000	Sigma Aldrich (GENXA931-1ML)
Anti-rat	Goat	1:5000	BioLegend (405405)

2.3.4 Luminex

Conditioned media from the samples used for transcript work were collected for Luminex analysis. The mouse personalised premixed magnetic multi-analyte kit was used in accordance with the manufacturer's instructions (R&D systems). All reagents and standards were included in the kit and prepared as outlined in the guidelines. Briefly, samples were diluted 1:2 with calibrator diluent (75µl in 75 µL). 10 µL of the pre-coated microparticle cocktail was added to each well of the 96 well microplates, followed by either 50 µL sample or 50 µL standard, sealed and placed on an orbital shaker (0.12 mm orbit at 800 ± 50 rpm) for 2 hours at room temperature. The plates were washed twice with 100 µL/ well wash buffer and then incubated with 50 µL/ well anti-biotin detector antibody for 1 hour at RT on the shaker (0.12 mm orbit at 800 ± 50 rpm). The plates were washed as previously described and 50 µl/ well of streptavidin-phycoerythrin was added and incubated for 30 minutes at room temperature. Microparticles were resuspended in 100 µL/ well of wash buffer and immediately read on a Bio-Rad analyser.

2.3.4.1 Analysis of results

The Luminex analysis was acquired on a Luminex 100 Bio-Rad instrument. Each microparticle bead region was designated as stated on the certificate of analysis. When beads are injected into the flow cell, a small number can aggregate and go through as doublets. To avoid this, the doublet discriminator channel measures the amount of light scatter from the particles that flow past the laser and specific gates were set between 8000 and 16,500 to ensure that

only beads of the correct size were measured. Mean Fluorescence intensity (MFI) was acquired. Total protein concentrations were determined using the Thermo Scientific Pierce ™ BCA Protein Assay Kit (Thermo Fisher Scientific) and were used for normalization of the data. Each bar represents an n of 3 and is plotted as mean ± SEM. Statistical tests included One Way ANOVA in conjunction with a Tukey's compare all comparisons test when comparing across MSCs from different sources. Students T test was used when comparing stimulated vs unstimulated within one tissue source.

2.4 *In Vivo* Procedures

2.4.1 Animal Welfare

All animals were housed within the Biological Central Research Facility. All experiments received ethical approval and were performed under the auspices of a UK Home Office License.

Mice used during this study were C57BL/6, which have fully functional innate and adaptive compartments of the immune system. 6–week old mice were obtained from Charles River Europe and before any procedure was carried out, mice were given 7 days within the Biological Central Research Facility for adjustment and settling. After experimental procedures, mice were euthanised using a recognised Schedule 1 technique.

2.4.2 Murine Air pouch Model

2.4.2.1 Induction and maintenance of the air pouch model

C57BL/6 female mice were put under general anaesthesia, inhaled isoflurane, before and during the procedure. To ensure that the air injected into the air pouch was sterile, the syringes were prepared in a sterile laminar flow hood. 3mL of sterile air was injected subcutaneously into the intracapsular area of the mouse to create an air pouch. After 3 days, a top-up of 3 mL sterile air was injected into the air pouch. A third top up of 1 mL sterile air was injected 2 days later, followed by an injection of $1x10^6$ resting or stimulated MSCs in 1 mL of

sterile PBS or sterile PBS alone (control animals) 24 hours later. Cells or PBS controls were left in the air pouch for 24 hours before mice were sacrificed.

2.4.2.2 Fluorescent labelling of MSCs

In vivo tracking studies require specialised probes that are nontoxic to living cells and do not alter the immune response if introduced into a host. Cell Tracker Green CMFDA (Invitrogen) was used to fluorescently label the cells. To stain the cells, culture media was removed and 5 μM warm Cell Tracker Working Solution was added and left incubating for 30 minutes at 37 ºC/ 5% CO_2/ 95% humidity. Cell Tracker Working Solution was prepared according to the manufacturer's instructions. Briefly, the lyophilised product was dissolved in DMSO to a final concentration of 10 mM, which was further diluted in serum free medium. After incubation with the Cell Tracker, the Cell Tracker Working Solution was removed, and cells were washed twice with PBS prior to injecting them into mice.

2.4.2.3 Dissection and preparation of the air pouch samples

Immediately after sacrifice, 3 mL of FACS buffer was injected into the air pouches of the mice and mice were gently shaken to allow the FACS buffer to mix throughout the air pouch to ensure an optimal retrieval of immune cells. The air pouch content was then drained and kept on ice until immunofluorescence staining was performed as outlined in Section 2.3.1.6.

To separate the overlying soft tissues from the air pouch membrane, a small incision was made into the dorsal skin overlying the air pouch to reveal the membrane, which was separated from the overlying skin. The membrane was then placed into 1 mL of Hank's balanced salt solution (Sigma) and kept on ice. To digest the membrane, 87.5 μg of liberase (Sigma) was added to each sample and was left incubating at 37 ºC for 1 hour on a thermo–shaker incubator at 900 rpm. Once membranes were fully digested, cells were passed through a 70 μm sterile cell strainer (Corning) to remove any remaining debris and create a single cell suspension. Cells were then washed twice in PBS and immunofluorescence staining was performed as explained in Section 2.3.1.6.

Chapter 3

Phenotyping MSCs

3 Phenotyping MSCs

3.1 Introduction and aims

Stem cells are cells which can both self–renew and differentiate into different multiple lineages depending on the potency of the cell. Based on their origin and their differentiation potential, stem cells can be divided into different categories such as embryonic stem cells, induced pluripotent stem cells or mesenchymal stromal cells. Stability, safety and a highly accessible stem cell source are requirements for the use of stem cells as cellular therapeutics for future clinical applications. The crucial limitation of stem cell graft survival is recognition by the recipient of alloantigen. However, MSCs avoid allogenic rejection due to the low levels of MHC Class I expression and the lack of MHC Class II and co-stimulatory molecules expression such as CD80, CD40 and CD86. This characteristic, in addition to their ability to self–renew and differentiate, has risen the biological and clinical interest of MSCs (Horwitz et al., 2005).

MSCs were first isolated from the bone marrow but they can be isolated from a wide range of tissues around the body including umbilical cord, cord blood, placenta, Wharton's jelly, dental pulp, periodontal ligament, adipose tissue and pancreas among others (Lv et al., 2014). However, tissue source of origin directs phenotype and biological activity. For example, differences in the expression of surface markers CD49d, CD54, CD34 and CD106 have been identified between bone marrow derived MSCs (BM MSCs) and adipose tissue derived MSCs (Ad MSCs) (De Ugarte et al., 2003). Regarding their differentiation potential, BM MSCs have been described to possess stronger osteogenic and lower adipogenic differentiation potential compared to Ad MSCs (Xu et al., 2017), while the ability to trans-differentiate to neural precursors is higher in Ad MSCs compared to bone marrow, skin and umbilical cord derived MSCs (Urrutia et al., 2019). The in vitro expansion capacity of cells is important for cell therapy and tissue origin has been described to have an impact on this, as umbilical cord derived MSCs have a higher proliferation capacity than BM MSCs (Baksh et al., 2004). Discrepancies in findings between studies are not uncommon within the MSC literature. As an example, the immunosuppressive capacity was measured by their ability to inhibit the activation and proliferation of T cells in two different studies (Ribeiro et al., 2013, Xishan et al., 2013) and Ribeiro et al. stated that Ad MSCs have

greater immunosuppressive ability compared to BM MSCs while Xishan et al. stated the opposite.

Due to different isolation/ enrichment protocols, culture conditions and the intrinsic variability among MSCs derived from different donors/ tissues the characteristics of MSCs differ, leading to controversial results through the literature. For this reason, with the aim of making studies easier to compare, the International Society for Cell Therapy (ISCT) proposed minimal criteria for defining human MSCs. These criteria include adherence to plastic under standard culture conditions; surface expression of the "stemness" markers CD105, CD73 and CD90 with lack of expression of CD45, CD11b, CD34 and MHC Class II; and trilineage mesenchymal differentiation into adipocytes, chondrocytes and osteocytes (Dominici et al., 2006). Conversely, although adherence and trilineage differentiation are characteristics of murine MSCs, the surface marker phenotype is not so well characterised, and variations might apply.

This chapter will focus on assessing the cells isolated from the bone marrow, islets of Langerhans and adipose tissue of mice to compare their phenotypes to ISCT criteria for human MSCs. Therefore, their morphology, surface molecule phenotype and differentiation potential were assessed in a standardised manner. The overall aim of this study was to determine the potential roles of MSCs within clinical settings, which are often inflammatory settings. For this reason, the morphology and surface molecule phenotype of MSCs from the three sources were assessed after 24 hours of inflammatory stimulation with a cocktail of inflammatory mediators containing a combination of IFN-γ, TNF-α and IL-1ß, LPS, LTA or Poly I:C. Therefore, the aim of this chapter was to fully characterise cells isolated from the bone marrow, adipose tissue and islets of Langerhans for MSC criteria and to understand the effect of tissue origin and inflammation on their phenotype. For this results chapter, and the ones that follow, a colour code has been used for all data sets, which is outlined in Figure 3-1.

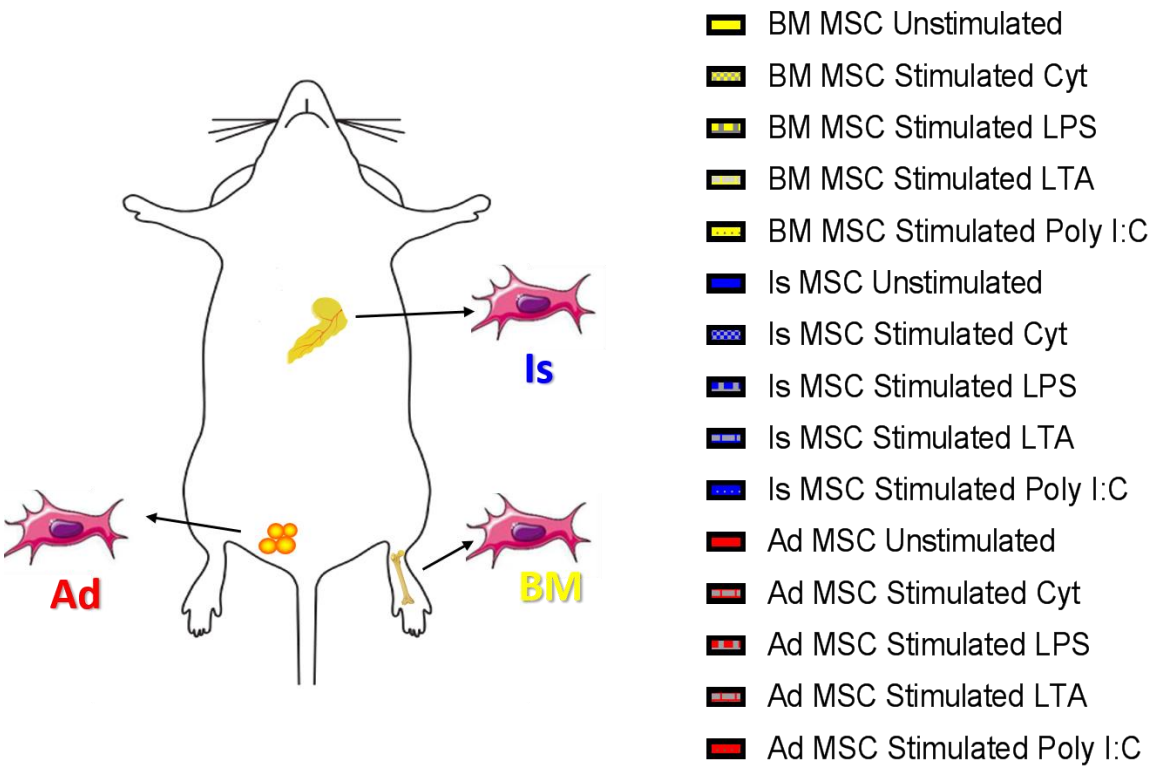


Figure 3-1. Schematic representation of the colour scheme used in all figures throughout this study.
The above diagram illustrates the colour scheme throughout this thesis, linking a specific tissue source of MSCs to a particular colour. When data are graphed, BM MSCs will always be represented in yellow, Is MSCs in blue and Ad MSCs in red. Under resting conditions data will be graphed as bars without a filling pattern, while different filling patterns will be used if cells have been under inflammatory conditions. Cytokine-mediated stimulation of cells will be represented by a grey checks filling; while LPS-, LTA- and Poly I:C-mediated stimulation of cells will be represented by grey squares, grey bricks and grey dots respectively.

Results

3.2 Physical morphology of MSCs

To assess the plastic adherence criteria established by the ISCT, after isolation of the cells from the bone marrow and adipose tissue, cells were seeded into 100 mm sterile Petri dishes and 5 days later were transferred into culture flasks and left to grow. Islets of Langerhans were directly plated in culture flasks. In all cases, once cells were seeded into culture flasks it was considered passage 1 (P1). Cells were grown up to passage 3 and their morphology was observed and monitored using a light-phase microscope. At P1, cells isolated from the bone marrow and adipose tissues showed a spindle-shaped morphology that was maintained through passage. However, cells grown from the islets of Langerhans

showed a cluster of small spherical cells where the islet was seeded, surrounded by spindle-shaped cells and transitioning cells in between these two populations with different morphologies. However, at passage 2 and 3 the only cells present in the flask showed a spindle–like morphology. It is unclear whether the small spherical cells were an epithelial population unable to proliferate or more likely, if an epithelial to mesenchymal transition was leading to the generation of MSCs. As shown in Figure 3-2, cells from all sources (excluding P1 in the cells isolated from the islets of Langerhans) were similar in size and shape. This satisfies the ISCT criteria of MSCs being plastic-adherent and spindle-shaped cells in standard culture conditions.

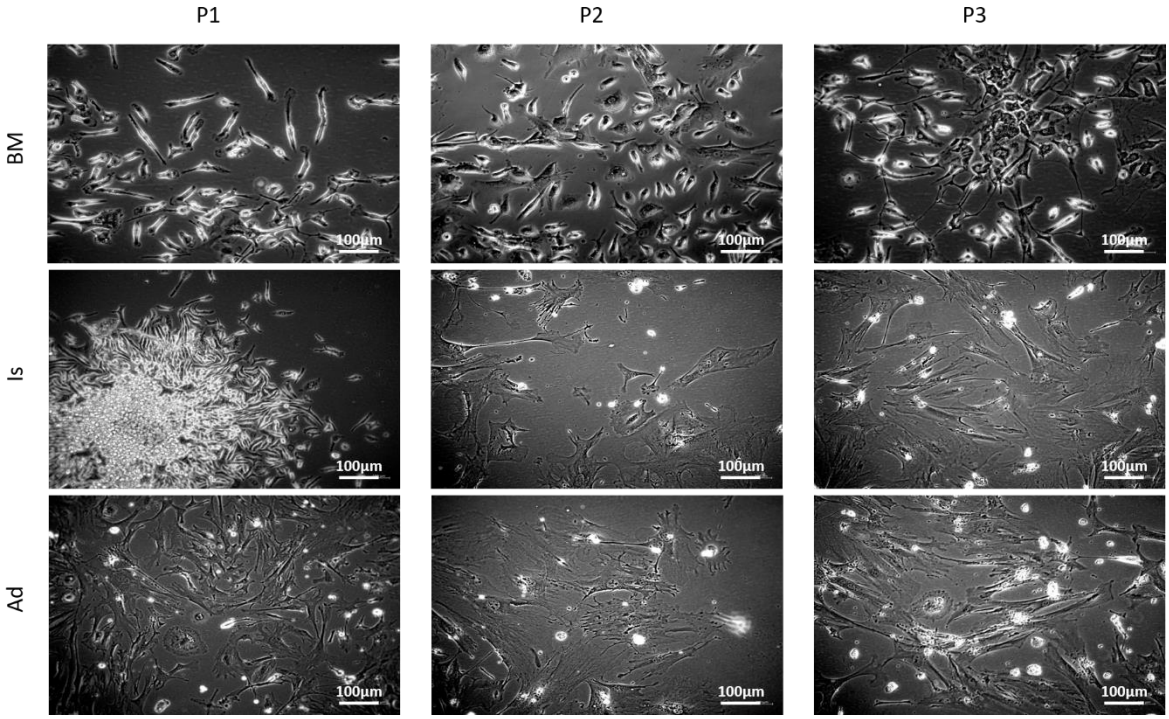


Figure 3-2. MSCs isolated from all tissue sources exhibit an MSC spindle-like morphology. MSCs were isolated from mice and grown until P3. Their morphology was observed and monitored using a light-phase microscope. All MSCs were adherent to plastic and possessed the typical spindle-like morphology.

3.3 Surface molecule phenotype of MSCs

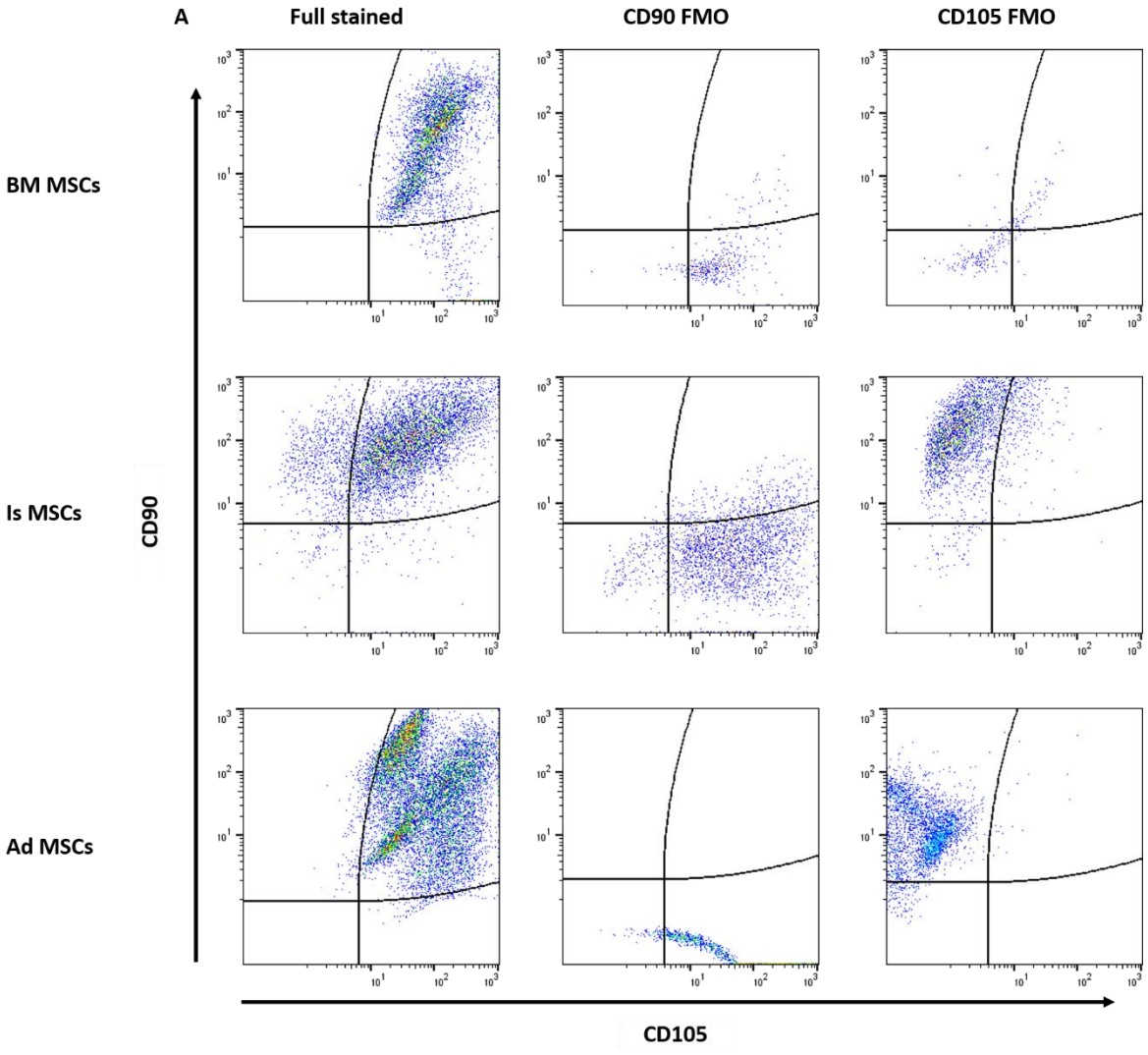
Flow cytometry was used to assess the surface phenotype of the cells isolated from the BM, Ad and Is at P3. The cocktail of antibodies used included the markers established by the ISCT to determine if cells are MSCs: CD90, CD105, CD73, MHC Class II, CD45, CD11b, CD19 and CD34. CD64 was used to further exclude monocytes/ macrophages, the most likely haematopoietic cell to be

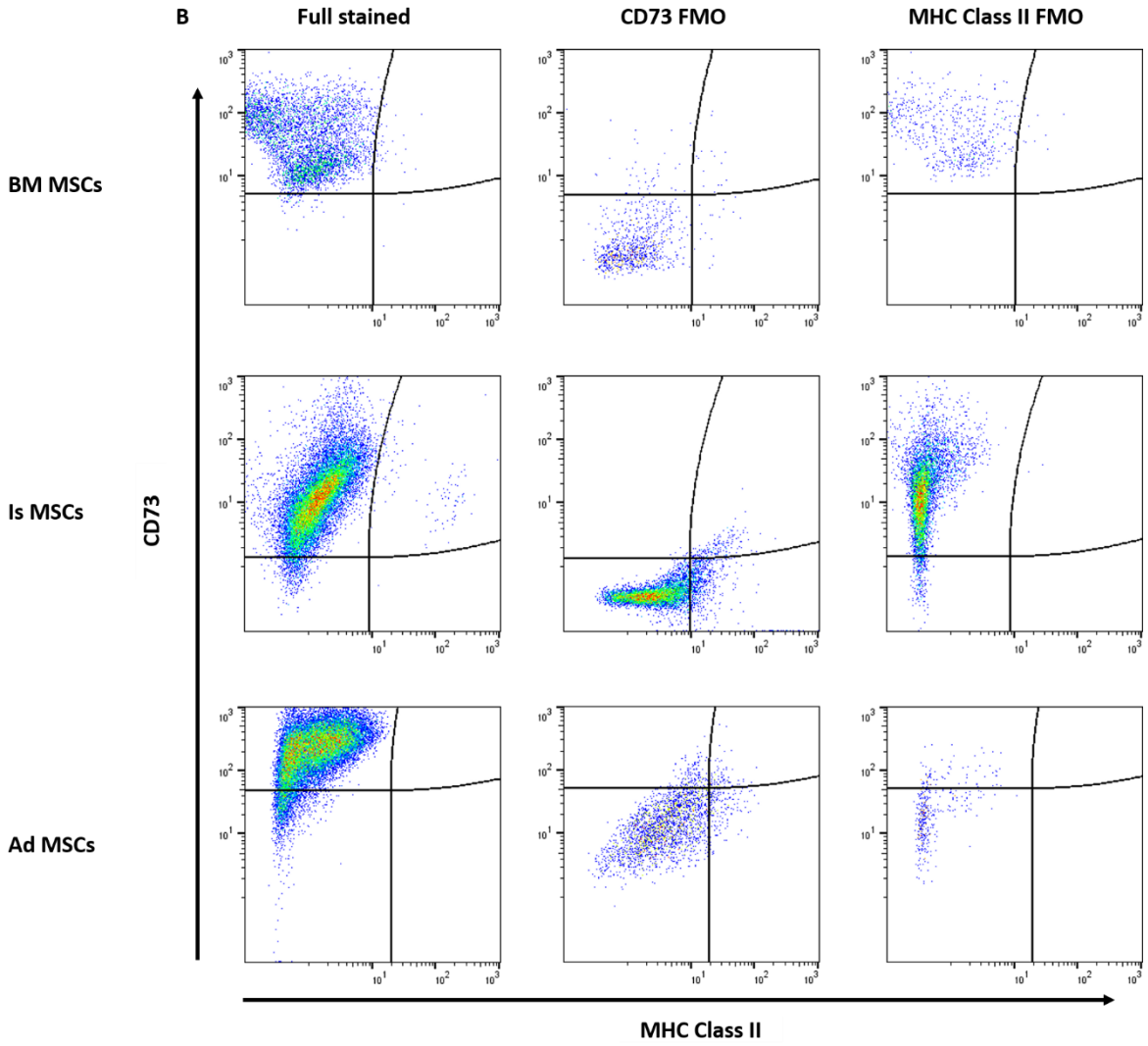
found in an MSC culture. Due to the lack of standardised protocols to isolate murine MSCs from these tissues, successful isolation of the cells from the different sources was obtained at different stages of the project and therefore, were analysed when available. For this reason, different controls, voltage and compensation settings were used for the analysis of the cells isolated from each tissue, which explains the gating differences among tissues. However, in all the cases, fluorescence minus one (FMO) controls allowed the accurate identification of positive and negative populations in full stained samples.

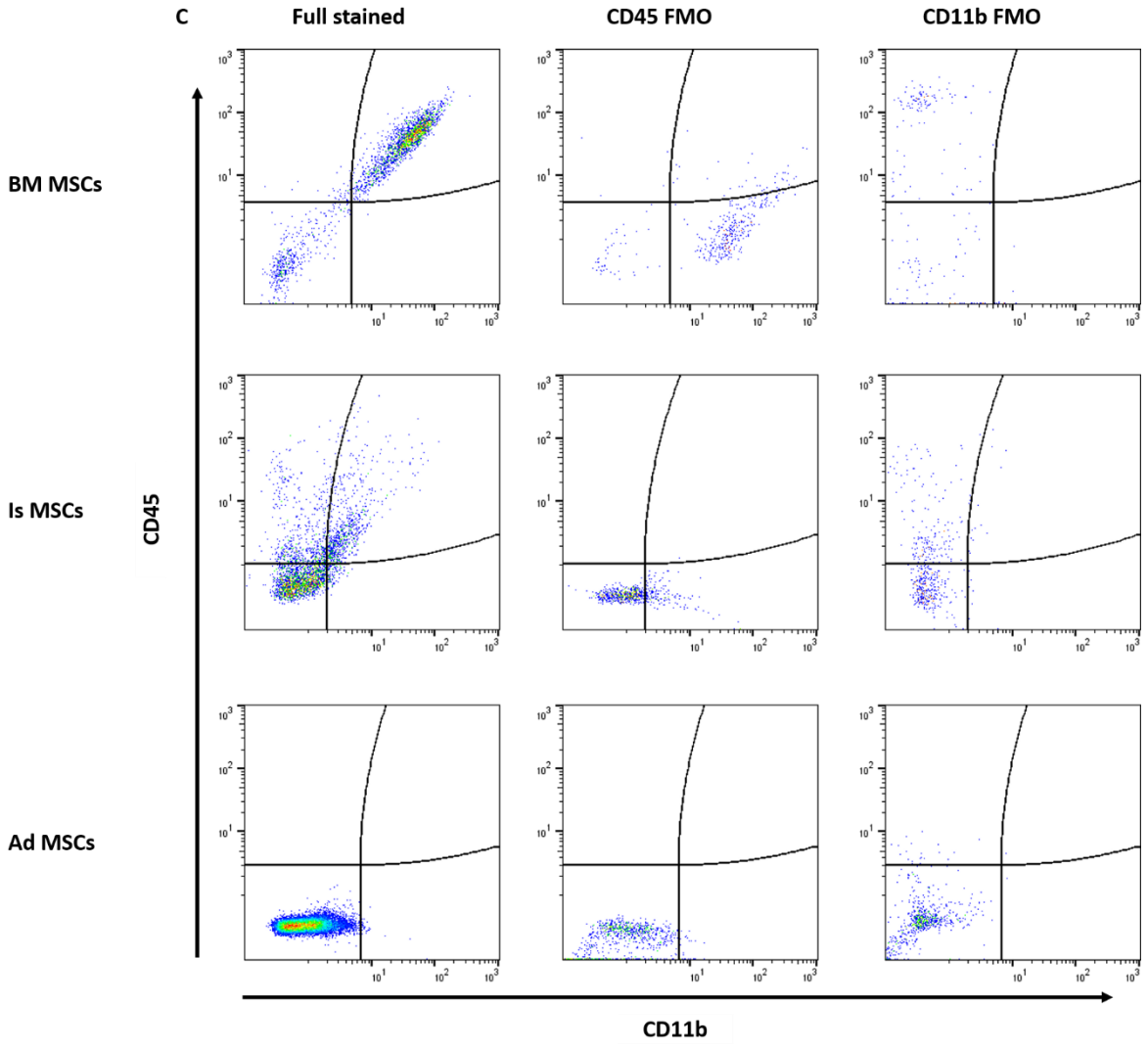
As shown in Figure 3-3 A and B, cells isolated from all three tissues are positive for the MSC markers CD90, CD105 and CD73. The percentage of live cells positive for each marker is graphed (Figure 3-3, F-N) to assess the effect on the tissue source on the surface phenotype of these cells. ~100% of the cells were positive for the MSC markers regardless of the tissue of origin. CD90, a glycophosphatidylinositol anchored conserved cell surface protein also known as Thy–1 cell surface antigen, was positively expressed by ~100% of the cells irrespectively of the tissue of origin (F). CD105, a type 1 membrane glycoprotein also known as endoglin, was also expressed by >98% of the cells with no significant differences in the percentage of positive cells when compared among tissue source (G). Similarly, CD73, an enzyme that converts adenosine monophosphate to adenosine, also known as ecto-5'-nucleotidase, was also very highly expressed (>98%) by cells from every tissue source (H).

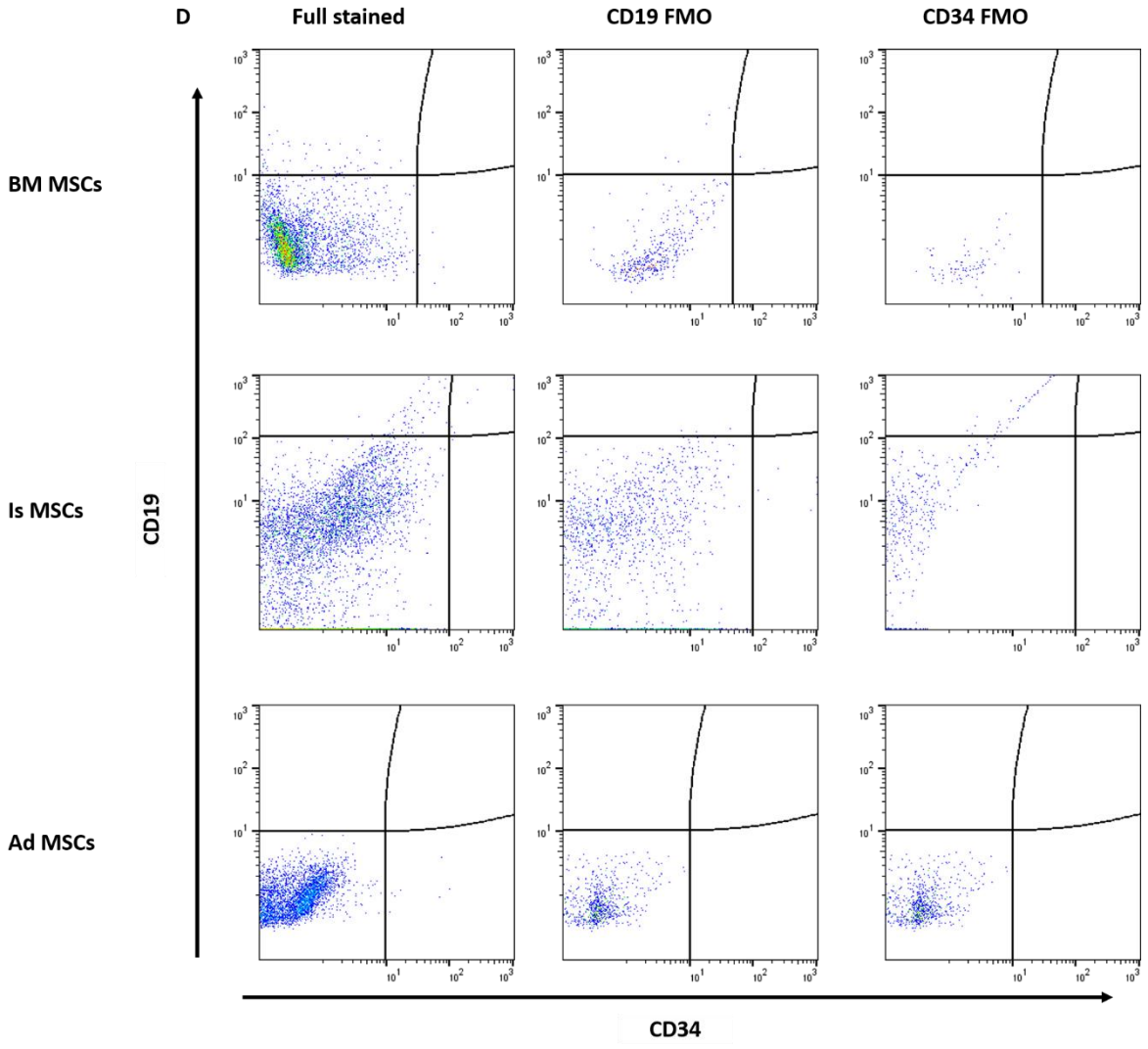
According to the ISCT, MSCs must not only express specific surface markers but have to lack the expression of others. All the cells were negative for the MHC Class II surface marker regardless of the tissue of origin (Figure 3-3, I). The percentage of live cells positive for the haematopoietic marker CD45 was <2% for cells isolated from the Ad, while it was ~30% for the cells isolated from the islets of Langerhans and ~85% for the cells isolated from the bone marrow (J). These numbers correlate quite nicely with the percentage of cells expressing the integrin CD11b as it was barely expressed by the cells isolated from the adipose tissue (<2%) while ~25% for the cells isolated from the islets of Langerhans and ~85% for the cells isolated from the bone marrow expressed it (K). Regarding the expression of the B-lymphocyte antigen CD19, all the cells were negative (<2%) for this marker regardless of the tissue of origin (L). Similarly, <2% of the cells

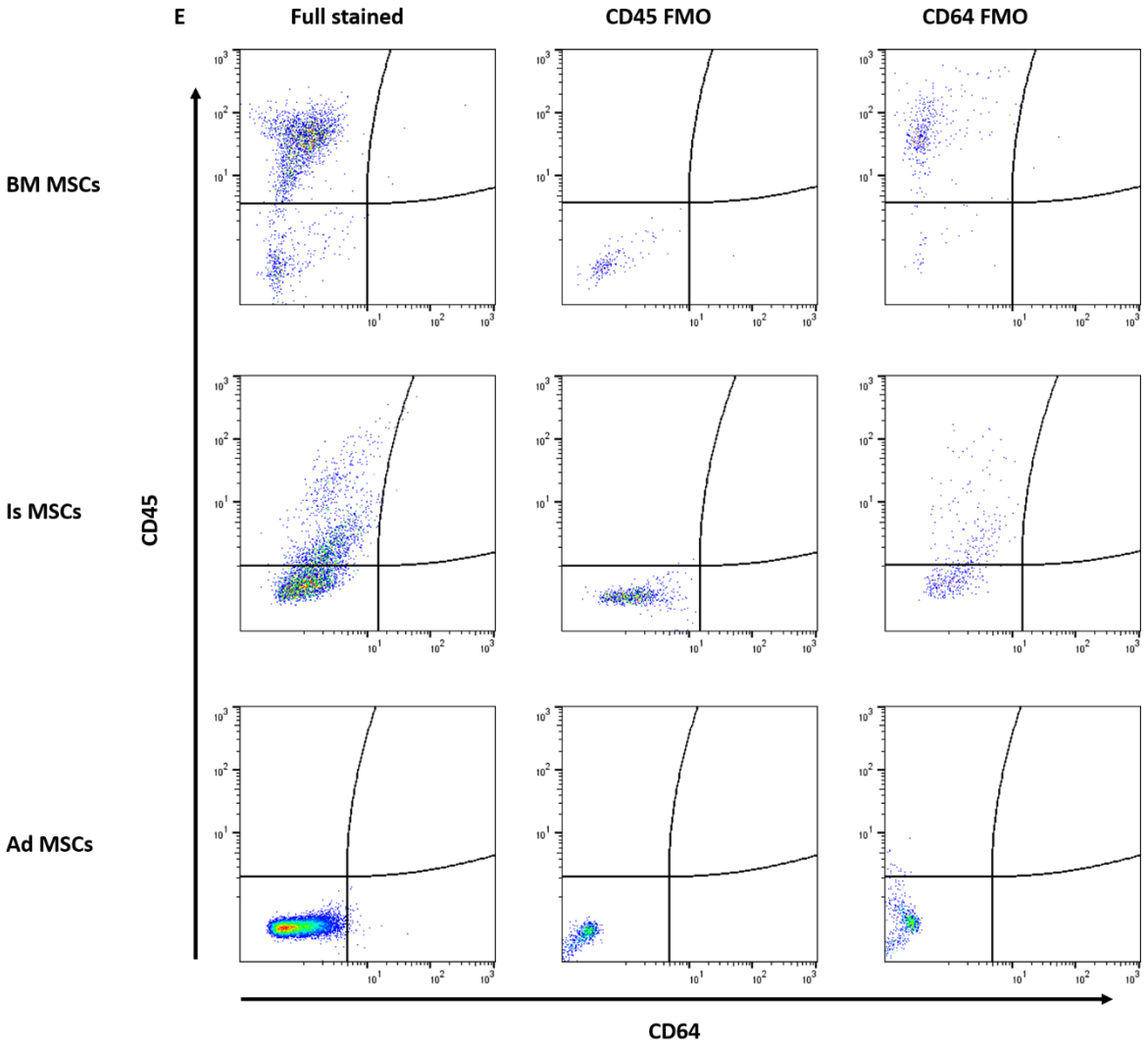
from the three tissue sources stained positive for the primitive haematopoietic progenitor marker CD34 (M). Due to the positive staining for CD45 and CD11b surface markers, cells from the three tissues were further characterised for the expression of CD64, an integral membrane glycoprotein that is constitutively found on macrophages and monocytes, and it was observed that the expression of this marker was minimal in the cells isolated from the three sources (N), suggesting that the isolated cells were not of myeloid origin.




B
Full stained
CD73 FMO
MHC Class II FMO
BM MSCs
Is MSCs
Ad MSCs
CD73
MHC Class II


C
Full stained
CD45 FMO
CD11b FMO
BM MSCs
Is MSCs
Ad MSCs
CD45
CD11b


D
Full stained
CD19 FMO
CD34 FMO
BM MSCs
Is MSCs
Ad MSCs
CD19
CD34


E
Full stained
CD45 FMO
CD64 FMO
BM MSCs
Is MSCs
Ad MSCs
CD45
CD64

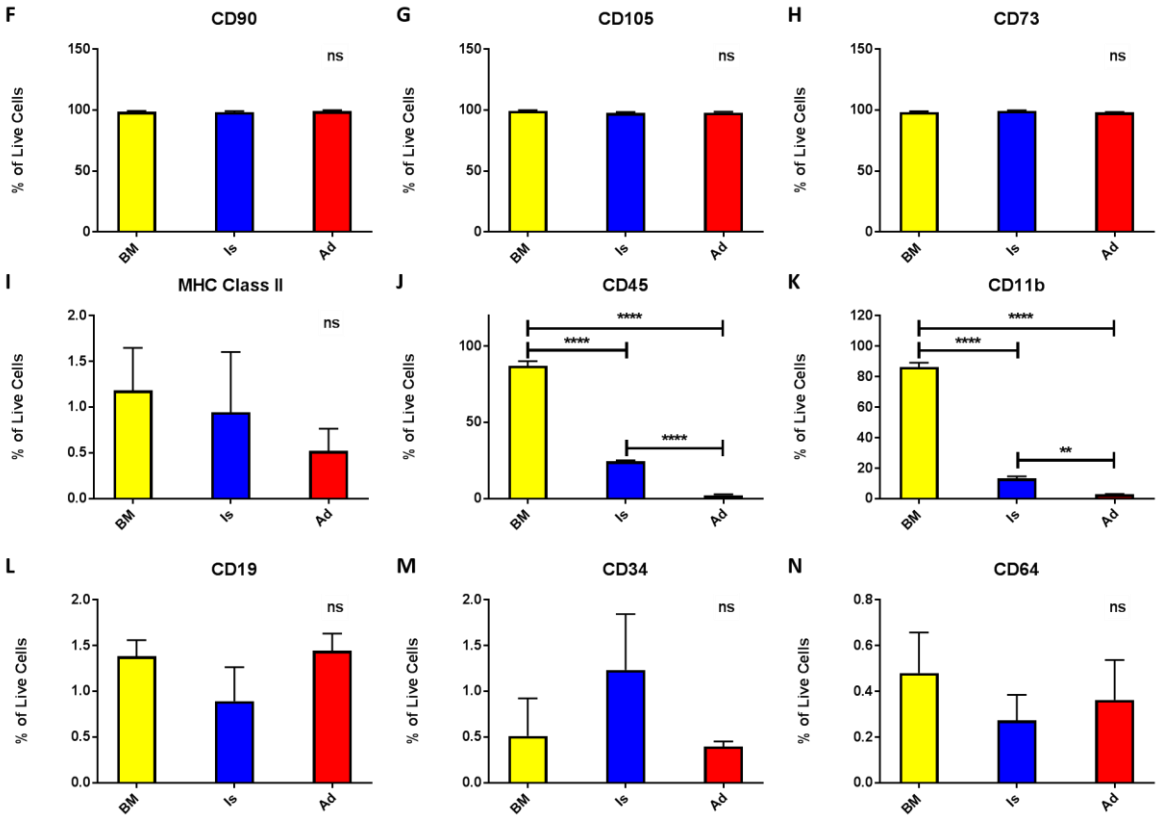

F
CD90
G
CD105
H
CD73
I
MHC Class II
J
CD45
K
CD11b
L
CD19
M
CD34
N
CD64
% of Live Cells
ns
****
**
BM
Is
Ad

Figure 3-3. Cells isolated from all tissues express MSC Markers.
MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. MSCs were then stained with a cocktail of antibodies to allow characterisation of MSCs by flow cytometry.

As explained in Chapter 2, FSC and SSC were used to gate on cells of correct size and granularity, doublets were excluded, and live cells were selected in all samples before assessing surface phenotype (**A-E**). Use of FMOs allowed the accurate identification of positive and negative populations in full stained samples. The% of live MSCs which stained positive for each of the tested markers is graphed to compare expression between MSC tissue sources (**F-N**). Each bar represents an n of 3 independent experiments and is graphed as mean ± SEM. ONE WAY ANOVA with Tukey's multiple comparison post-test analysis was used for statistical assessment of differences between MSC sources. $p = 0.05$ was considered the limit for statistical significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3.4 Differentiation potential of MSCs

To determine the trilineage differentiation potential of the cells isolated from the BM, Is and Ad, cells were cultured with appropriate differentiation factors to guide them through a specific differentiation pathway.

Cells isolated from the BM (Figure 3-4, A.i), Is (Figure 3-4, B.i) and Ad (Figure 3-4, C.i) were able to differentiate into adipocytes as the majority of the cells stained positive for fatty acid binding protein 4 (FABP4). Undifferentiated control samples were stained with anti-FABP4 to ensure that cells were not expressing FABP4 prior to differentiation (A.iv, B.iv, C.iv). As a control for positive staining, isotype controls (A.ii, B.ii, C.ii) and no-primary antibody controls (A.iii, B.iii, C.iii) were used. The lack of fluorescence in the isotype and no-primary controls and in the undifferentiated cells suggests that positive staining was specific for differentiation induced FABP4 expression and that cells isolated from the BM, Is and Ad were fully capable of adipogenic differentiation.

Cells isolated from the BM (Figure 3-5, A.i), Is (Figure 3-5, B.i) and Ad (Figure 3-5, C.i) were able to differentiate into chondrocytes as most of the cells stained positive for collagen II. Undifferentiated control samples were stained with anti-collagen II antibody to ensure that cells were not expressing collagen II prior to differentiation (A.iv, B.iv, C.iv). As a control for positive staining, isotype controls (A.ii, B.ii, C.ii) and no-primary antibody controls (A.iii, B.iii, C.iii) were used. The lack of fluorescence in the isotype and no-primary controls and in the undifferentiated cells suggests that positive staining was specific for

differentiation-induced collagen II expression and that cells isolated from the BM, Is and Ad were fully capable of differentiating into chondrocytes.

Similarly to adipogenic differentiation, cells isolated from the BM (Figure 3-6, A.i), Is (Figure 3-6, B.i) and Ad (Figure 3-6, C.i) were able to differentiate into osteocytes as the majority of the cells stained positive for osteopontin. Undifferentiated control samples were stained with anti–osteopontin antibody to ensure that cells were not expressing osteopontin prior to differentiation (A.iv, B.iv, C.iv). As a control for positive staining, isotype controls (A.ii, B.ii, C.ii) and no–primary antibody controls (A.iii, B.iii, C.iii) were used. The lack of fluorescence in the isotype and no–primary controls and in the undifferentiated cells suggests that positive staining was specific for differentiation induced osteopontin expression and that cells isolated from the BM, Is and Ad were fully capable of differentiating into adipocytes.

Cells isolated from the BM, Is and Ad could differentiate into adipocytes, chondrocytes and osteocytes as confirmed by positive staining of specific tissue markers. This satisfies the trilineage differentiation potential established by the ISCT.

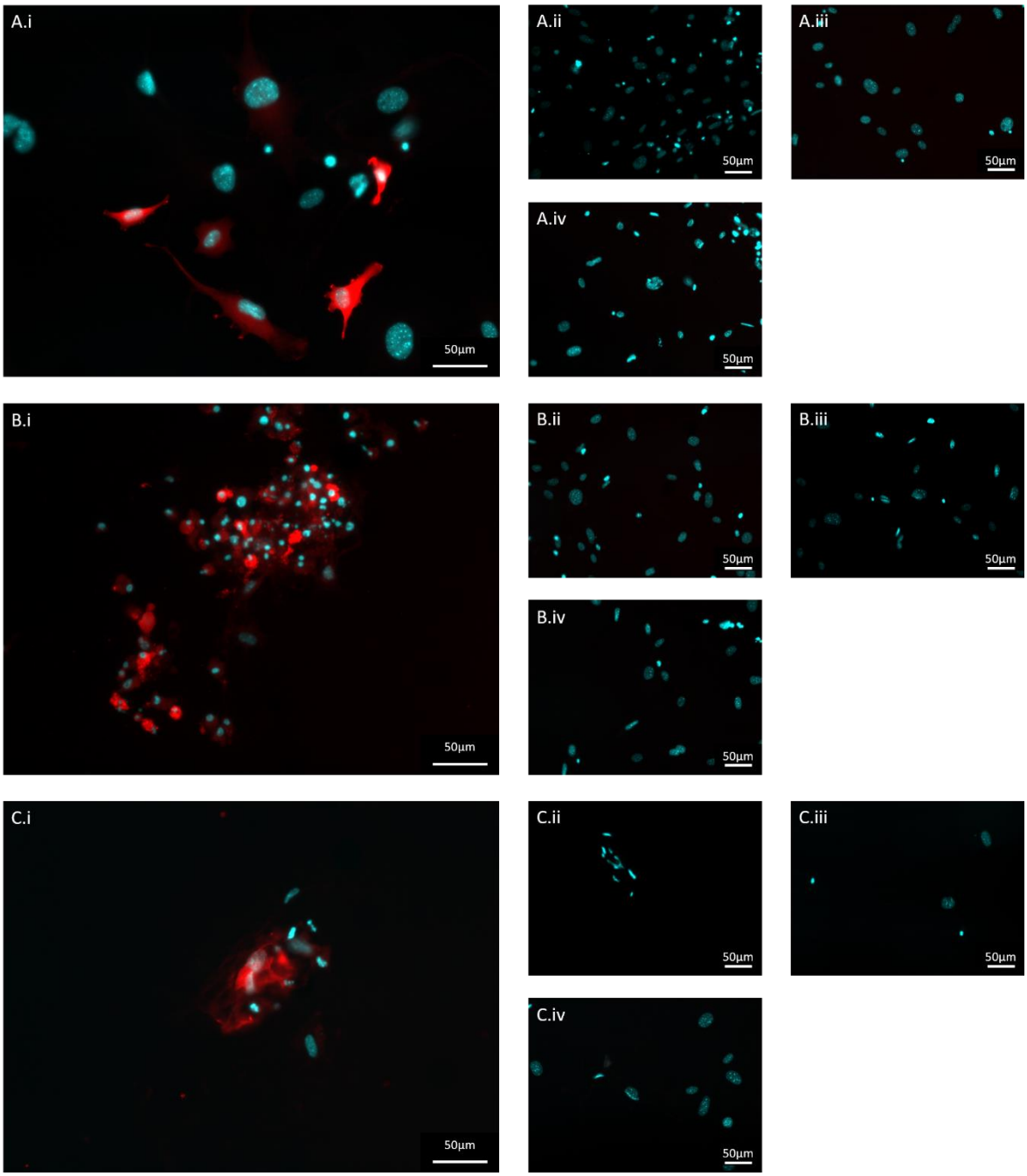


Figure 3-4. MSCs can successfully differentiate into adipocytes.
For adipogenesis, BM (**A.i**), Is (**B.i**) and Ad (**C.i**) MSCs were seeded at $2.1x10^4$ per cm^2 and grown in 4-well Nunc™ Lab-Tek™ Chamber Slides. When MSCs reached 100% confluency, differentiation factors were added every 2-3 days for 14-21 days. Undifferentiated control samples (**A.iv, B.iv, C.iv**) were maintained in medium without differentiation factors. After 14-21 days of differentiation, cells were stained with anti-FABP4 (RED), except for isotype controls (**A.ii, B.ii, C.ii**) and no-primary controls (**A.iii, B.iii, C.iii**). All samples were stained with specific fluorescent secondary antibodies. DAPI (BLUE) marks the cell nuclei.

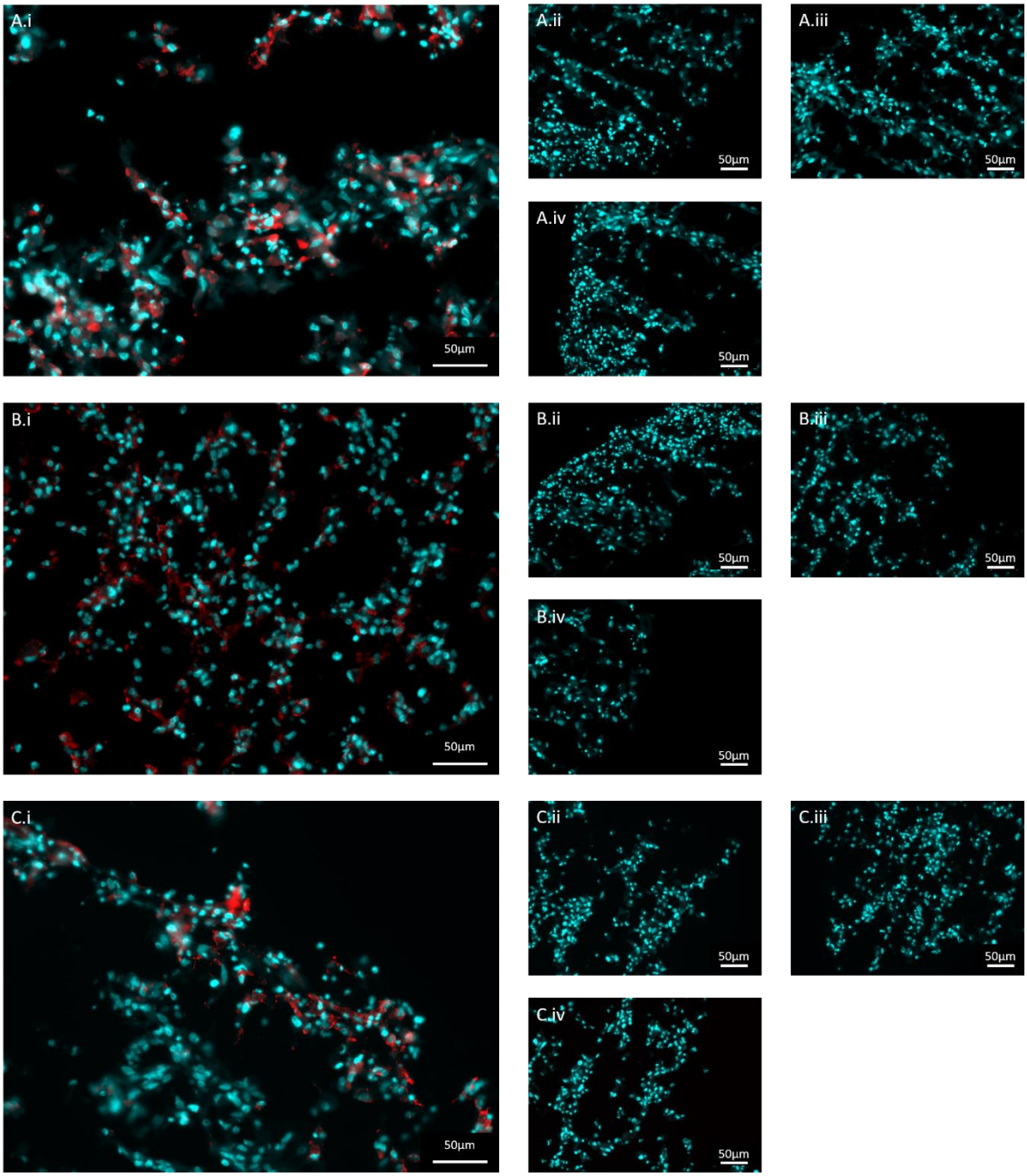


Figure 3-5. MSCs can successfully differentiate into chondrocytes.
For chondrogenesis, BM (**A.i**), Is (**B.i**) and Ad (**C.i**) MSCs were seeded at $2.5x10^5$ per cm^2, spun down in a 15 mL falcon tube and left culturing in spherical balls within the falcon in differentiation medium. Differentiation medium was changed every 2-3 days. Undifferentiated control samples (**A.iv, B.iv, C.iv**) were maintained in medium without differentiation factors. After 14-21 days of differentiation, cells were stained with anti-Collagen II (RED), except for isotype controls (**A.ii, B.ii, C.ii**) and no-primary controls (**A.iii, B.iii, C.iii**). All samples were stained with specific fluorescent secondary antibodies. DAPI (BLUE) marks the cell nuclei.

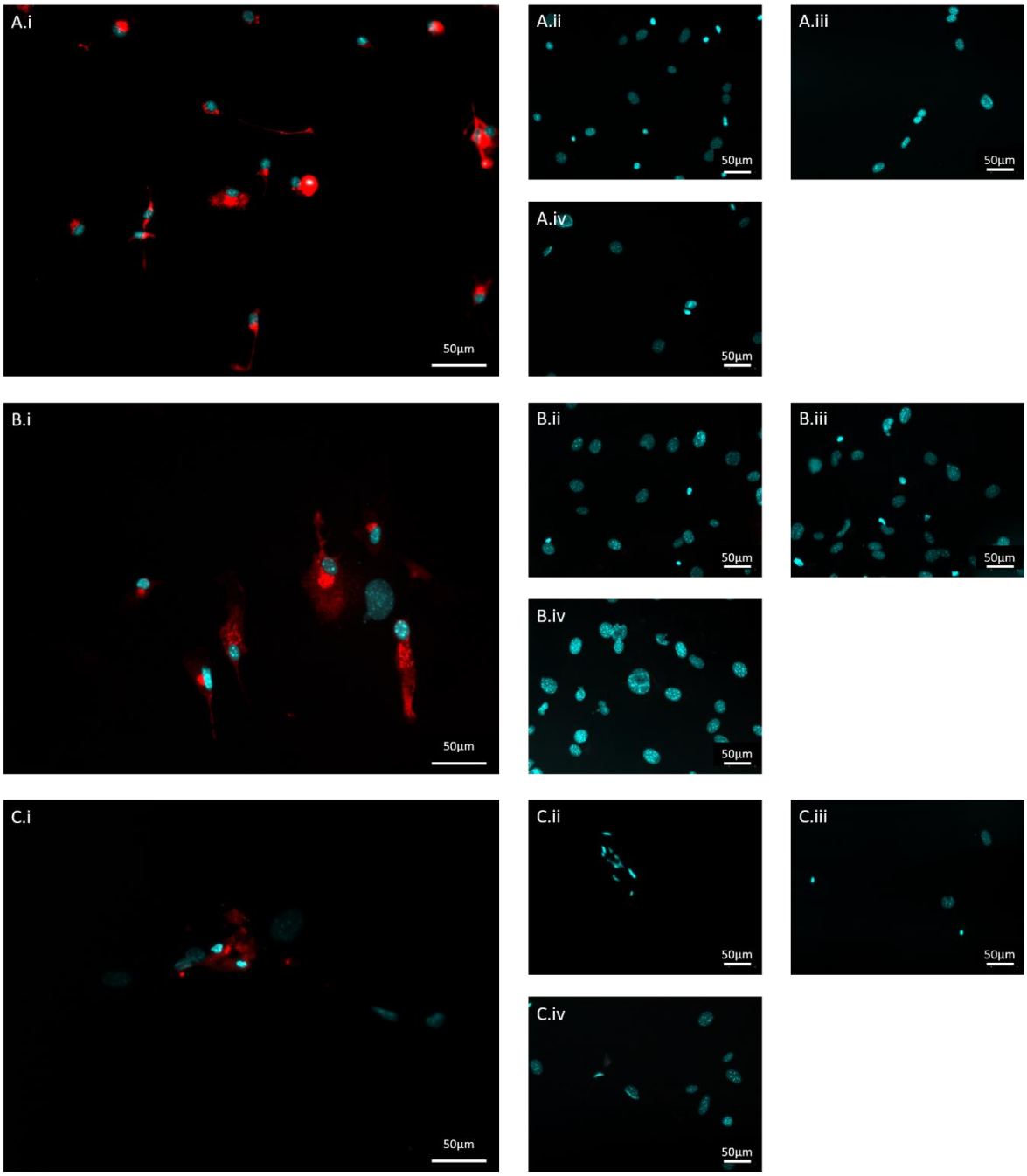


Figure 3-6. MSCs can successfully differentiate into osteocytes.
For osteogenesis, cells were seeded at 4.2x10^3 per cm^2 and grown in 4-well Nunc™ Lab-Tek™ Chamber Slides. When MSCs reached 100% confluency, differentiation factors were added every 2-3 days for 14-21 days. Undifferentiated control samples (**A.iv, B.iv, C.iv**) were maintained in medium without differentiation factors. After 14-21 days of differentiation, cells were stained with anti-osteopontin (RED), except for isotype controls (**A.ii, B.ii, C.ii**) and no-primary controls (**A.iii, B.iii, C.iii**). All samples were stained with specific fluorescent secondary antibodies. DAPI (BLUE) marks the cell nuclei.

3.5 Validation of CD45 expression in Islet derived MSCs

As observed in Figure 3-3, ~30% of the Is MSCs and ~85% of the BM MSCs are CD45 positive. According to the ISCT, in order to consider a human cell population as

MSCs, cells must be CD45 negative; however, surface marker phenotype is not well characterised in the mouse. Induction of CD45 expression on murine BM MSCs has been described (Yeh et al., 2006); anyhow, this event has not been documented in Is MSCs. For this reason, further analysis of the CD45 positive and negative populations within the Is MSCs was required, as well as validation of the CD45 expression in case the positive staining was an artefact.

To assess and compare the morphology, size and granularity of these two populations CD45 MACS MicroBeads were used. CD45 positive and negative cells were separated and distributed into new flasks at a 3500 cells/ cm^2 concentration and left growing. As a control, CD45 positive and negative cells were put back together in a 25:75 (1:3) proportion to assess the effect of the isolation on the cells. Cell morphology was observed and monitored using a light-phase microscope and both populations possessed the typical spindle-like morphology (Figure 3-7).

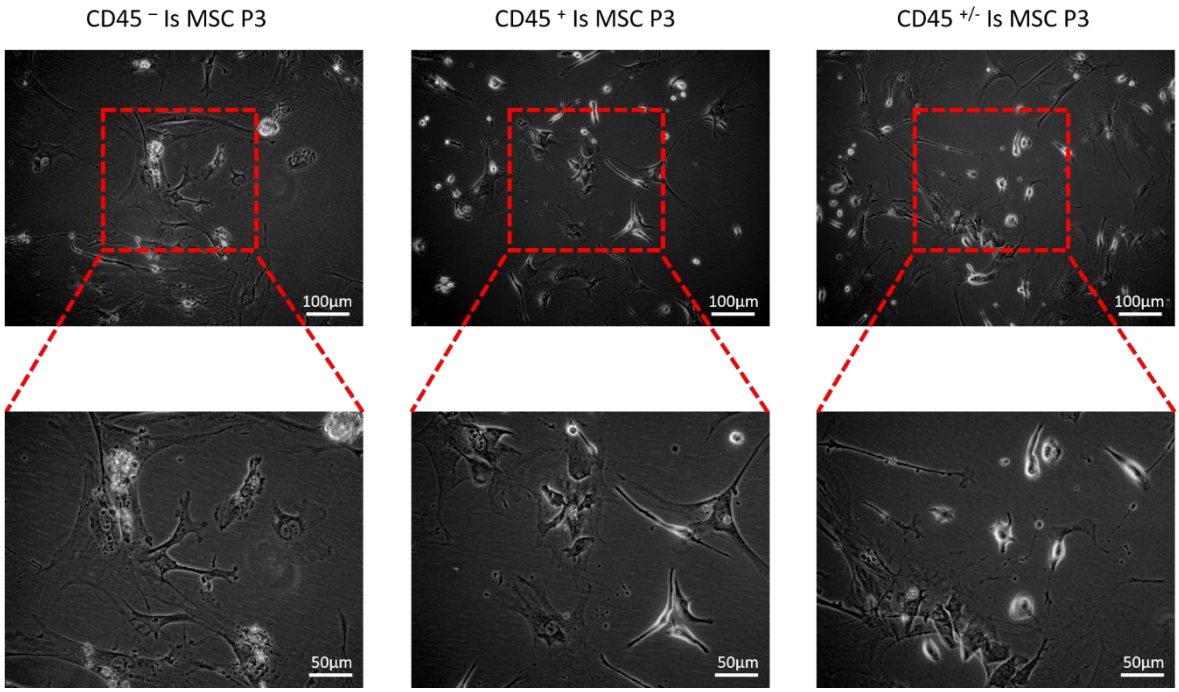


Figure 3-7. CD45 positive and negative Is MSCs exhibit MSC spindle-like morphology. MSCs were isolated from mice and grown until P2. CD45 microbeads were then used to isolate the CD45 positive cells. After isolation, CD45 positive and negative cells were distributed into new flasks at a 3500 cells/ cm^2 concentration and left growing. As a control, CD45 positive and negative cells were put back together in a 25:75 proportion to assess the effect of the isolation on the cells. Cell morphology was observed and monitored using a light-phase microscope. Both populations were adherent to plastic and possessed the typical spindle-like morphology.

Using flow cytometry, the parameters forward scatter (FSC) and side scatter (SSC) were used to measure the size and granularity (respectively) of both populations. The size of a cell is measured by the amount of laser light that can pass around the cell, while granularity is measured by the amount of light that bounces off particles within the cell. This way, size and complexity of a cell are easily measured. CD45 negative cells had a larger FSC (Figure 3-8, A) and SSC (Figure 3-8, B) compared to the CD45 positive population. To assess the effect of CD45 depletion on size and granularity, isolated CD45 positive and negative populations were compared to un-depleted populations. These results suggest that there are two different populations with different size and granularity. In order to perform statistical analysis and clarify the plotted data in histograms, mean fluorescent intensities of FSC (Figure 3-8 C) and SSC (Figure 3-8 D) values were graphed.

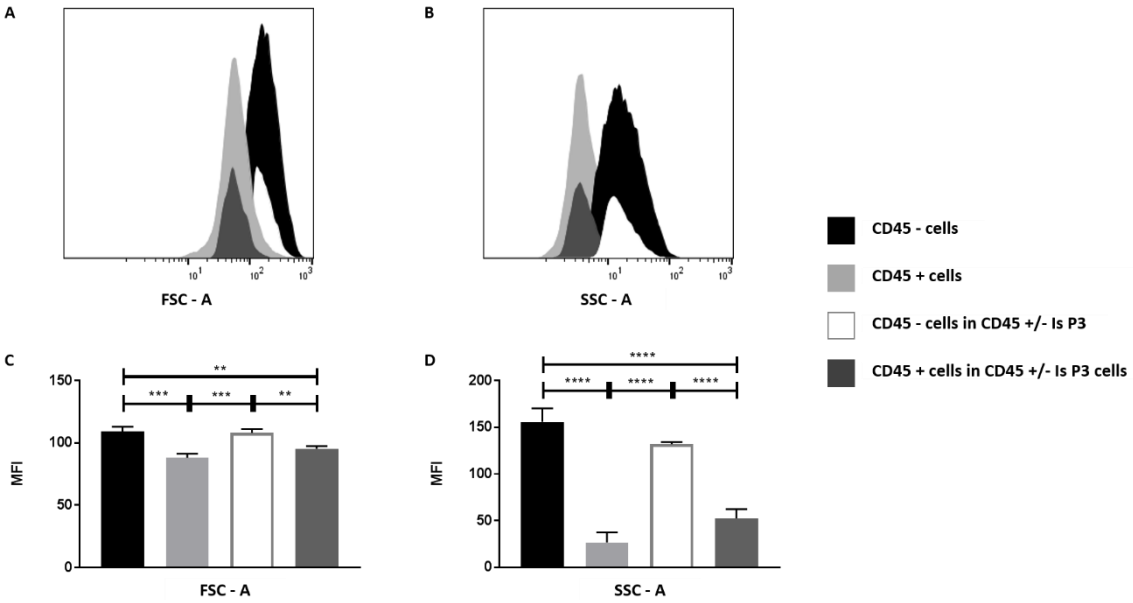


Figure 3-8. CD45 positive and negative Is MSCs differ in size and granularity.
Following the experimental set up of Figure 3-18, flow cytometry was used to measure FSC and SSC to assess size (**A** and **C**) and granularity (**B** and **D**) of CD45 positive and negative Is MSCs. Each bar represents an n of 3 independent experiments and is graphed as mean ± SEM. ONE WAY ANOVA with Tukey's multiple comparison post-test analysis was used for statistical assessment of differences between MSC sources. p = 0.05 was considered the limit for statistical significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Therefore, the next question to address was whether the CD45 positive cells were MSCs or a contaminating population able to express MSC markers. To address this, isolated CD45 positive and negative populations were differentiated in to all three lineages to assess their potential. As shown in Figure 3-9, both

islet-derived cell populations can successfully differentiate into adipocytes, chondrocytes and osteocytes, which suggests that both populations can be considered MSCs.

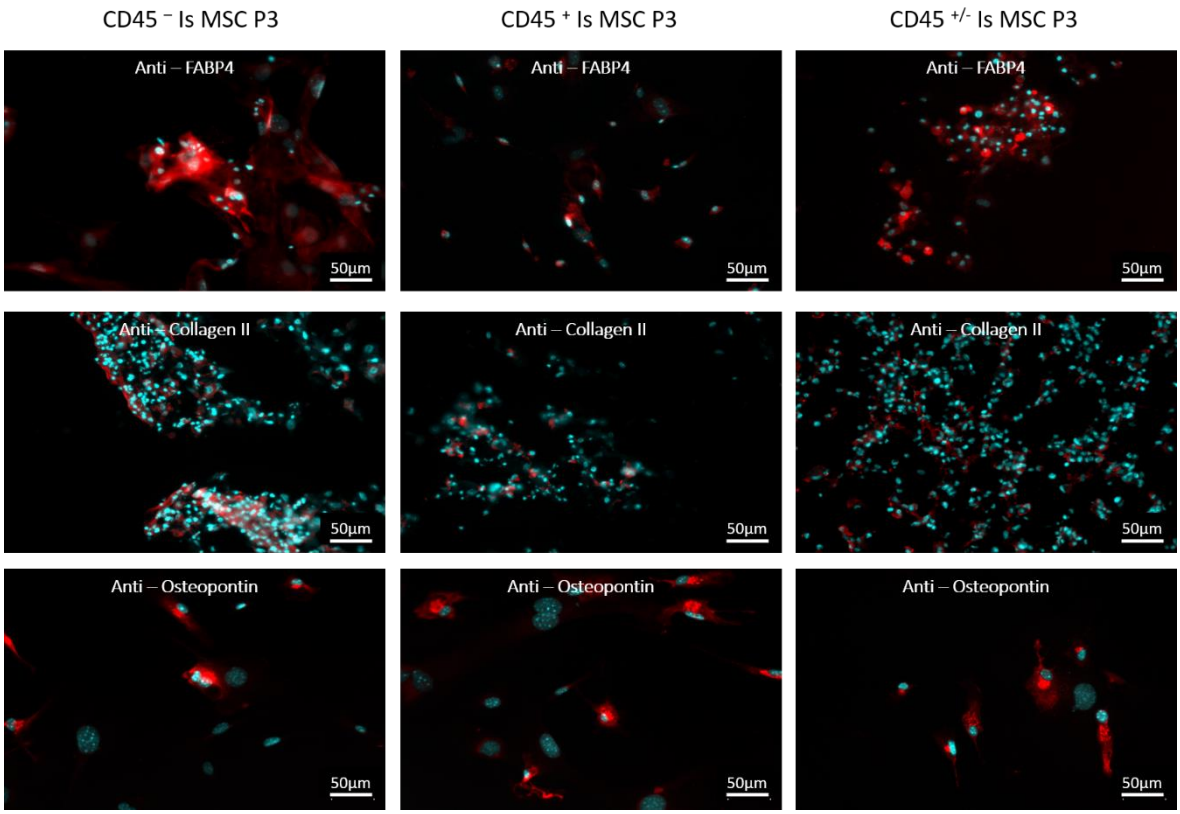


Figure 3-9. Both Is derived MSC populations can successfully differentiate into adipocytes, chondrocytes and osteocytes.
The experiment set up for adipogenesis is identical as the one in Figure 3-4, the experiment set up for chondrogenesis is explained in Figure 3-5 while the osteogenic differentiation is explained in Figure 3-6. All samples were stained with specific unlabelled primary antibodies and fluorescent secondary antibodies. DAPI (BLUE) marks the cell nuclei.

Lastly, validation of CD45 positive staining was performed to ensure that the antibodies were truly interacting with CD45 and that the positive staining was not an artefact. CD45 RNA (Figure 3-10, A) and protein (Figure 3-10, B) levels were measured in CD45 negative and positive populations. As a negative control 3T3 mouse embryo fibroblasts were used while splenocytes were considered as positive control for CD45 expression at both RNA and protein levels. These findings confirm that there is an islet derived MSC population that expresses CD45.

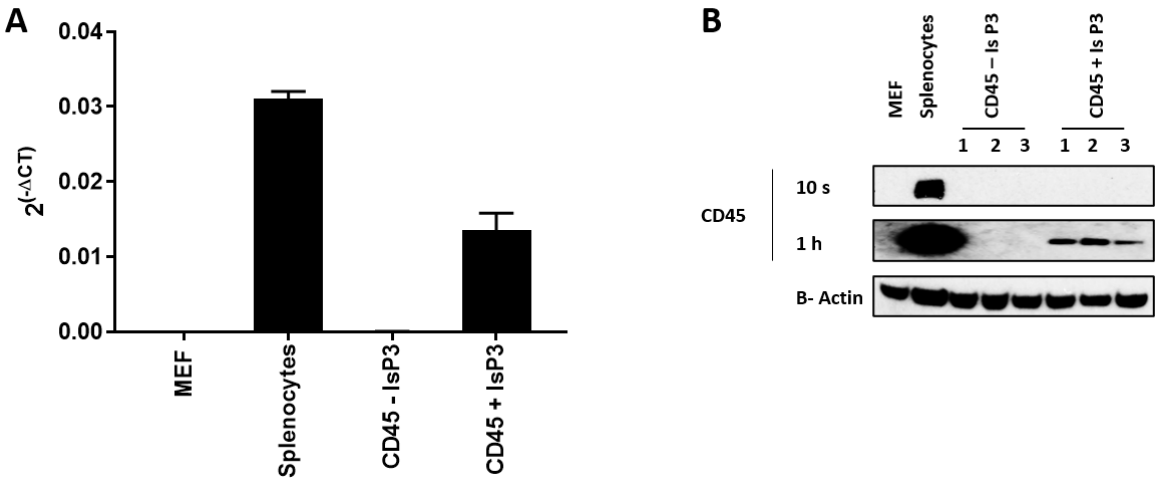


Figure 3-10. Validation of the expression of CD45 protein in Is MSCs.
MSCs were isolated from mice and grown until P2. CD45 microbeads were then used to isolate the CD45 positive cells. After isolation, CD45 positive and negative cells were distributed into new flasks at a 3500 cells/ square^2 concentration and left growing. Once 80% confluence was reached, cells were harvested and prepared for RNA (**A**) and protein analysis (**B**). As a negative control of CD45 expression 3T3 mouse embryo fibroblasts were used while splenocytes were used as positive control.

3.6 Analysis of the epithelial origin of islet derived MSCs

The origin of MSCs isolated from pancreatic exocrine tissue remains unclear and the epithelial to mesenchymal transition process is one of the possible mechanisms suggested to explain the origin of these cells. This process is based on the decrease in the expression of the adhesion molecules between epithelial cells, EpCAM among others, followed by an increase in the mesenchymal markers, such as vimentin. As observed in Figure 3-2, cells grown from the islets of Langerhans showed a cluster of small spherical cells where the islet was seeded surrounded by spindle–shaped cells, with transitioning cells in between these two populations with different morphologies. However, at later passages the only cells present in the flask showed a spindle–like morphology. For this reason, it was important to determine if the small spherical cells were an epithelial population unable to proliferate or more likely, if an epithelial to mesenchymal transition was leading to the generation of MSCs.

3.6.1 Co-expression of epithelial and mesenchymal markers through passage

Islets of Langerhans were isolated and between 10 to 20 islets or the cells obtained by the disaggregation of 10 to 20 islets were seeded in 4-well Nunc™ Lab-Tek™ Chamber Slides and left growing for 10 days. Cells were then fixed, permeabilised and stained for epithelial (EpCAM) and mesenchymal (vimentin) specific markers (Figure 3-11). As a control for positive staining, isotype controls for both EpCAM and vimentin were used. All slides were imaged with a Zeiss epifluorescence microscope using the appropriate fluorescent channels and magnifications. The lack of fluorescence in the isotype controls suggests that positive staining was specific for EpCAM and vimentin expression and that cells isolated from the islets of Langerhans co-express epithelial and mesenchymal traits.

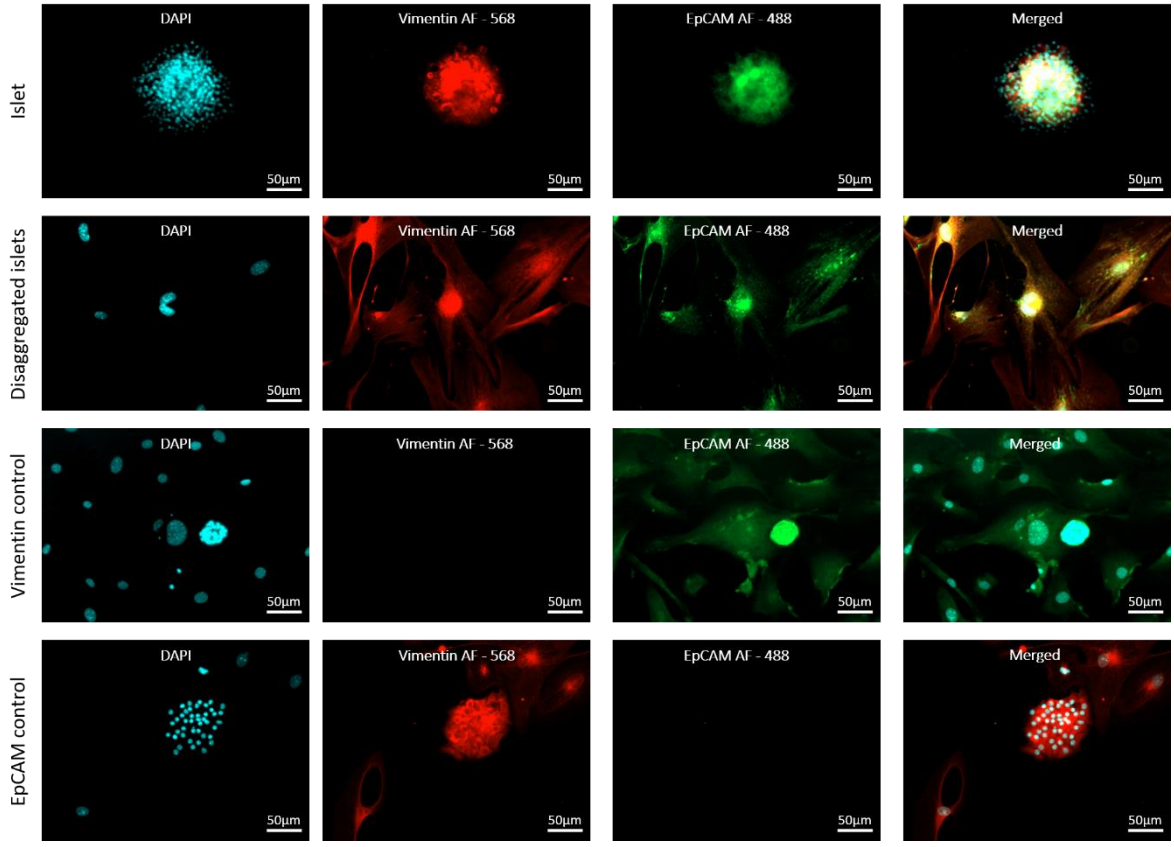


Figure 3-11. Cells isolated from the islets of Langerhans co-express epithelial and mesenchymal markers.
Between 10 and 20 islets or the cells obtained from the disaggregation of 10 to 20 islets were seeded in 4-well Nunc™ Lab-Tek™ Chamber Slides and left growing for 10 days. Cells were then stained with EpCAM (GREEN) and Vimentin (RED), except for isotype controls (Vimentin control and EpCAM control). All samples were stained with specific fluorescent secondary antibodies. DAPI (BLUE) marks the cell nuclei. All slides were imaged with a Zeiss epifluorescence microscope using the appropriate fluorescent channels and magnifications. Images were prepared using Zen software.

Co-expression of mesenchymal antigens by epithelial cells within the pancreas has been described as an artefact of cell culture (Seeberger et al., 2009), for this reason, islets of Langerhans were isolated, disaggregated and stained with a cocktail of antibodies to study their epithelial origin by flow cytometry. After viability exclusion, two populations of different size were observed. The smaller population was highly positive for EpCAM and negative for vimentin while the larger co-expressed both markers (Figure 3-12, A). ~80% of the cells were only expressing EpCAM while no cells were just vimentin positive (Figure 3-12, B). In addition, from the cells expressing EpCAM, ~20% of the cells were vimentin positive (Figure 3-12, C).

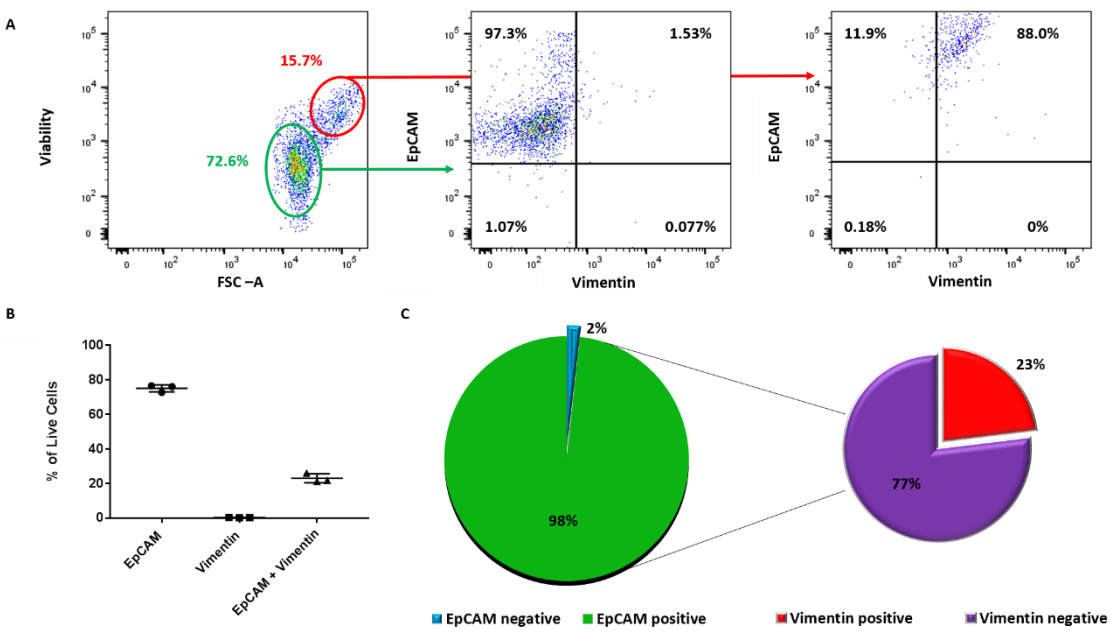


Figure 3-12. Cells isolated from the islets of Langerhans co-express epithelial and mesenchymal markers.
Islets of Langerhans were disaggregated and stained with a cocktail of antibodies to study their epithelial origin by flow cytometry. After viability exclusion, two populations were observed which were analysed for EpCAM and Vimentin expression (**A**). The% of live cells which stained positive for each of the tested markers is graphed (**B**) and represented as pie-charts (**C**).

As observed in Section 3.2, epithelial like cells were only observed at P1. For that reason, we wanted to determine if cells would still be expressing EpCAM at passage 3. Is MSCs were grown in 4-well Nunc™ Lab-Tek™ Chamber Slides and left growing for 10 days. Cells were then fixed, permeabilised and stained for EpCAM and vimentin specific markers (Figure 3-13). As a control for positive staining, isotype controls for both EpCAM and vimentin were used. All slides were imaged with a Zeiss epifluorescence microscope using the appropriate fluorescence

channels and magnifications. The lack of fluorescence in the isotype controls suggests that positive staining was specific for EpCAM and vimentin expression and that passage 3 Is MSCs co-express epithelial and mesenchymal traits.

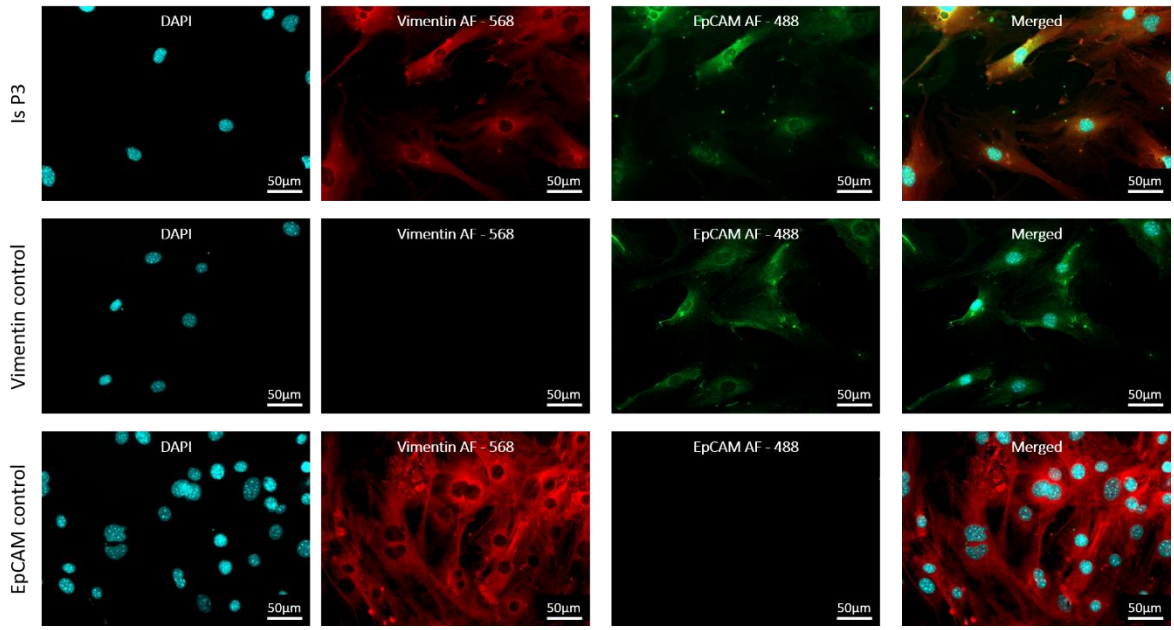


Figure 3-13. Is MCS co-express epithelial and mesenchymal markers at P3.
Is MSCs were grown in 4-well Nunc™ Lab-Tek™ Chamber Slides and left growing until 80% confluence was reached. Cells were then stained with EpCAM (GREEN) and Vimentin (RED), except for isotype controls (Vimentin control and EpCAM control). All samples were stained with specific fluorescent secondary antibodies. DAPI (BLUE) marks the cell nuclei. All slides were imaged with a Zeiss epifluorescence microscope using the appropriate fluorescent channels and magnifications. Images were prepared using Zen software.

3.6.2 Co-expression of CD45 and epithelial markers

As determined in Section 3.5, there is a CD45 positive population within the Is MSCs. For this reason, we wanted to determine if there was a correlation between the epithelial origin of these MSCs and the CD45 expression. Islet of Langerhans were disaggregated and stained with a cocktail of antibodies to study their EpCAM and CD45 status by flow cytometry. After viability exclusion, cells were gated according to their EpCAM and vimentin expression as before, and those populations were gated for CD45 expression (Figure 3-14, A). ~90% of the cells were expressing CD45 (Figure 3-14, B). In addition, CD45 was co-expressed with both EpCAM and vimentin, with ~20% of the cells co-expressing EpCAM, vimentin and CD45 (Figure 3-14, C).

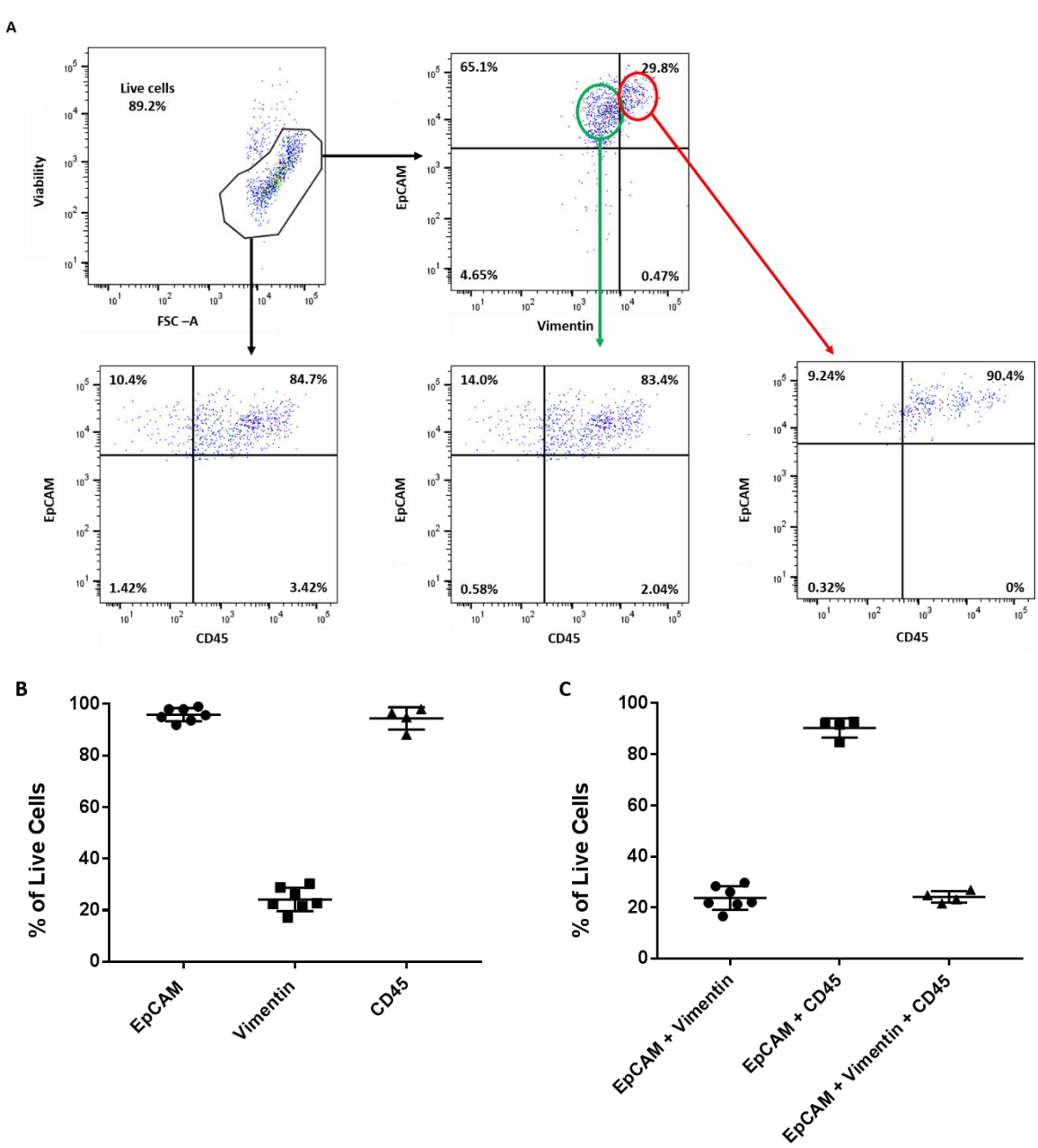


Figure 3-14. ~90% of the cells isolated from the islets of Langerhans are CD45 positive and co-express EpCAM.
Islets of Langerhans were disaggregated and stained with a cocktail of antibodies to study their epithelial origin by flow cytometry. After viability exclusion, cells were assessed for CD45, EpCAM and Vimentin expression (**A**). The% of live cells which stained positive for each of the tested markers (**B**) and the co-expression of them (**C**) has been graphed.

As stated in Section 3.3, ~30% of the cells were CD45 positive at passage 3. To correlate CD45 expression with EpCAM expression, P3 Is MSCs were stained with a cocktail of antibodies. ~99% of the cells were CD73 positive, even the ~20% that was still EpCAM positive. EpCAM positive and negative cells were gated and their CD45 expression was studied (Figure 3-15, A). ~95% of the EpCAM negative cells were CD45 negative while ~93% of the EpCAM positive cells were CD45 cells

(Figure 3-15, B), suggesting an unknown correlation between the epithelial to mesenchymal transition process and CD45 levels in the islets of Langerhans.

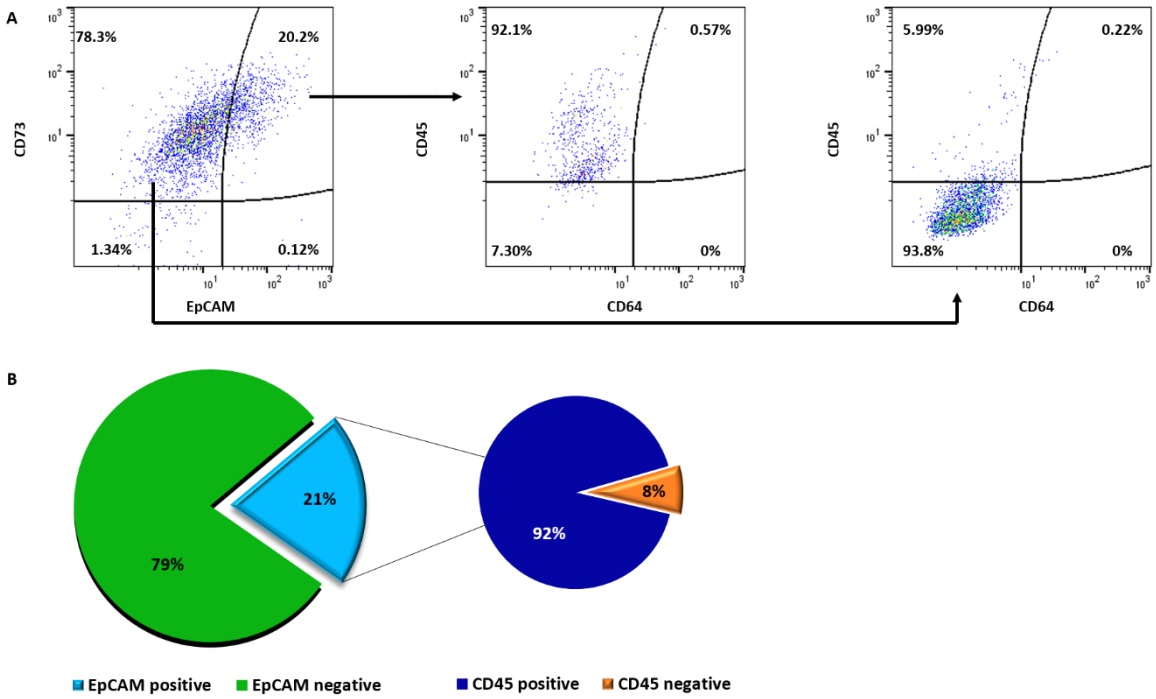


Figure 3-15. CD45 positive Is MSC population expresses the epithelial marker EpCAM at P3. Is MSCs were grown in culture until P3. MSCs were then stained with a cocktail of antibodies, to allow characterisation of MSCs by flow cytometry (**A**). The% of live cells which stained positive for each of the tested markers has been represented as pie-charts (**B**).

Disaggregation of the islets of Langerhans showed that ~95% of the cells within the islets were CD45 positive, while after culture of those cells, more precisely at P3, ~30% of the cells were CD45 positive. To explain this, Ki67 proliferation marker was studied. Ki67 is a nuclear protein that is involved in ribosomal RNA transcription and therefore, can be used as a proliferation marker. P3 Is MSCs were stained with a cocktail of antibodies, viable cells were gated and live cells were analysed for their CD45 and Ki67 levels (Figure 3-16, A). To appropriately gate the Ki67 negative cells an isotype control was used (Figure 3-16, B). As shown in Figure 3-16, CD45 positive cells are not proliferating in culture efficiently as their Ki67 levels are barely detectable (Figure 3-16, C).

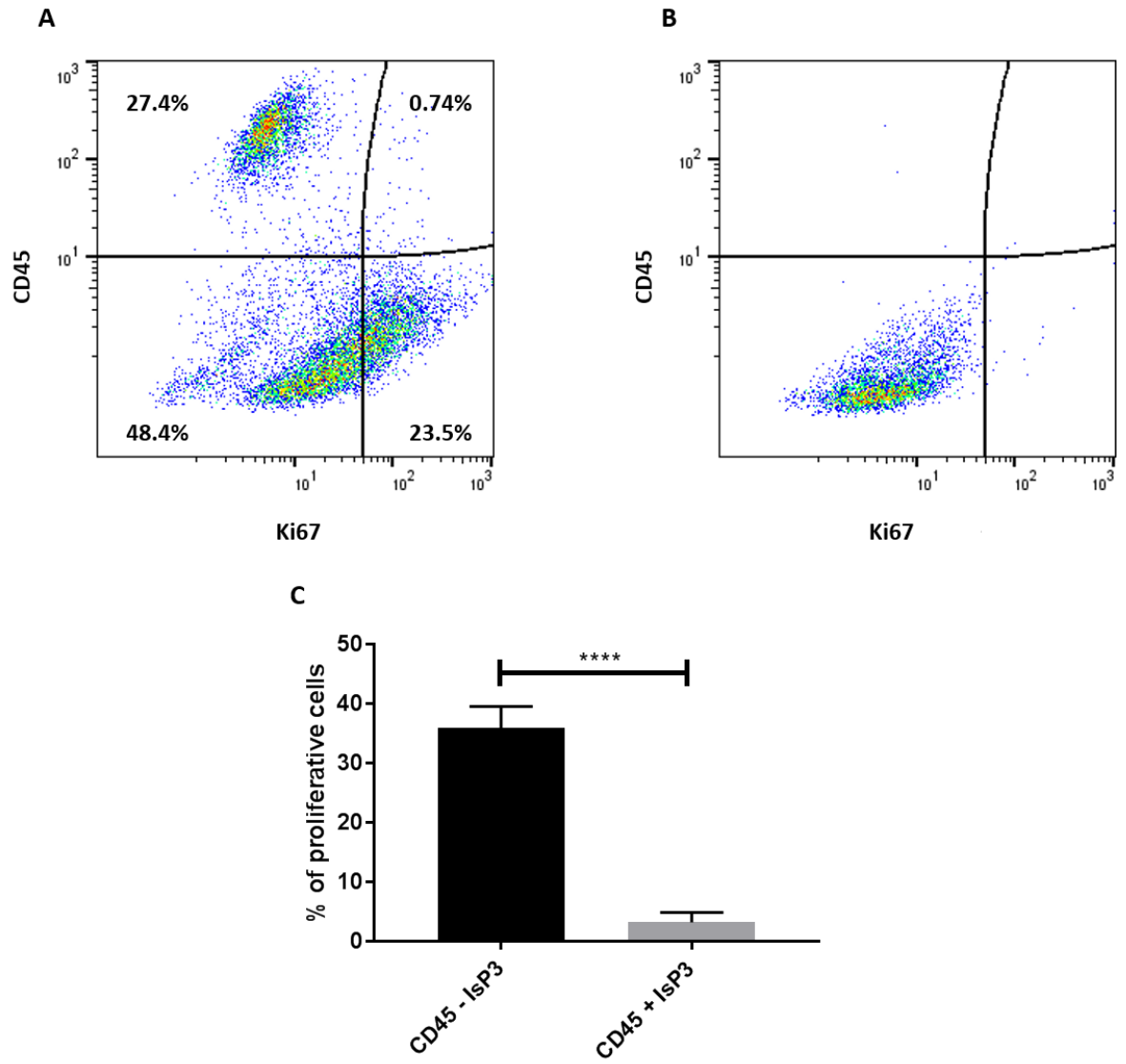


Figure 3-16. CD45 positive population is replication-defective.
Is MSCs were grown in culture until P3. MSCs were then stained with a cocktail of antibodies, to allow characterisation of MSCs by flow cytometry (**A**). Positive staining was measured using isotype controls and gates were drawn appropriately (**B**). Percentage of proliferative cells in each population was determined and graphed (**C**). Each bar represents an n of 3 independent experiments and is graphed as mean ± SEM. Mann-Whitney test analysis was used for statistical assessment. p = 0.05 was considered the limit for statistical significance; * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.

3.7 Phenotype of MSCs during Inflammation

MSCs are able to modulate the activity of the host immune responses, which makes them a very promising cell therapy in the treatment of chronic inflammatory diseases. As MSCs are going to be infused into pre-existing inflammatory environments, it is extremely important to understand the effect of inflammation on the phenotype of MSCs. Different inflammatory agents elicit different patterns of responses in the cells of the immune system; therefore,

seems reasonable to suppose that different inflammatory agents could lead to differences in the morphology and surface molecule phenotype of licensed MSCs. Thus, MSCs were stimulated in four different ways; MSCs isolated from one mouse donor were grown until P2 and split into 5 flasks and allowed to grow until 80% confluence was reached. Cells were then stimulated with a cocktail of cytokines involved in systemic inflammation (40ng/ml of TNF-α, IL-1ß and IFN-ϒ), 100 ng/mL LPS, 100 ng/mL LTA or 4 µg/mL Poly I:C for 24 hours. Even if homeostasis is hard to mimic *in vitro*, as control one flask was maintained in MSC culture medium and this is referred as resting MSCs, while stimulated MSCs are considered licensed MSCs- this terminology will be used throughout.

3.7.1 Size and granularity of MSCs in resting vs inflammatory conditions

Using flow cytometry, forward scatter (FSC) and side scatter (SSC) parameters were used to measure the size and granularity (respectively) of BM, Is and Ad MSCs under resting and inflammatory conditions (Figure 3-17). When MSCs were under non-stimulatory conditions, BM MSCs were significantly smaller than Is and Ad MSCs (BM = 103.75 [±2.47], Is = 120.33 [±2.05], Ad = 114.00 [±3.26]) (Figure 3-17, A). Inflammatory stimulation did not influence the size except in LTA-mediated stimulation of Is MSCs, where the FSC decreased from 120.33 (±2.05) to 106.33 (±2.86). Tissue origin and inflammation both influenced MSC granularity as the differences in granularity were statistically significant among the three different tissue derived MSCs in resting conditions (BM = 231.00 [±4.32], Is = 133.33 [±2.86], Ad = 121.00 [±2.94]) (Figure 3-17, B). Cytokine-mediated stimulation produced a statistically significant decrease of granularity in Ad MSCs, from 121.00 (±2.94) to 100.66 (±2.49). LPS-mediated stimulation produced a decrease in granularity in both BM and Is MSCs as SSC values from 231.00 (±4.32) to 214.00 (±4.54) and from 133.33 (±2.86) to 111.33 (±2.05), respectively. LTA and Poly I:C-mediated stimulation produced no significant changes in the granularity of the cells from any tissue (compared to the control).

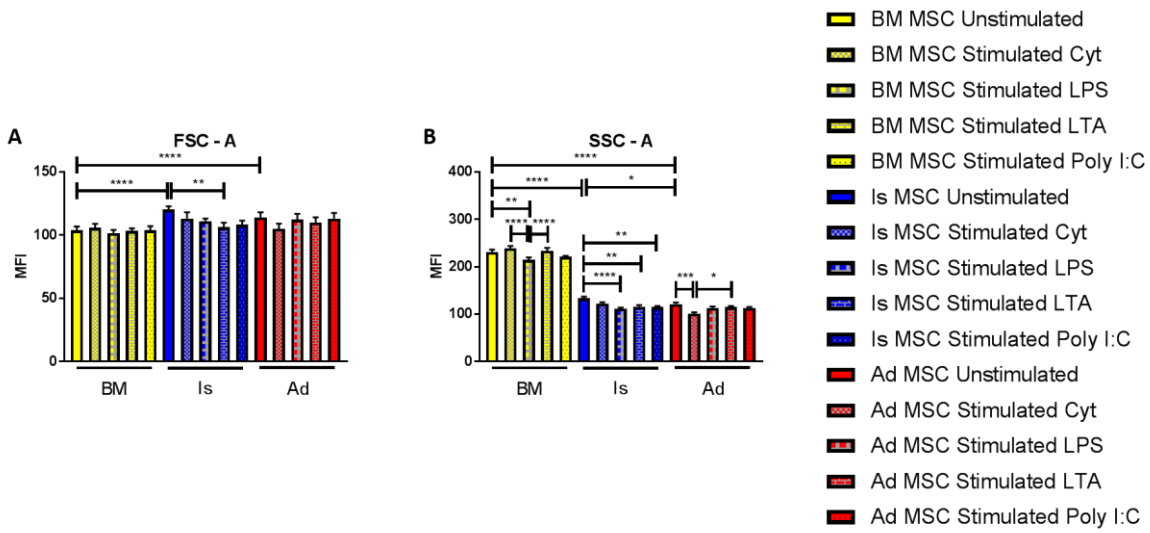


Figure 3-17. Size and granularity of MSCs under inflammatory stimulation.
Following the experimental set up of Figure 3-18, flow cytometry was used to measure FSC and SSC to assess size (**A**) and granularity (**B**) of MSCs during resting and inflammatory conditions. Each bar represents an n of 3 independent experiments and is graphed as mean ± SEM. ONE WAY ANOVA with Tukey's multiple comparison post-test analysis was used for statistical assessment of differences between MSC sources and licensing agent. Significant differences are marked with the appropriate number of asterisks. $p = 0.05$ was considered the limit for statistical significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3.7.2 Surface molecule phenotype of MSCs in resting vs inflammatory conditions

Representative dot plots illustrate the percentage of Is MSCs expressing the markers CD73, CD146, CD166, CD271 and MHC Class I and MHC Class II during resting and inflammatory conditions (Figure 3-18, A). Positive staining was determined by the use of fluorescent minus one controls. The percentage of live BM, Is and Ad MSCs expressing these markers under resting and licensed conditions are graphed in Figure 3-18, B-G.

Tissue source and licensing did not affect the number of live cells expressing CD73 as >97% of live cells were CD73 positive irrespective of tissue source or licensing condition. Under resting conditions, 98.80% (± 0.70) of BM MSCs, 98.53% (± 0.57) of Is MSCs and 99.16% (± 0.33) of Ad MSCs stained positive for CD73 (Figure 3-18, B). The percentage of CD73 positive MSCs increased in a non-significant manner after cytokine-mediated licensing in the BM (98.80% [± 0.70] to 99.14% [± 0.22]), Is (98.53% [± 0.57] to 99.00% [± 0.41]) and Ad MSCs (99.16% [± 0.33] to 99.46% [± 0.47]). Licensing with LPS (BM = 98.47% [± 0.49], Is = 98.61% [± 0.54], Ad = 98.83% [± 0.30]), LTA (BM = 98.38% [± 0.58], Is = 98.90% [±

0.49], Ad = 98.96% [± 0.20]), and Poly I:C (BM = 98.60% [± 0.32], Is = 98.9% [± 0.35], Ad = 98.40% [± 0.24]), led to a non-significant decrease in the number of CD73 positive MSCs. Median fluorescence intensity (MFI) was used to describe the median intensity and level of anti-CD73 antibody binding (Figure 3-19, A). Tissue source of MSCs did not affect the level of CD73 expression as under resting conditions the MFIs were as following: BM MSCs = 43 (± 3.26); Is MSCs = 44.66 (± 2.05) and Ad MSCs = 38.86 (± 2.21). No statistically significant differences in the expression levels of CD73 were observed comparing resting to licensed BM (Cytokines = 48 [± 1.20], LPS = 45.66 [± 4.98], LTA = 43 [± 4.54], Poly I:C = 40.33 [± 3.29]), Is (Cytokines = 44.33 [± 3.39], LPS = 49.66 [± 5.24], LTA = 45 [± 2.94], Poly I:C = 47.66 [± 4.10]) or Ad MSCs (Cytokines = 38.86 [± 3.72], LPS = 34.35 [± 2.21], LTA = 34.08 [± 2.58], Poly I:C = 34.12 [± 1.65]).

The cell surface glycoprotein CD146 is another marker commonly used for MSC identification (Junker et al., 2010). Even if >92.6% of MSCs from the three sources were positive for CD146, tissue source had an effect on the number of live cells expressing CD146 as BM MSCs had significantly fewer cells expressing this marker compared to Is and Ad MSCs (BM = 92.64% [± 1.44], Is = 98.21% [± 0.74], Ad = 94.73% [± 2.73]) (Figure 3-18, C). Licensing of BM MSCs led to an increase of the percentage of CD146 expressing cells (Cytokines = 93.06% [± 1.20], LPS = 95.20% [± 1.02], LTA = 93.53% [± 0.96], Poly I:C = 93.13% [± 1.23]) while licensing of Ad MSCs led to a decrease of the percentage of CD146 expressing cells (Cytokines = 81.16% [± 1.62], LPS = 91.63% [± 1.32], LTA = 86.53% [± 0.38], Poly I:C = 86.70% [± 4.98]). In the case of Is MSCs, cytokine-mediated stimulation led to an increase of the percentage of CD146 expressing cells (Is = 98.21% [± 0.74] to 98.40% [± 0.53]) while LPS (96.20% [± 0.82]), LTA (98.15% [± 0.26]) and Poly I:C (97.38% [± 0.89]) licensing led to a decrease. These differences in percentage of live cells expressing CD146 were further confirmed by MFI analysis (Figure 3-19, B) of CD146. Under resting conditions, differences in the expression levels of CD146 were significant among MSCs from the three tissues (BM = 21.33 [± 3.29], Is = 44 [± 3.74], Ad = 78.33 [± 2.05]). Licensing of BM MSCs led to an increase of the level of CD146 expression in all the cases but when licensed with the cytokine cocktail (Cytokines = 20.66 [± 1.69], LPS = 27.33 [± 2.86], LTA = 22.33 [± 2.86], Poly I:C = 24.5 [± 2.27]). Licensing of Is (Cytokines = 34.66 [± 3.29], LPS = 35.33 [± 2.86], LTA = 38.66 [±

2.05], Poly I:C = 40.33 [± 2.49]) and Ad MSCs (Cytokines = 57 [± 2.16], LPS = 69.66 [± 2.86], LTA = 64 [± 2.44], Poly I:C = 70.66 [± 2.86]) led to a decrease in the median fluorescence intensity of CD146. Licensing only produced significant differences in Ad MSCs at both percentage of live cells expressing CD146 and CD146 expression levels.

The membrane glycoprotein ALCAM, also known as CD166, is also a marker of MSCs, however, its expression levels are significantly variable. The percentage of ALCAM positive MSCs in resting conditions showed high variations from source to source with Ad MSCs expressing the highest number of live positive cells (35.56% [± 1.90]), followed by BM MSCs (22.82% [± 0.72]) and a very low number of cells expressing this marker in Is MSCs (0.47% [± 0.07]) (Figure 3-18, D). Cytokine licensing led to a decrease in the percentage of CD166 expressing MSCs from the BM (20.55% [± 0.52]), Is (0.39% [± 0.14]) and Ad (27.9% [± 2.90]), as well as LPS (BM = 17.99% [± 0.87], Is = 0.36% [± 0.15], Ad = 26.43% [± 3.21]) and Poly I:C (BM = 21.34% [± 1.78], Is = 0.39% [± 0.10], Ad = 31.76% [± 2.82]) licensing. LTA licensing produced an increase in the percentage of BM MSCs expressing CD166 (from 22.82% [± 0.72] to 25.15% [± 1.86]), while it produced a decrease in the percentage of Is (from 0.47% [± 0.07] to 0.39% [± 0.13]) and Ad (from 35.56% [± 1.90] to 29.36% [± 3.44]) MSCs expressing this marker. These differences in percentage of live cells expressing CD166 among tissue of origin and inflammatory agent were further confirmed by MFI analysis of CD166 (Figure 3-19, C). Under resting conditions, differences in the expression levels of CD146 were statistically significant among MSCs from all three tissues (BM = 15.76 [± 2.15], Is = 3.86 [± 0.99], Ad = 1.63 [± 0.34]). Cytokine and Poly I:C licensing led to a decrease in the expression levels of CD166 in MSCs from the BM (Cytokine = 15.06 [± 2.34]; Poly I:C = 14.86 [± 1.48]), Is (Cytokine = 3.68 [± 0.91]; Poly I:C = 3.46 [± 0.44]) and Ad (Cytokine = 1.50 [± 0.14]; Poly I:C = 1.2 [± 0.29]). LPS and LTA licensing led to an increase in the expression levels of CD166 in MSCs from the BM (LPS = 16.56 [± 2.47]; LTA = 17.36 [± 3.02]), Is (LPS = 4.13 [± 1.16]; LTA = 3.46 [± 0.53]) and Ad (LPS = 4.09 [± 3.61]; LTA = 1.25 [± 0.27]). Licensing produced statistically no significant differences in MSCs from any tissue source at both percentage of life cells expressing CD166 and CD166 levels.

The low-affinity nerve growth factor receptor, also known as CD271, is a marker of bone marrow MSCs with enhanced differentiation capacity for bone repair; it is expressed at variable levels in MSCs from different sources (Kohli et al., 2019). The percentage of CD271 positive MSCs in resting conditions showed very high and statistically significant variations from source to source where BM MSCs had the highest number of positive cells (40.21% [± 2.79]), followed by Ad MSCs, which had a low number of cells expressing this marker (3.71% [± 0.67]) and a very low number of Is MSCs positive for the expression of CD271 (0.41% [± 0.02]) (Figure 3-18, E). MSC licensing produced no significant differences in the percentage of MSCs positive for this marker from any tissue source except for BM MSCs after 24 hours of cytokine cocktail, LPS or LTA licensing, but not Poly I:C licensing. Licensing of BM MSCs with cytokines (39.9% [± 1.95]), LPS (33.36% [± 2.50]) and Poly I:C (38.73% [± 4.45]) produced a decrease of the percentage of positive cells expressing this marker, while LTA licensing produced an increase (from 40.21% [± 2.79] to 41.09% [± 2.57]). Licensing of the Is MSCs produced an increase of the CD271 positive cells percentage (Cytokines = 0.52% [± 0.05], LPS = 0.51% [± 0.01], LTA = 0.57% [± 0.02], Poly I:C = 0.55% [± 0.07]) while Ad MSCs licensing produced a decrease of CD271 positive cells percentage (Cytokines = 3.01% [± 0.40], LPS = 1.71% [± 0.17], LTA = 1.33% [± 0.10], Poly I:C = 2.83% [± 0.71]). These differences in percentage of live cells expressing CD271 among tissue of origin and inflammatory agent were further confirmed by MFI analysis (Figure 3-19, D) of CD271. Under resting conditions, BM MSCs had significantly higher expression levels of CD271 compared to Is and Ad MSCs (BM = 15.76 [± 2.15], Is = 3.86 [± 0.99], Ad = 1.63 [± 0.34]). MSC licensing had no effect in the expression levels of CD271 in Is (Cytokines = 4.29 [± 1.18], LPS = 4.75 [± 1.19], LTA = 4.63 [± 1.06], Poly I:C = 4.4 [± 0.29]) and Ad MSCs (Cytokines = 1.1 [± 0.24], LPS = 1.11 [± 0.13], LTA = 1.08 [± 0.19], Poly I:C = 1.55 [± 0.22]) and only had a significant effect in LPS-mediated licensing in BM MSCs (Cytokines = 42.66 [± 2.49], LPS = 39.16 [± 1.43], LTA = 41.56 [± 4.37], Poly I:C = 42.33 [± 3.39]).

Under resting conditions, <2% of the MSCs isolated from the three tissues were positive for MHC Class II. Despite the low number of cells expressing this marker, tissue source had an effect on the number of live cells expressing MHC Class II as Is MSCs (0.24% [± 0.07]) had significantly fewer positive cells for this marker compared to BM (1.64% [± 0.39]) and Ad MSCs (1.94% [± 0.38]) (Figure 3-18, F).

BM MSC licensing by cytokines, LTA and Poly I:C led to an increase of the percentage of cells expressing MHC Class II (from 1.64% [± 0.39] to 5.14% [± 0.28], 2.02% [± 0.24] and 1.85% [± 0.18] respectively), while LPS licensing led to a decrease of the percentage of cells expressing MHC Class II, from 1.64% (± 0.39) to 1.54% (± 0.28); only cytokine-mediated licensing produced a statistically significant change in the percentage of BM MSCs expressing this marker. Licensing of Is MSCs led to a statistically non-significant decrease of the percentage of cells expressing MHC Class II (Cytokines = 0.19% [± 0.06], LPS = 0.12% [± 0.03], LTA = 0.16% [± 0.04], Poly I:C = 0.12% [± 0.02]). In the case of Ad MSCs, licensing of these cells led to a statistically non-significant decrease of the percentage of cells expressing MHC Class II when stimulated with cytokines (from 1.94% [± 0.38] to 1.36% [±0.28]), while LPS, LTA and Poly I:C licensing led to a statistically non-significant increase of the percentage of cells expressing MHC Class II (LPS = 1.97% [± 0.30], LTA = 1.53% [± 0.33], Poly I:C = 1.76% [± 0.28]). These differences in percentage of live cells expressing MHC Class II among tissue of origin and inflammatory agent were further confirmed by MFI analysis (Figure 3-19, E). Under resting conditions, tissue source of origin had no significant effect on the expression levels of MHC Class II (BM = 2.63 [± 0.77], Is = 2.4 [± 0.37], Ad = 1.15 [± 0.09]). Licensing of MSCs produced no significant alteration on the median fluorescence intensity of the MHC Class II antibody in MSCs isolated from the BM (Cytokines = 6.56 [± 0.56], LPS = 3.52 [± 0.69], LTA = 3.17 [± 0.50], Poly I:C = 3.36 [± 0.60]), Is (Cytokines = 2.7 [± 3.38], LPS = 3.38 [± 0.53], LTA = 2.72 [± 0.49], Poly I:C = 2.16 [± 0.24]) and Ad (Cytokines = 1.20 [± 0.29], LPS = 1.33 [± 0.27], LTA = 1.56 [± 0.14], Poly I:C = 1.23 [± 0.21]) with the exception of cytokine-mediated licensing in BM MSCs (from 2.63 [± 0.77] to 6.56 [± 0.56]).

Under resting conditions, the percentage of MHC Class I positive MSCs showed high variations from source to source with BM MSCs expressing the highest number of live positive cells (25.21% [± 2.15]), followed by Is MSCs (10.01% [± 1.14]) and almost an inexistent number of cells expressing this marker in Ad MSCs (0.68% [± 0.02]) (Figure 3-18, G). All the licensing agents were able to produce a statistically significant increase in the percentage of positive cells for MHC Class I in the BM MSCs; this increase was highest when cells were treated with LPS (Cytokines = 48.5% [± 1.96], LPS = 78.95% [± 2.43], LTA = 28.49% [±

1.34], Poly I:C = 30.73% [± 1.82]). Within the Is MSCs, all the stimulatory agents were able to produce a statistically significant increase in the percentage of cells expressing this marker (Cytokines = 80.34% [± 2.93], LPS = 24.073% [± 2.27], LTA = 11.376% [± 1.39], Poly I:C = 13.56% [± 2.07]. Very few cells within the Ad MSCs were positive for MHC Class I and only cytokine produced licensing increased this percentage in a statistically significant manner, from 0.68% (±0.02) to 23.9% (±3.52); LPS-mediated licensing led to a 0.76% [± 0.10] of the cells expressing the marker while LTA and Poly I:C produced a slight decrease of the percentage of cells expressing the marker (LTA = 0.66% [±0.11], Poly I:C = 0.56% [±0.08]). These differences in percentage of live cells expressing MHC Class I among tissue of origin and inflammatory agent were further confirmed by MFI analysis (Figure 3-19, F). Despite the high variation in the percentage of MSCs positive for MHC Class I among tissue source, under resting conditions tissue source of origin had no significant effect on the expression levels of MHC Class I (BM = 24.93 [± 2.80], Is = 19.42 [± 2.99], Ad = 16 [± 0.81]). Cytokine and LPS-mediated licensing produced a statistically significant increase in the expression levels of MHC Class I in BM MSCs (Cytokines = 41.97 [± 4.52], LPS = 71.27 [± 6.50], LTA = 33.27 [± 4.00], Poly I:C = 30.73 [± 1.82]). In the case of Is MSCs, only cytokine-mediated licensing produced a statistically significant increase in the expression levels of anti-MHC Class I (Cytokines = 46.03 [± 4.00], LPS = 22.68 [± 3.48], LTA = 18.54 [± 5.12], Poly I:C = 17.56 [± 4.44]). On the other hand, licensing of Ad MSCs produced no effect in the expression levels of MHC Class I (Cytokines = 21 [± 1.63], LPS = 16 [± 0.81], LTA = 14.33 [± 1.24], Poly I:C = 15 [± 2.44]).

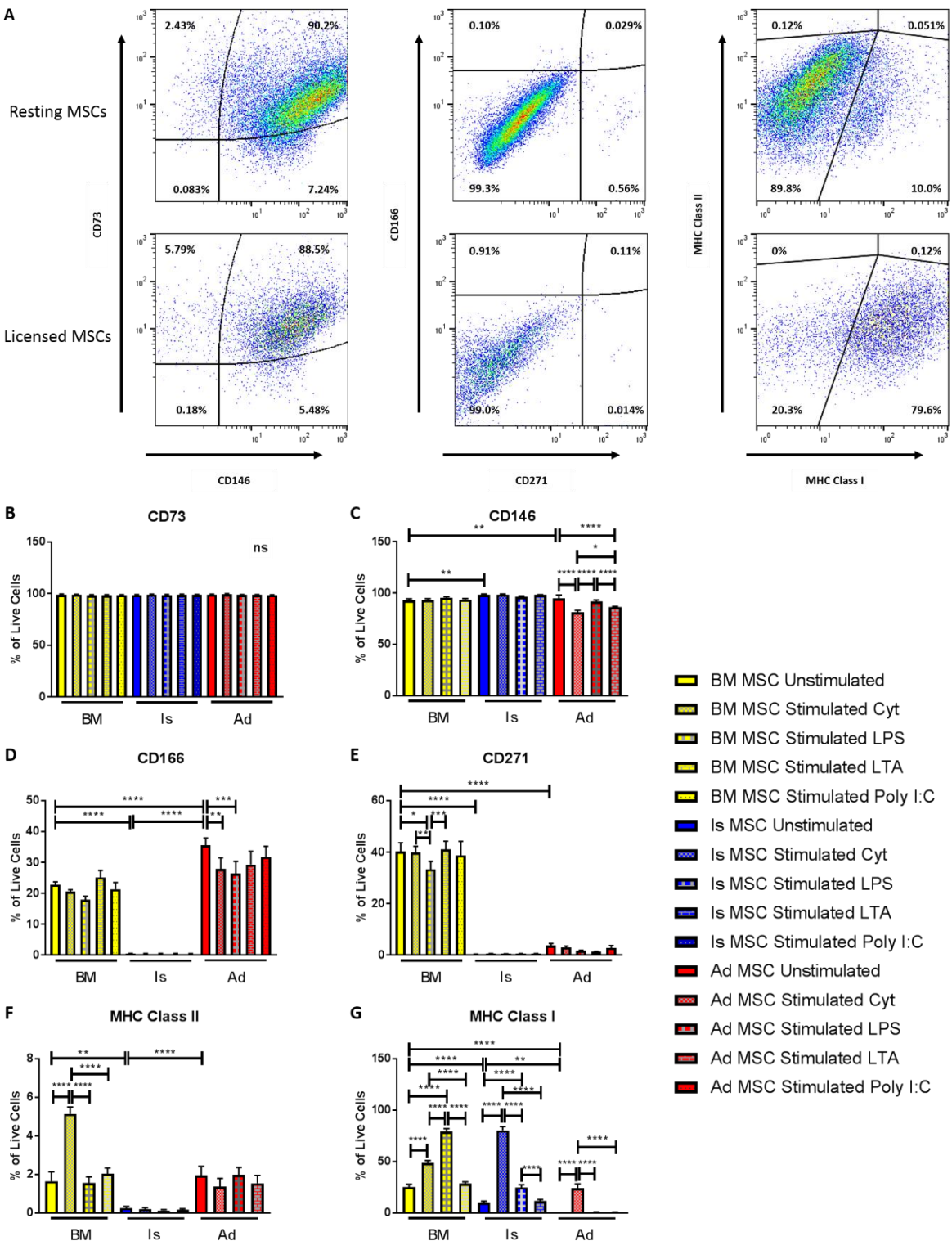


Figure 3-18. Surface molecule phenotype of MSCs following inflammatory stimulation. MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with either a cocktail of cytokines (40 ng/mL of IFN-Ɣ, TNF-α and IL-1β), 100 ng/mL LPS, 100 ng/mL LTA or 4μg/mL Poly I:C. Unstimulated cells were left growing in MSC culture medium as a control. 24 hours after stimulation, MSCs were stained with a cocktail of antibodies, to allow characterisation of MSCs by flow cytometry (**A**). The% of live MSCs which stained positive for each of the tested markers is graphed to compare expression between MSC tissue sources (**B-G**). Each bar represents an n of 3 independent experiments and is graphed as mean ± SEM. ONE WAY ANOVA with Tukey's multiple comparison post-test analysis was used for statistical assessment of differences between MSC sources and licensing agent. Significant differences are marked with the appropriate number of asterisks. p = 0.05 was considered the limit for statistical significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

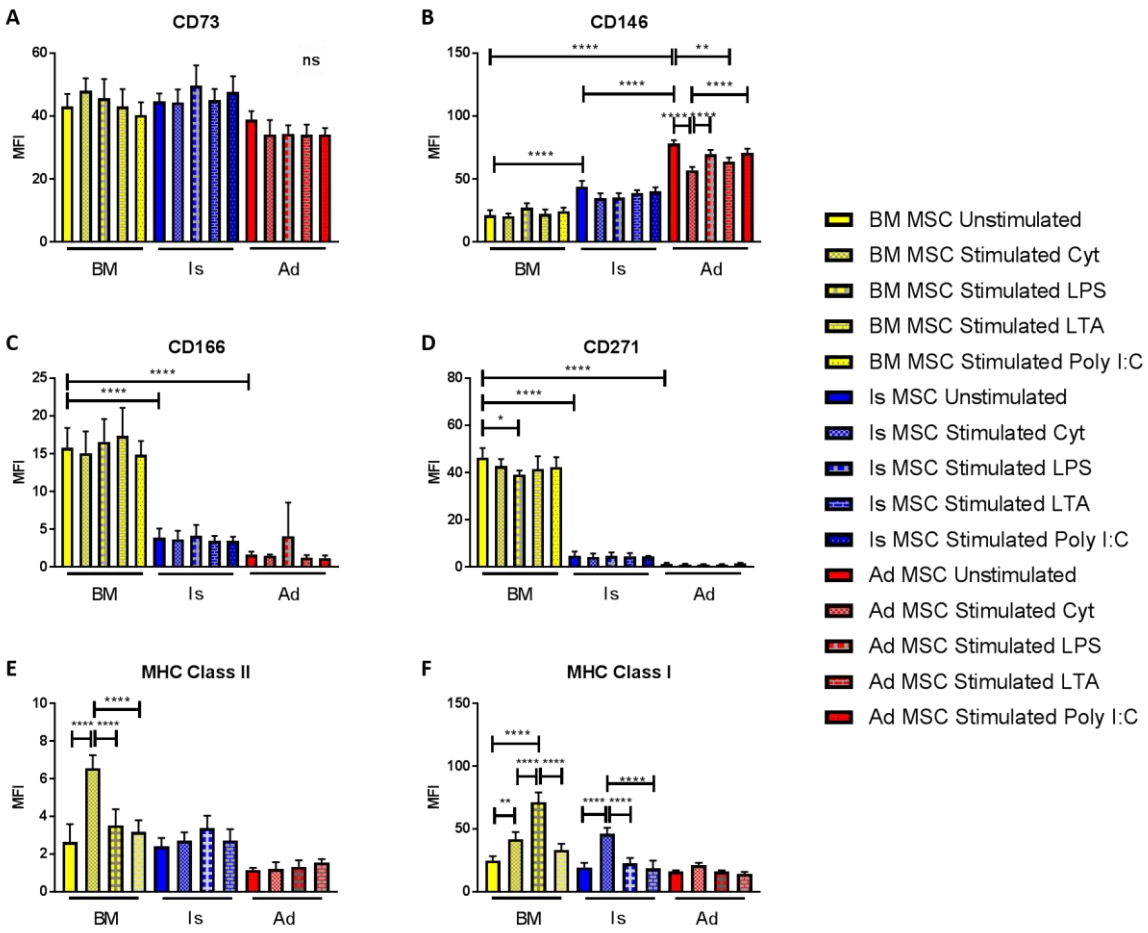


Figure 3-19. MFI of surface molecules expressed on MSCs following inflammatory stimulation.
Data shown in Figure 3-18 has been represented as mean fluorescence intensity (A-F). Each bar represents an n of 3 independent experiments and is graphed as mean ± SEM. ONE WAY ANOVA with Tukey's multiple comparison post-test analysis was used for statistical assessment of differences between MSC sources and licensing agent. Significant differences are marked with the appropriate number of asterisks. $p = 0.05$ was considered the limit for statistical significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

In summary, CD73 surface marker staining detected the highest percentage of positive cells (> 97%) of all markers tested and there was no significant change in its expression after 24 hours of stimulation. CD146 staining detected a high percentage of positive cells (>92.6%) by MSCs from every tissue source, where Is MSCs had highest percentage of cells staining positive for this marker and Ad MSCs had the highest expression levels of CD146. 24 hours of stimulation did not produce any significant effect on the percentage of cells expressing CD146 in BM and Is MSCs but produced a decrease in the percentage of cells expressing this marker on Ad MSCs. CD166 and CD271 showed tissue specific expression levels, where BM MSCs had the highest percentage of positive cells and Ad MSCs the

lowest percentage of cells expressing these markers; inflammatory stimulation had little effect on these markers as it produced no change in the percentage of cells expressing this marker in Is MSCs and very subtle changes in BM and Ad MSCs after 24 hours cytokine- and LPS-mediated inflammation. Despite MHC Class II expression levels being very low in MSCs from every source, there were statistically significant differences in the percentage of positive cells among tissue sources. 24 hours of stimulation with the different licensing agents produced no statistically significant variations except for cytokine-mediated stimulation in BM MSCs. The percentage of positive cells for MHC Class I was affected by tissue source; however, this percentage increased in MSCs from the three tissues after cytokine stimulation and in BM and Is MSCs after LPS stimulation. LPS-mediated licensing produced the highest increase in the percentage of positive cells for MHC Class I in BM MSCs while Is MSCs responded better to cytokine-mediated stimulation.

3.8 Discussion and conclusions

During the past decades, MSCs have been extensively investigated both in basic and clinical research; however, there is a lot of inconsistency and controversy in this topic. Despite the efforts of the ISCT to establish minimum requirements for what constitutes a mesenchymal stromal cell in humans, there are still significant sources of variability during the manufacture of these cells, including isolation protocols, culture conditions and the intrinsic variability among source material. In order to reduce manipulation and contamination risk and to create a cellular product for clinical applications that could comply with Good Manufacturing Practice (GMP) standards, the aim of this thesis was to establish a standardised easily reproducible methodology for isolation and culture of murine MSCs that produced consistent results. A further aim of this chapter was to determine if the developed methodologies for murine MSC isolation and culture produced cells that satisfied the minimal criteria established by the ISCT to be considered MSCs. It is important to mention that ISCT criteria were established for the identification of human MSCs and although adherence and trilineage differentiation are characteristics of murine MSCs, the surface marker phenotype

of murine MSCs is not well characterised. In fact, induction of CD45 expression on murine BM MSCs has already been described (Yeh et al., 2006).

Moreover, we aimed to determine if tissue of origin could influence the phenotype of mouse MSCs. Finally, as the ultimate aim is to use MSCs as cellular therapeutics where they would most likely be infused in inflammatory environments, and as pro-inflammatory stimulation leads to the production of immunomodulatory factors by MSCs, the phenotype of MSCs was also assessed under different inflammatory conditions (Krampera, 2011).

Since their discovery in 1976, MSCs have been described as plastic adherent, spindle-shaped cells; therefore, the ISCT established this morphology as their first criteria for defining MSCs (Horwitz et al., 2005). BM and Ad MSCs satisfied this criterion from isolation to later passage (P3), while Is MSCs did not show this morphology until P2. Is MSCs were isolated from islets of Langerhans. Islets of Langerhans are clusters of 5 mixed populations of endocrine cells, more precisely, α-cells, β-cells, δ-cells, ε-cells and γ-cells, which produce glucagon, insulin, somatostatin, ghrelin and pancreatic polypeptide respectively. ß-cells have been described to undergo an EMT process when expanded in monolayer culture, giving rise to MSCs (Moreno-Amador et al., 2018), which would explain the mixture of morphologically distinct populations observed at P1.

ISCT states that these spindle–shaped cells must be able to differentiate into adipocytes, chondrocytes and osteocytes (Dominici et al., 2006), criterion that was fulfilled by cells isolated from all three tissues.

Lastly, in order to qualify as MSCs, multipotent spindle–shaped cells must express an array of mesenchymal markers including CD90, CD105, CD73 and lack the B cell marker CD19, the primitive haematopoietic progenitor marker CD34, the pan–leukocyte marker CD45 and the monocyte/ macrophage marker CD11b. It is important to keep in mind that ISCT criteria were established for human MSCs; thus, variations might apply, and have been described, for murine MSCs. Sca-1 is the most recognised haematopoietic stem cell marker in mice but for consistency reasons with the ISCT criteria CD34 primitive haematopoietic progenitor marker was used for haematopoietic contamination exclusion. Ideally, these three criteria should be enough to identify MSCs; however,

fibroblasts are plastic adherent spindle-shaped cells that are positive for the expression of the surface markers CD73, CD90 and CD105 and that have proven tripotency (Haniffa et al., 2009). For this reason, other markers are often used to identify MSCs such as CD146, which has weak expression on fibroblasts and high for MSCs (Lv et al., 2014), CD166 and CD271. However, the expression of these markers is not consistent among tissue source and the percentage of positive cells expressing these surface markers fluctuate through passage (Kang et al., 2016, Maleki et al., 2014).

CD90, also known as Thy-1, is a glycophosphatidylinositol-anchored protein that was first discovered in mouse T cells but is predominantly expressed on subsets of fibroblast, lymphocytes and stem cells (Sauzay et al., 2019). As a cell surface glycoprotein, its role is related to cell-cell and cell-matrix interactions within an inflammatory and wound healing context (Leyton and Hagood, 2014). MSCs isolated from the BM, Is and Ad expressed very high levels of CD90 with more than 98% of the cells being positive for this marker regardless of tissue source. CD105, also known as Endoglin, is a component of the TGF-β receptor complex expressed in endothelial cells, haematopoietic stem cells and MSCs, which is involved in blood vessel development, erythropoiesis and myelopoiesis (Cho et al., 2001) and neovascularisation (Fonsatti et al., 2003).

Human MSCs have been stated to be CD105 positive, however, there are discrepancies regarding the CD105 phenotype in murine MSCs. Anderson et al. stated that CD105 is induced in a subpopulation of murine MSCs early upon *in vitro* culture giving rise to $CD105^+$ and $CD105^-$ populations of MSCs that have almost identical surface marker phenotypes ($CD29^+CD44^+Sca1^+$MHC Class I^+ and $CD45^-CD11b^-CD31^-$) but which vary in their differentiation and immunoregulatory properties (Anderson et al., 2013). In addition, culture medium influences expression levels of CD105; serum-supplemented media contains TGF-β, which induces CD105 expression leading to the downregulation of its surface expression (Mark et al., 2013). Despite MSCs having been described to have variable levels of CD105, tissue source of origin was not found to produce a change in the surface expression of CD105 as more than 98% of BM, Is and Ad MSCs stained positive for this marker.

Similarly, CD73 was also expressed by cells from every tissue source with more than 98% of the cells expressing this marker. CD73, an enzyme that converts adenosine monophosphate to adenosine, has been linked to the regulatory phenotypes of T and NK cells, as well as to MSCs. Extracellular ATP acts on many immune cells to promote inflammation while the ATP metabolite adenosine is mostly an anti-inflammatory molecule, which makes CD73 essential for the modulation of the immune response (Regateiro et al., 2013). In addition, CD73 is involved in tissue injury (Ryzhov et al., 2019), lymphocytes adhesion to the epithelium (Linden and Cekic, 2012) and is a key regulatory molecule for cancer cell proliferation, migration and invasion *in vitro* and tumour angiogenesis and immune escape *in vivo* (Chen et al., 2019). The high number of positive cells for CD90, CD105 and CD73 in MSCs from all three tissue sources suggests an immunomodulatory role within the context of inflammation.

Even if the positive expression of these markers enables the characterisation of MSCs, the expression of these markers is not MSC exclusive, and it is essential to ensure that they lack other cell-type associated markers. As CD90, CD105 and CD73 are all present in haematopoietic stem cells, ensuring that MSCs lack the primitive haematopoietic progenitor marker CD34, a transmembrane phosphoglycoprotein expressed by haematopoietic stem cells, was essential. B cells might adhere to MSCs during isolation and remain viable in culture; therefore, B cell exclusion was done by CD19 B cell marker analysis. CD73 is expressed by T cells and macrophages and monocytes which are the cell types most likely to be found in MSC cultures. Thus, the pan-leukocyte marker CD45 and the monocyte and macrophage marker CD11b are essential for exclusion of these cells.

The percentage of live cells positive for CD45 was <2% for Ad MSCs, ~30% for Is MSCs and ~85% BM MSCs while the percentage of cells positive for CD11b was ‹2% for Ad MSCs, ~25% for Is MSCs and ~85% for BM MSCs. Although induction of CD45 expression on murine BM MSCs had already been described (Yeh et al., 2006), cells in the current study were further characterised for expression of CD64, which is only expressed in macrophages and monocytes, and it was observed that the expression of this marker was minimal in the cells isolated from the three sources. Thus, it may be concluded that the isolated cells were not of myeloid

origin. In addition, macrophages and other leukocytes lack the trilineage potential, which further confirms that these cells were not from myeloid origin.

MSCs are similar to fibroblasts as they have a similar morphology and are plastic adherent (Haniffa et al., 2009). Moreover, human dermal fibroblasts have also been shown to have tripotency (Junker et al., 2010) and immunoregulatory functions similar to MSCs (Haniffa et al., 2007) and to express most MSC markers such as CD105, CD73 and CD90 (Schwab et al., 2008). Fibroblast contamination is a potential impediment for regenerative medicine due to their senescence and transformation upon long-term expansion (Zhou et al., 2006); which makes proper MSC isolation essential. CD146 expression has found to be weak for fibroblasts and high for MSCs (Lv et al., 2014) and can therefore be used for discrimination between these two cell types. BM, Is and Ad MSCs all had a very high percentage of positive cells for CD146 (BM = 92.64% [±1.44], Is = 98.21% [±0.74], Ad = 94.73% [±2.73]), which definitely shows that the cells isolated from these tissue sources can be considered MSCs.

Isolation of murine MSCs from exocrine pancreatic culture has been reported in the literature; however, to my knowledge, no studies have reported isolation of murine MSCs from adult islets of Langerhans. Human Is MSCs have previously been isolated and characterised but CD45 expression had not been characterised in Is MSCs before. Analysis of the CD45 positive cells isolated from the islets of Langerhans confirmed the presence of a CD45 positive population, which was smaller in size, and less complex in granularity, than the CD45 negative Is MSCs, and which had the potential to differentiate into adipocytes, chondrocytes and osteoblasts.

There is controversy regarding the origin of Is MSCs. BM MSCs were suggested to migrate to the pancreas contributing to pancreatic regeneration and turnover (Song et al., 2013). However, after transplantation of green fluorescent protein (GFP) positive, sex-mismatched bone marrow into mice, no GFP positive ß-cells were found, which challenged this hypothesis (Lechner et al., 2004). Epithelial to mesenchymal transition, a process in which epithelial cells change their phenotype to become mesenchymal cells, has been suggested as a mechanism leading to the generation of MSCs from cultured endocrine pancreas (Moreno-Amador et al., 2018, Gershengorn et al., 2004). EMT would explain the different

morphologies observed when assessing the cell morphology of Is MSCs at P1. The small, polarised, epithelial cells start decreasing the expression of the adhesion molecules between epithelial cells, losing their apical-basal polarity and becoming spindle in shape while an increase in the mesenchymal markers takes place. During EMT, cells do not necessarily exist in “pure” epithelial or mesenchymal states; there are hybrid populations that share mixed features, which are termed as the intermediate cell states (ICSs). At P1, polarised and spindle shaped cells could be observed, while at later passages only the spindle shaped cells were present. Immunocytochemistry and flow cytometry analysis confirmed that soon after isolation these cells were positive for EpCAM and that only a small percentage, ~20% of the cells, were co-expressing vimentin, the mesenchymal marker. However, at P3, most of the cells had completed the EMT process with only ~20% of ICSs co-expressing EpCAM and vimentin, which confirms the hypothesis of pancreatic MSCs having an EMT origin. While the exact functions of ICSs are unclear, they have important roles in embryogenesis, tissue development, and pathological processes such as cancer metastasis. In addition, ICSs have been described to have greater potency/ stemness than “pure” mesenchymal cells, which makes them better for reprogramming (Sha et al., 2019).

MSCs are being evaluated as cellular therapeutics for inflammatory conditions and diseases, for this reason, understanding their phenotype under these conditions is of significance importance as it could provide preferential tissue sources for MSC isolation for cell therapeutics (Galipeau, 2016).

T cells are able to eliminate infected cells by closely interacting with other cells and discriminating the ones that have been affected by a pathogenic threat. Antigen presentation is crucial for discrimination and is carried by the major histocompatibility complex, which displays on the cell surface information about the different antigens that the cells are processing or have ingested. On the one hand, endogenous antigens, which can be self or foreign, are presented to cytotoxic T lymphocytes as peptides bound to MHC Class I molecules, which is expressed on all nucleated cells; while antigens engulfed into endocytic compartments are presented to helper T cells (CD4) as peptides bound to MHC Class II molecules, which are expressed in antigen presenting cells such as B

cells, monocytes, macrophages and dendritic cells and on epithelial cells following inflammatory signals. In this way, T cells can interact with these presented peptides to identify and eliminate cells expressing microbial genes, mutant sequences or foreign polymorphic genes from transplants. As nucleated cells, MSCs express MHC Class I molecules, however, they trigger a very weak cellular and humoral allogenic immune response due to their minimal expression of MHC Class II and the absence of co-stimulatory molecules CD40, CD40 ligand, CD80 and CD86 (Ryan et al., 2005). In line with the literature, BM, Is and Ad MSCs express MHC Class I under resting conditions which was upregulated after MSC licensing (Schu et al., 2012). In addition, the expression of MHC Class II on BM, Is and Ad MSCs was very low under resting conditions but as expected (Griffin et al., 2013), was slightly upregulated after MSC licensing. Nevertheless, MHC Class II levels were still low, which suggests an ability to escape alloreactive $CD4^+$ T cells recognition.

CD73 expression levels were not affected by licensing or tissue source and were very high. One study suggests that the role of CD73 in MSCs is related to attenuating $CCR2^+$ macrophage infiltration and upregulating several anti-inflammatory genes producing a pronounced anti-inflammatory activity (Tan et al., 2019).

Regarding CD146, MCAM, is a commonly used MSC marker with reported variable expression depending on MSC tissue of origin (Kang et al., 2016). However, here we found little variation, as all MSC sources showed very high expression of CD146, Ad MSCs having the highest expression and BM MSCs the lowest. Considering the percentage of live cells expressing this marker, MSC licensing slightly increased CD146 expression in BM MSCs while the overall trend in Is and Ad MSCs was a decrease of expression of this marker after licensing. One study suggests that CD146 is involved in MSC trans-endothelial migration, therefore the lower the levels of CD146 the easier it could be for the cells to be retained within the inflammatory site and immunomodulate it (Ode et al., 2011).

Like CD146, CD166 is a marker of MSCs described to have very variable levels among MSCs (Szepesi et al., 2016). In line with the literature, the percentage of MSCs positive for CD166 under resting conditions showed extensive variation from source to source with Ad MSCs expressing the highest levels among the

compared sources of MSCs. This large variation was exaggerated when MSCs were maintained under inflammatory conditions, however the overall trend of MSC CD166 expression was to downregulate when stimulated. CD166, also known as activated leukocyte cell adhesion molecule, is involved in cell adhesion and migration so as with CD146, its downregulation could be a mechanism used by MSCs to minimise their migration and be retained within the inflammatory site to fulfil their immunomodulatory role (Swart, 2002).

CD271 is a universal marker for MSCs; however, its expression has been linked with a specific subpopulation of trabecular bone-associated MSCs (Attar et al., 2013), which correlates with the fact of BM MSCs express significantly high levels of this marker and very low expression of CD271 by Ad and Is MSCs. BM and Ad MSCs downregulated CD271 expression after stimulatory licensing while Is MSCs slightly upregulated their almost inexistent CD271 levels. CD271 is a marker of interest as it is involved in cell growth and differentiation; $CD271^{+}$ cells have increased multipotency therefore methodologies to induce its expression in MSCs, as MSC co-culture with endothelial cells, are being developed, (Bellagamba et al., 2018).

One of the routes of delivery of MSCs into patients is intravenous infusion, but tracking of the cells shows that only a small percentage of the cells home and remain in the target tissue which could be due to MSC trapping in the lung after intravenous delivery (Fischer et al., 2009, Schrepfer et al., 2007). However, MSCs are able to exert their anti-inflammatory role without homing to target inflammatory sites in significant numbers and despite the fact that the cells disappear within short periods of time (Kurtz, 2008). The size of the cells could be an essential factor involved in the lung trapping, for this reason, the size of MSCs was compared among tissue sources and after licensing. BM MSCs are slightly smaller than Is and Ad MSCs, which suggests that they could be a better source of MSC for intravenous delivery. Inflammatory licensed MSCs have been described to exhibit an increased immunomodulation in inflammatory environments due to upregulation of anti-inflammatory molecule expression (Cassano et al., 2018). Some of these molecules are stored in granules, for this reason, we compared the granularity of MSCs from different tissues before and after stimulation. Under resting conditions, BM MSCs were the most granular

cells. Stimulation of MSCs produced little variation or decreased the granularity of MSCs from every tissue.

3.8.1 Conclusions

Altogether, the expression of the widely used MSC markers CD90, CD105 and CD73 remained constant among tissue source and inflammatory stimulus. Cells isolated from the bone marrow and islets of Langerhans were $CD45^+$ $CD11b^+$ and, for this reason, did not meet ISCT criteria. However, fibroblast and monocyte/ macrophage contamination were excluded from all the cultures. ISCT criteria were established for the identification of human MSCs and the surface marker phenotype of murine MSCs is not so well characterised. For this reason, based on plastic adherence, trilineage differentiation potential and CD90, CD105 and CD73 expression, we determined that cells isolated from the bone marrow, islets of Langerhans and adipose tissue were MSCs regardless of CD45 and CD11b expression. Moreover, specific criteria to enable the identification of murine MSCs should be established. CD146, CD166 and CD271 levels varied among tissue source, which suggests that MSC tissue of origin has an effect on migratory and differentiation potential. More importantly, MHC Class I and II molecules were upregulated under inflammatory licensing, with BM MSCs having the highest MHC Class II levels and Is MSCs having the highest MHC Class I levels after stimulation. Regarding the use of MSC as cellular therapeutics, these results might have implications in selection of tissue source of origin for treatment. In respect of size and granularity, BM MSCs are the smallest and most granular among the compared MSCs, which makes them, so far, the best candidates for cell therapy.

Chapter 4

Profiling of MSC chemokine and chemokine receptor expression at the transcript and protein level

4 Profiling of MSC chemokine and chemokine receptor expression at the transcript and protein level

4.1 Introduction and aims

In Chapter 3, thorough characterisation determined that cells isolated from adipose tissue satisfied the criteria of the ISCT and can therefore be considered MSCs. Cell isolated from the bone marrow and islets of Langerhans retain the haematopoietic markers CD45 and CD11b, and thus, do not meet ISCT criteria for human MSCs, but nonetheless, are MSCs. The overall aim of this thesis was to study the behaviour of BM, Is and Ad MSCs by studying their interaction with the immune system in order to better understand their regenerative and immunomodulatory potential to be used as cellular therapeutics. According to the United States National Library of Medicine, as of 26th of July of 2019, MSCs have received nine cell therapy approvals and one tissue engineering approval and 8133 studies are being carried out examining their efficacy in a variety of clinical applications such as bone, myocardium, kidney and liver repair and co-transplantation with haematopoietic stem cells among others (Horwitz et al., 1999, Baron et al., 2010, Mao et al., 2017, Ezquer et al., 2017, Saberi et al., 2019).

One critical aspect of MSC use as cellular therapeutics is related to the delivery method. The optimal method should provide the highest regenerative benefit with the lowest side effects. The most used routes of MSC administration, outside tissue-engineering-based methods, are direct injection into the tissue of interest and systemic infusion, both intravenously or intra-arterially. Systemic infusion is much less invasive than direct administration while direct injection should have the advantage of a much more precise localization of the cells. However, an engraftment study using three different administration methods in cardiac disease showed that only 1 to 5% of delivered cells engraft within the target site regardless of the delivery route (Freyman et al., 2006). In addition of being less invasive, systemic infusion enables easy access to oxygen and nutrients, which is why systemic infusion is often the preferred method for MSC delivery (Sarkar et al., 2011). Systemic infusion of MSCs leads to engraftment of MSCs mostly in the lungs, which is likely generated by the small capillary size,

the large size of the cells and their strong adhesion properties. However, MSCs can also be found in the spleen, liver, bone marrow, thymus, kidney and skin, seconds or minutes after IV injection, but, it is still unknown if MSCs migrate specifically to these organs or if they just get trapped (Devine et al., 2003, Fischer et al., 2009, Freyman et al., 2006, Schrepfer et al., 2007).

MSC entrapment represents a major bottleneck to reach the full therapeutic potential of MSC-based therapies. In order to improve this situation, better understanding of *in vivo* migration to target tissues and cell persistence within them is essential. In the presence of inflammation, 1 to 5% of delivered cells are able to escape the lung entrapment and migrate and home within the target site, which suggests that chemotactic stimuli could be guiding infused MSCs to sites of inflammation (Rustad and Gurtner, 2012). Chemokines are master regulators in immune cell trafficking under resting and inflammatory conditions; for this reason, we hypothesised that chemokines and their receptors should be involved in the homing of these cells to, and persistence at, sites of inflammation. Human and murine MSCs have been described to constitutively express chemokines and their receptors. CCR1, CCR7, CXCR2, CXCR4, CXCR6 and CX3CR1 are all important in the context of homing but the expression of these markers varies among reports in the literature due to tissue source of the cells, cell culture methods and passage number of the cells. Murine BM MSCs have been described to express CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR9, CCR10, CXCR3, CXCR4 and CXCR7 and to have functional CCR3, CCR4, CCR5, CCR7, CCR10 and CXCR4 (Alexeev et al., 2013). Ad MSCs have higher CXCR4 expression and migration capacity than BM MSCs and more interestingly, the chemokine receptor profile is sensitive to time in culture as the expression of chemokine receptors CCR1, CCR7, CXCR1, CXCR2, CXCR4 and CX3CR1 was decreased after passage (Heirani-Tabasi et al., 2017). In addition, expression of chemokine receptors and ligands can be upregulated by cytokine-mediated stimulation (Croitoru-Lamoury et al., 2007). All this together suggests that the tissue source of origin of the MSCs is associated with differential chemokine receptor expression and therefore, different homing potential to sites of inflammation, which could be of relevance when used as cellular therapeutics.

In a similar way, *in vitro* cultured MSCs have been proven to secrete a wide range of chemokines, such as CCL2, CCL3, CCL4, CCL5, CCL20, CXCL1, CXCL2, CXCL5, CXCL8, CXCL12 and CX3CL1, and to recruit cells from the immune system; for this reason, we consider that understanding MSC chemokine secretion will enable us to predict the interaction of these cells with the immune system and therefore, their immunomodulatory potential in an *in vivo* setting (Chen et al., 2008, Ren et al., 2008, Meirelles Lda et al., 2009).

As chemokine receptors seem to be involved in MSC homing to sites of inflammation, and chemokine ligands attract leukocytes towards them, we hypothesised that chemokines could have an extremely important role in the immunomodulatory potential of MSCs as they have the potential to orchestrate the migration and positioning of immune cells within the tissues. Therefore, the aims of this chapter were to i) characterise chemokine and chemokine receptor transcript and protein levels in MSCs, ii) identify the effect of MSC tissue source of origin in the expression of these molecules and iii) study how MSC licensing alters MSC chemokine and chemokine receptor transcription and expression.

Results

Throughout this results section, the transcription and expression profile of chemokines and their receptors is described in detail in each section under resting and inflammatory conditions. Transcriptional profiling enabled the identification of the chemokines that had undergone the most significant variations at transcriptional level upon stimulation. The secretion profile of those chemokines by MSCs from the three sources was then analysed under resting and stimulatory conditions.

4.2 Analysis of the effect of a single inflammatory stimulus on the transcription of chemokines and chemokine receptors

To understand the mechanisms involved in MSC migration and immunomodulation, it was important to understand their chemokine and chemokine receptor expression. To do this, the most relevant chemokines and their receptors in the MSCs context were selected based on previous work carried out by Dr Kayleigh Thirlwell on human MSCs (Thirlwell, 2018), and their transcription was assessed. BM, Is and Ad MSCs were transcriptionally assessed under resting and inflammatory (40 ng/ mL of TNF-α, IL-1ß and IFN-ϒ) conditions to determine the effect of tissue source and/ or inflammatory conditions on the MSC chemokine and chemokine receptor transcriptional profile.

Transcription data were normalised to the house keeping gene *beta-2 microglobulin* (*B2M*) to consider variations in RNA quality and quantity. As this study did not have a reference sample, data are represented as $2^{(-\Delta CT)}$, which enables the visualization of expression levels of specific genes normalised to *B2M* for each sample. Due to the nature of normalisation, genes that generated a CT of 35 or above resulted in $2^{(-\Delta CT)}$ less than or equivalent to ~0.0001 and are marked with a red box on the following graphs and are likely not transcribed at meaningful levels by MSCs (Thirlwell, 2018).

4.2.1 Transcription of CC chemokines under resting and inflammatory conditions

Under resting conditions, BM, Is and Ad MSCs transcribed very few, if any, CC chemokines with the exception of *CCL2*, which was transcribed by all tissue source MSCs at similar levels (Figure 4-1, A), and *CCL7*, which was very highly expressed by BM MSCs (E). A pattern of transcriptional upregulation after stimulation was observed in *CCL2* (A), *CCL3* (B), *CCL5* (D), *CCL7* (E), *CCL11* (G), *CCL19* (H) and *CCL20* (I). However, these chemokines were upregulated differentially in MSCs according to their tissue of origin. *CCL9* (F) was the only chemokine that was downregulated after 24-hour cytokine-mediated stimulation. Fold changes of transcriptional regulation upon cytokine-mediated licensing of MSCs are specified in Table 4-1.

BM MSCs expressed the highest levels of *CCL2* under resting and inflammatory conditions, while Is MSCs had the highest levels of *CCL3* under resting and inflammatory conditions. Inflammatory stimulation resulted in a substantial and significant upregulation of both *CCL2* and *CCL3* transcripts in MSCs from all sources. MSCs from every source did not show any statistically significant variation in their *CCL4* levels after stimulation; these levels were much more consistent (at a low level) in the BM MSCs (C). *CCL5* transcript levels were very low under resting conditions but inflammatory stimulation led to a marked increase in expression levels in BM and Is MSCs. As previously mentioned, *CCL7* was expressed at very substantial levels under resting conditions in all MSCs; however, tissue of origin of MSCs had an effect in the transcript levels as BM MSCs expressed much higher levels than Is and Ad MSCs. Inflammatory stimulation led to a substantial and significant upregulation of *CCL7* transcripts by BM, Is and Ad MSCs. *CCL9* transcripts were the highest in BM MSCs and *CCL9* was the only chemokine in which transcript levels decreased after inflammatory stimulation. *CCL11* and *CCL19* had similar levels of transcription under resting conditions and inflammatory stimulation produced an upregulation of these chemokines in MSCs from all sources; however, stimulated BM MSCs had the highest transcript levels of *CCL11,* while *CCL19* was expressed in higher amounts by licensed Is MSCs. *CCL20* showed a similar pattern of transcriptional regulation to *CCL5*, with very low levels of transcript under resting conditions and substantial levels after stimulation. BM MSCs had the highest levels of *CCL20* transcription, closely followed by Ad MSCs, while Is MSCs had much lower levels.

To sum up, MSCs from all sources expressed a variety of CCL chemokines, which could be involved in the recruitment of immune cells such as monocytes, macrophages, NK cells, eosinophils and B cells. Under resting conditions, MSCs transcribed very low levels of CCLs, except for *CCL2* and *CCL7*; however, inflammatory stimulation resulted in a marked upregulation of CCL chemokine transcription by all tissue sources of MSCs except *CCL9*, which was downregulated.

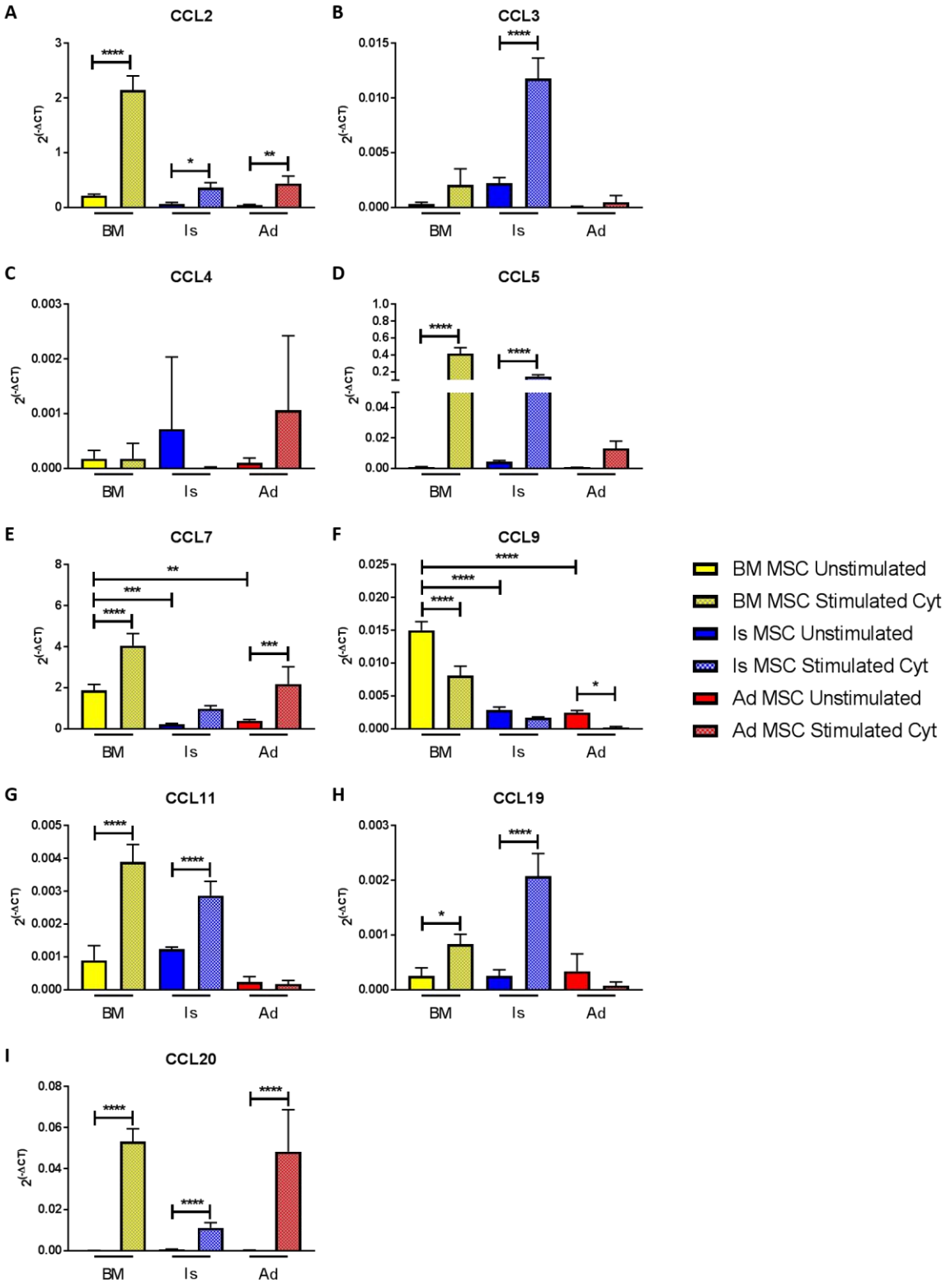


Figure 4-1. Inflammation and MSC tissue origin impacts CC chemokine transcript levels in MSCs.

MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with a cocktail of cytokines, 40 ng/ mL of IFN-Ɣ, TNF-α and IL-1β, for 24 hours. Unstimulated cells were left growing in MSC culture medium as a control. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate CCL transcripts in BM, Is and Ad MSCs under resting and inflammatory conditions. Each bar represents an n of 3 independent experiments and is graphed as mean ± SEM. Data are normalised to the housekeeping gene *B2M* and expressed as $2^{(-\Delta CT)}$. Appropriate statistical analysis was performed and includes Students paired T test between one MSC tissue source (Resting vs Inflammatory

Conditions) and One Way ANOVA with Tukey's multiple comparisons post-test to compare all MSC sources. Statistically significant differences are marked with the appropriate number of asterisks. p = 0.05 was considered the limit for statistical significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Table 4-1. Fold change in CC chemokine transcript levels of cytokine-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.
Following the experimental set up explained in Figure 4-1, fold change in transcript levels of CC chemokine is represented as mean of fold change ± standard deviation. Statistically significant differences are marked with a colour code, where $p < 0.05$ is represented by green, $p < 0.01$ is represented by orange, $p < 0.001$ is represented by blue and $p < 0.0001$ is represented by red.

Gene	BM MSCs	Is MSCs	Ad MSCs
CCL2	10.30 ± 2.11	5.71 ± 0.97	10.32 ± 2.23
CCL3	11.71 ± 12.16	5.44 ± 0.86	9.98 ± 12.59
CCL4	0.65 ± 0.54	0.11 ± 0.09	13.47 ± 9.79
CCL5	438.68 ± 60.22	33.15 ± 3.06	20.46 ± 5.93
CCL7	2.20 ± 0.41	4.91 ± 1.13	5.69 ± 1.98
CCL9	0.54 ± 0.10	0.59 ± 0.07	0.08 ± 0.05
CCL11	5.91 ± 3.71	2.31 ± 0.25	10.04 ± 16.53
CCL19	4.27 ± 2.11	9.89 ± 3.94	0.20 ± 0.14
CCL20	631.34 ± 148.34	19.27 ± 2.37	1443.42 ± 1440.99

4.2.2 Transcription of CXC chemokines under resting and inflammatory conditions

Under resting conditions, BM, Is and Ad MSCs transcribed very few, if any, CXC chemokines with the exception of *CXCL1*, which was highly transcribed by BM MSCs (Figure 4-2, A). A pattern of transcriptional upregulation was observed in *CXCL1* (A), *CXCL2* (B), *CXCL5* (C), *CXCL10* (D) and *CXCL16* (G). However, these chemokines were upregulated differentially in MSCs according to their tissue of origin. *CXCL12* (E) and *CXCL13* (F) were the only chemokines that were downregulated after 24-hour cytokine-mediated stimulation. Fold changes of transcript upon cytokine-mediated licensing of MSCs are shown in Table 4-2. BM MSCs expressed high levels of *CXCL1* transcript under resting conditions while Is and Ad MSCs barely expressed any transcript; however, inflammatory stimulation of the cells produced a marked upregulation of *CXCL1* in cells from all tissues, with BM MSCs expressing very high levels of *CXCL1*. Expression of *CXCL2* was almost undetectable during resting conditions but inflammatory stimulation produced a significant upregulation in BM and Is MSCs; Ad MSCs upregulated their *CXCL2* transcript levels even if it was in a statistically non-significant way. *CXCL5*

levels were almost undetectable under resting conditions in cells from all the tissue sources. Inflammatory stimulation produced 45 times higher levels of *CXCL5* transcript levels, generating 5.91 times more expression of *CXCL5* than the housekeeping gene *B2M*. *CXCL10* levels were very low in MSCs from all the tissue sources under resting conditions, and inflammatory stimulation only produced an increase in the amount of transcript in Is and Ad MSCs; Is MSCs were able to generate the highest levels of *CXCL10*. Regarding *CXCL12*, during resting conditions, BM MSCs were able to produce significantly higher transcript levels than Is and Ad MSCs, which produced very little. After inflammatory stimulation, MSCs isolated from the three tissue sources produced lower levels of *CXCL12* transcript, where this downregulation was only significant in BM MSCs. *CXCL13* levels were very low under both resting and inflammatory conditions in BM, Is and MSCs and is likely not transcribed in MSCs. Lastly, *CXCL16* transcripts were barely detectable in MSCs during resting conditions but inflammatory licensing led to a pattern of upregulation of this transcript, with Ad MSCs being the cells expressing the highest levels and the only MSCs in which upregulation was statistically significant.

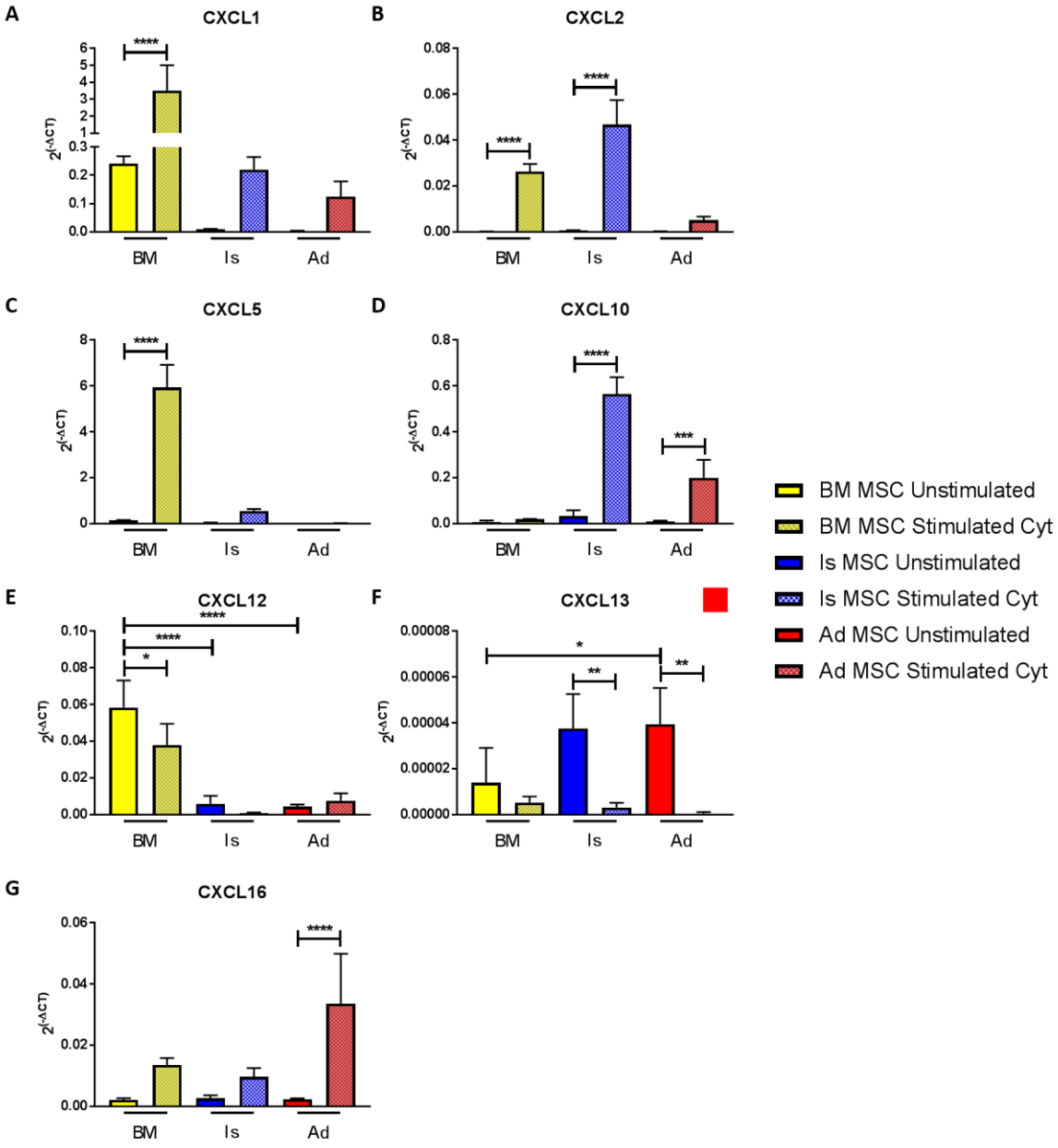


Figure 4-2. Inflammation and MSC tissue origin impacts CXC chemokine transcript levels in MSCs.

MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with a cocktail of cytokines, 40 ng/ mL of IFN-Ɣ, TNF-α and IL-1β, for 24 hours. Unstimulated cells were left growing in MSC culture medium as a control. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate CXCL transcripts in BM, Is and Ad MSCs under resting and inflammatory conditions. Each bar represents an n of 3 independent experiments and is graphed as mean ± SEM. Data are normalised to the housekeeping gene *B2M* and expressed as $2^{(-\Delta CT)}$. Appropriate statistical analysis was performed and includes Students paired T test between one MSC tissue source (Resting vs Inflammatory Conditions) and One Way ANOVA with Tukey's multiple comparisons post-test to compare all MSC sources. Statistically significant differences are marked with the appropriate number of asterisks. p = 0.05 was considered the limit for statistical significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Table 4-2. Fold change in CXC chemokine transcript levels of cytokine-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.
Following the experimental set up explained in Figure 4-2, fold change in transcript levels of CXC chemokine is represented as mean of fold change ± standard deviation. Statistically significant differences are marked with a colour code, where $p < 0.05$ is represented by green, $p < 0.01$ is represented by orange, $p < 0.001$ is represented by blue and $p < 0.0001$ is represented by red.

Gene	BM MSCs	Is MSCs	Ad MSCs
CXCL1	15.14 ± 7.11	27.31 ± 3.28	33.65 ± 12.33
CXCL2	197.83 ± 66.14	89.07 ± 12.03	31.85 ± 12.57
CXCL5	45.52 ± 8.46	28.30 ± 13.55	38.20 ± 16.28
CXCL10	5.44 ± 5.06	25.92 ± 15.32	34.32 ± 15.31
CXCL12	0.65 ± 0.10	0.15 ± 0.05	1.91 ± 1.21
CXCL13	6.74 ± 9.51	0.09 ± 0.06	0.01 ± 0.01
CXCL16	6.78 ± 1.15	3.80 ± 0.47	14.66 ± 6.23

4.2.3 Transcription of CC chemokine receptor expression under resting and inflammatory conditions

Under resting conditions, BM, Is and Ad MSCs do not appear to be transcribing CC chemokine receptors except for *CCR1* (Figure 4-3, A) and *CCR5* (Figure 4-3, E). *CCR2* (B), *CCR3* (C), *CCR4* (D), *CCR6* (F), *CCR7* (G), *CCR8* (H), *CCR9* (I) and *CCR10* (J) generated a CT of 35 or above, resulting in $2^{(-\Delta CT)}$ less than or equivalent to ~0.0001, which suggests that are likely not transcribed at meaningful levels by MSCs and are marked with a red box on the following graphs. Fold changes in transcript levels upon cytokine-mediated licensing of MSCs are specified in Table 4-3.

CCR1 expression was very low in BM and Ad MSCs during resting conditions and transcript levels did not increase after inflammatory stimulation (Figure 4-3, A). Is MSCs, on average, showed higher *CCR1* transcript levels during resting conditions but extensive variations were seen among the samples. However, after inflammatory stimulation, Is MSCs expressed higher transcript levels than MSCs isolated from other tissues. Similarly, *CCR5* transcript levels were very low in BM and Ad MSCs under resting conditions while Is MSCs had higher levels but with a bigger deviation among samples. Inflammatory stimulation produced a small upregulation of *CCR5* transcript levels in Is MSCs, but again, due to large variation this increase was not significant, and the expression levels were still quite low, which may not translate into any biological effect.

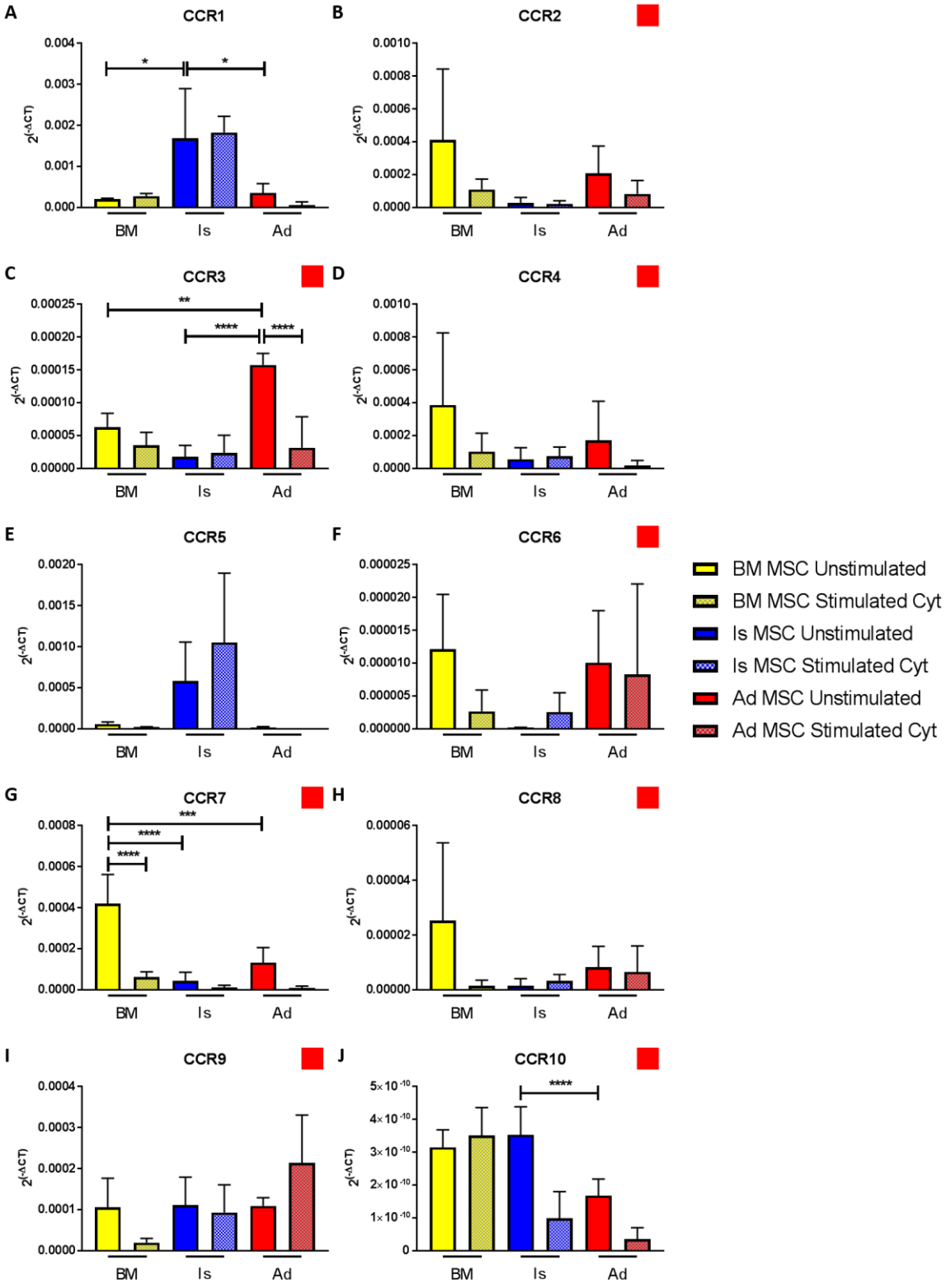


Figure 4-3. Inflammation and MSC tissue origin does not have a big impact on CC chemokine receptor transcript levels in MSCs.
MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with a cocktail of cytokines, 40 ng/ mL of IFN-Ɣ, TNF-α and IL-1β, for 24 hours. Unstimulated cells were left growing in MSC culture medium as a control. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate CCR transcripts in BM, Is and Ad MSCs under resting and inflammatory conditions. Each bar represents an n of 3 independent experiments and is graphed as mean ± SEM. Data are normalised to the housekeeping gene *B2M* and expressed as $2^{(-\Delta CT)}$. Appropriate statistical analysis was performed and includes Students paired T test between one MSC tissue source (Resting vs Inflammatory Conditions) and One Way ANOVA with Tukey's multiple comparisons post-test to compare all MSC sources. Statistically significant differences are marked with the appropriate number of asterisks. p

= 0.05 was considered the limit for statistical significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Table 4-3. Fold change in CC chemokine receptor transcript levels of cytokine-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source. Following the experimental set up explained in Figure 4-3, fold change in transcript levels of CC chemokine receptors is represented as mean of fold change ± standard deviation. Statistically significant differences are marked with a colour code, where $p < 0.05$ is represented by green, $p < 0.01$ is represented by orange, $p < 0.001$ is represented by blue and $p < 0.0001$ is represented by red.

Gene	BM MSCs	Is MSCs	Ad MSCs
CCR1	1.38 ± 0.35	3.12 ± 3.77	0.42 ± 0.56
CCR2	0.58 ± 0.35	1.34 ± 1.05	0.34 ± 0.24
CCR3	0.58 ± 0.26	5.25 ± 6.04	**0.18 ± 0.22**
CCR4	0.37 ± 0.26	5.79 ± 5.67	0.14 ± 0.20
CCR5	0.38 ± 0.11	5.84 ± 6.92	0.05 ± 0.05
CCR6	0.19 ± 0.16	19.89 ± 19.64	0.09 ± 0.05
CCR7	**0.15 ± 0.03**	0.39 ± 0.31	0.05 ± 0.05
CCR8	0.15 ± 0.16	29.07 ± 26.60	47.15 ± 81.36
CCR9	0.22 ± 0.11	0.88 ± 0.74	2.14 ± 1.24
CCR10	1.17 ± 0.40	0.25 ± 0.15	0.22 ± 0.16

4.2.4 Transcription of CXC chemokine receptors expression under resting and inflammatory conditions

As described for CC chemokine receptors, BM, Is and Ad MSCs do not appear to be transcribing CXC chemokine receptors except for *CXCR6* (Figure 4-4, F). *CXCR1* (A), CXCR2 (B), *CCR3* (C), *CXCR4* (D) and *CXCR5* (E) generated a CT of 35 or above, resulting in $2^{(-\Delta CT)}$ less than or equivalent to ~0.0001, which suggests that are likely not transcribed at meaningful levels by MSCs and are marked with a red box on the following graphs. Fold changes of transcript levels upon cytokine-mediated licensing of MSCs are detailed in Table 4-4.

Under resting conditions, BM MSCs have the highest transcript levels of *CXCR6* and Is MSCs the lowest (Figure 4-4, F). Inflammatory stimulation produced a downregulation of *CXCR6* transcript in BM and Is MSCs, while Ad MSCs did not show a clear pattern due to the big variation among samples.

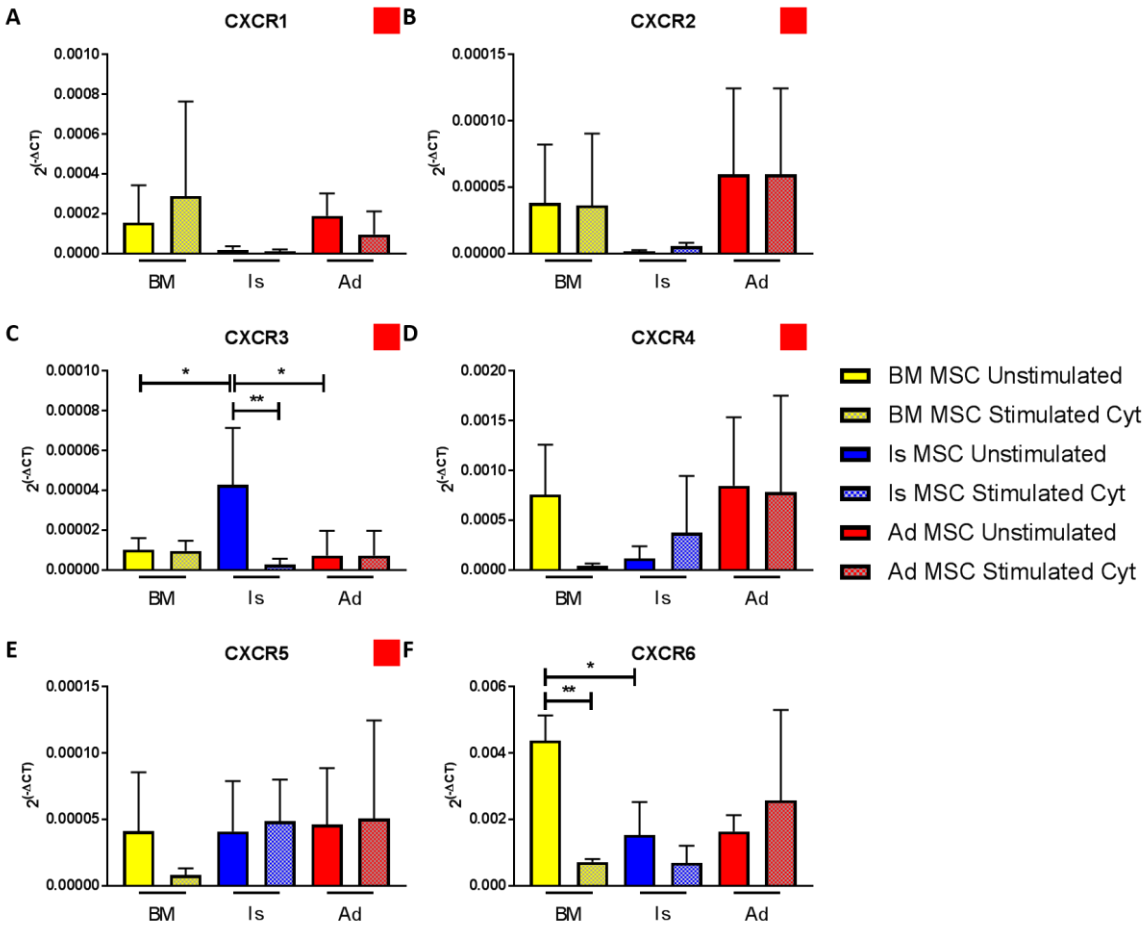


Figure 4-4. Inflammation and MSC tissue origin does not have a big impact on CXC chemokine receptor transcript levels in MSCs.
MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with a cocktail of cytokines, 40 ng/ mL of IFN-Ɣ, TNF-α and IL-1β, for 24 hours. Unstimulated cells were left growing in MSC culture medium as a control. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate CXCR transcripts in BM, Is and Ad MSCs under resting and inflammatory conditions. Each bar represents an n of 3 independent experiments and is graphed as mean ± SEM. Data are normalised to the housekeeping gene *B2M* and expressed as $2^{(-\Delta CT)}$. Appropriate statistical analysis was performed and includes Students paired T test between one MSC tissue source (Resting vs Inflammatory Conditions) and One Way ANOVA with Tukey's multiple comparisons post-test to compare all MSC sources. Statistically significant differences are marked with the appropriate number of asterisks. p = 0.05 was considered the limit for statistical significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Table 4-4. Fold change in CXC chemokine receptor transcript levels of cytokine-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.
Following the experimental set up explained in Figure 4-4, fold change in transcript levels of CXC chemokine receptors is represented as mean of fold change ± standard deviation. Statistically significant differences are marked with a colour code, where $p < 0.05$ is represented by green, $p < 0.01$ is represented by orange, $p < 0.001$ is represented by blue and $p < 0.0001$ is represented by red.

Gene	BM MSCs	Is MSCs	Ad MSCs
CXCR1	3.20 ± 3.07	0.79 ± 0.32	0.36 ± 0.34
CXCR2	2.57 ± 1.88	13.00 ± 18.19	3.92 ± 3.63
CXCR3	2.62 ± 3.47	0.12 ± 0.13	1.00 ± 0.00
CXCR4	0.06 ± 0.01	6.44 ± 7.50	3.05 ± 3.65
CXCR5	1.35 ± 1.92	2.42 ± 2.30	3.39 ± 4.35
CXCR6	0.17 ± 0.04	0.61 ± 0.65	1.51 ± 1.33

4.2.5 Transcription of atypical chemokine receptor expression under resting and inflammatory conditions

BM, Is and Ad MSCs do not appear to be transcribing *ACKR2* (Figure 4-5, B) and *ACKR3* (Figure 4-5, C) as they generated a CT of 35 or above, resulting in $2^{(-\Delta CT)}$ less than or equivalent to ~0.0001, which suggests that are likely not transcribed at meaningful levels by MSCs and are marked with a red box on the following graphs. Fold changes of transcript levels upon cytokine-mediated licensing of MSCs are specified in Table 4-5.

Under resting conditions, no significant differences were found in the transcript levels of *ACKR1* among the MSCs isolated from the three different sources and inflammatory stimulation did not produce a clear pattern of down or upregulation, which is quite likely to be due to the large variation among samples (Figure 4-5, A). During resting conditions, *ACKR4* transcript levels were significantly higher in the MSCs isolated from the BM compared to Is and Ad MSCs. Inflammatory stimulation produced a significant downregulation in BM MSCs and no effect in Is and Ad MSCs (D).

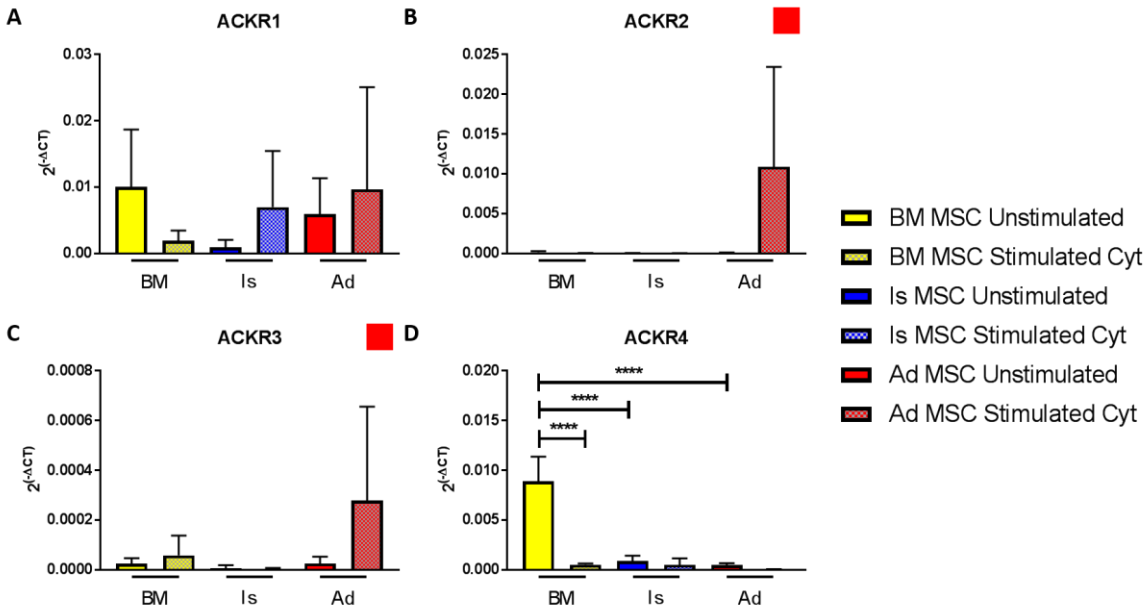


Figure 4-5. Inflammation and MSC tissue origin impacts *ACKR4* transcript levels in MSCs. MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with a cocktail of cytokines, 40 ng/mL of IFN-Ɣ, TNF-α and IL-1β, for 24 hours. Unstimulated cells were left growing in MSC culture medium as a control. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate ACKR transcripts in BM, Is and Ad MSCs under restingand inflammatory conditions. Each bar represents an n of 3 independent experiments and is graphed as mean ± SEM. Data are normalised to the housekeeping gene *B2M* and expressed as $2^{(-\Delta CT)}$. Appropriate statistical analysis was performed and includes Students paired T test between one MSC tissue source (Resting vs Inflammatory Conditions) and One Way ANOVA with Tukey's multiple comparisons post-test to compare all MSC sources. Statistically significant differences are marked with the appropriate number of asterisks. p = 0.05 was considered the limit for statistical significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Table 4-5. Fold change in ACKR transcript levels of cytokine-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.
Following the experimental set up explained in Figure 4-5, fold change in transcript levels of ACKRs is represented as mean of fold change ± standard deviation. Statistically significant differences are marked with a colour code, where $p < 0.05$ is represented by green, $p < 0.01$ is represented by orange, $p < 0.001$ is represented by blue and $p < 0.0001$ is represented by red.

Gene	BM MSCs	Is MSCs	Ad MSCs
ACKR1	0.21 ± 0.08	21.39 ± 22.02	1.60 ± 1.66
ACKR2	1.46 ± 2.39	9.87 ± 15.63	272.83 ± 278.35
ACKR3	19.85 ± 31.88	19.31 ± 25.31	74.91 ± 108.19
ACKR4	**0.06 ± 0.01**	0.97 ± 1.32	0.12 ± 0.04

4.2.6 Transcription of XCL and CX3C chemokine expression, their receptors and the receptors for the stimulatory agents under resting and inflammatory conditions

CX3CL1 transcript level variations were not detected in the MSCs isolated from the different sources under resting conditions; however, tissue of origin did

affect the transcript levels after inflammatory stimulation. BM MSC showed an upregulation of *CX3CL*1 even if it was not statistically significant, Is MSCs transcript levels showed no variation after stimulation and lastly, *CX3CL1* transcript levels were upregulated by Ad MSC in a statistically significant manner after inflammatory stimulation (Figure 4-6, A). The chemokine receptors *CX3CR1* (Figure 4-6, B) and *XCR1* (Figure 4-6, C) generated a CT of 35 or above, resulting in $2^{(-\Delta CT)}$ less than or equivalent to ~0.0001, which suggests that they are likely not transcribed by MSCs at meaningful levels and are therefore marked with a red box on the following graphs. Fold changes of transcript levels upon cytokine-mediated licensing of MSCs are specified in Table 4-6.

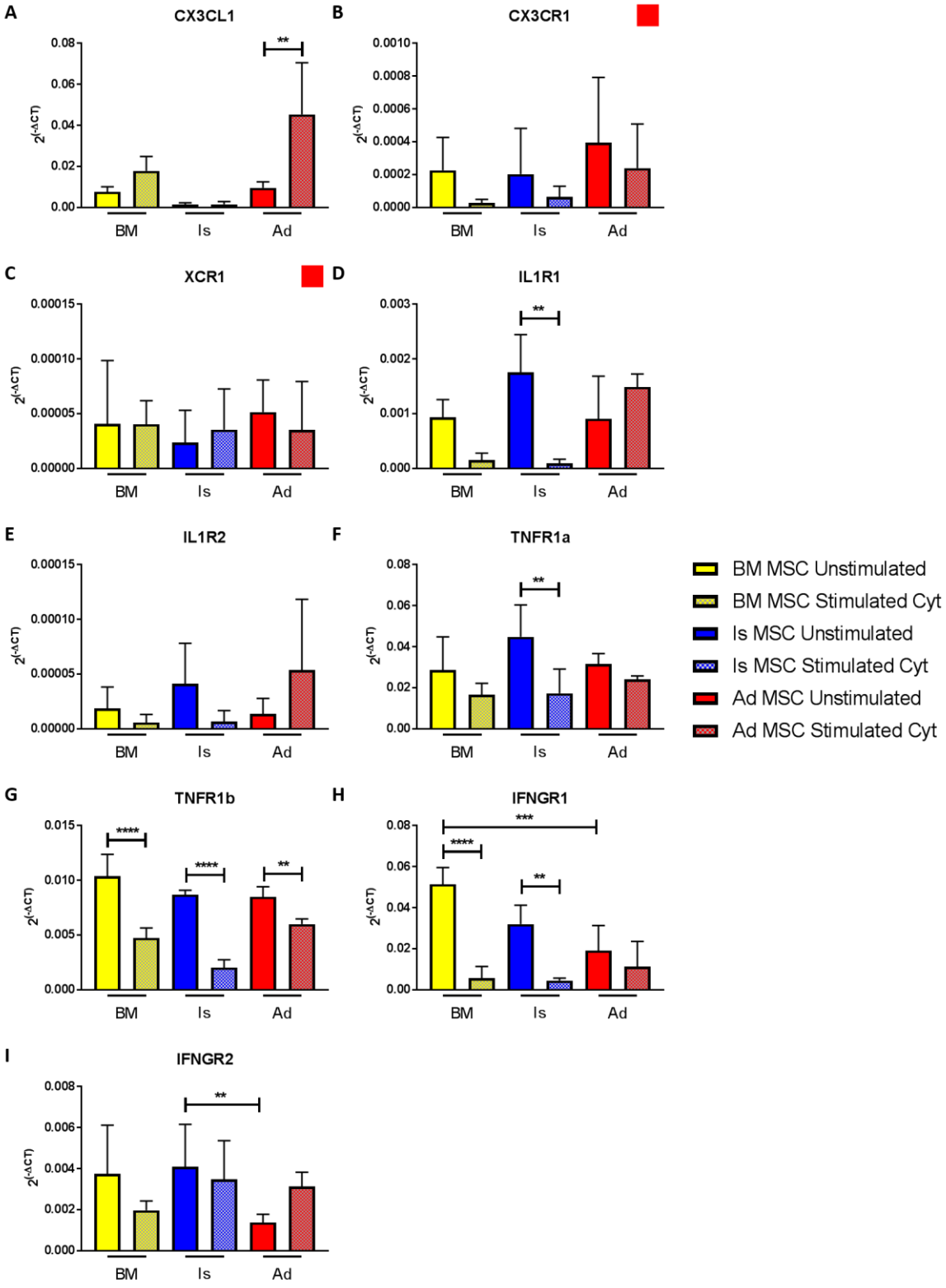


Figure 4-6. Inflammation and MSC tissue origin impacts CX3CL1 transcript levels in MSCs. MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with a cocktail of cytokines, 40 ng/ mL of IFN-Ɣ, TNF-α and IL-1β, for 24 hours. Unstimulated cells were left growing in MSC culture medium as a control. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate transcripts in BM, Is and Ad MSCs under resting and inflammatory conditions. Each bar represents an n of 3 independent experiments and is graphed as mean ± SEM. Data are normalised to the housekeeping gene *B2M* and expressed as $2^{(-\Delta CT)}$. Appropriate statistical analysis was performed and includes Students paired T test between one MSC tissue source (Resting vs Inflammatory Conditions) and One Way ANOVA with Tukey's multiple comparisons post-test to compare all MSC sources. Statistically significant differences are marked with the appropriate number of asterisks. p

= 0.05 was considered the limit for statistical significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Table 4-6. Fold change in XCL and CX3C chemokines, their receptors and the receptors of the stimulatory agents' transcript levels of cytokine-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.
Following the experimental set up explained in Figure 4-6 fold change in transcript levels of XCL and CX3C, their receptors and the receptors of the stimulatory agents is represented as mean of fold change ± standard deviation. Statistically significant differences are marked with a colour code, where $p < 0.05$ is represented by green, $p < 0.01$ is represented by orange, $p < 0.001$ is represented by blue and $p < 0.0001$ is represented by red.

Gene	BM MSCs	Is MSCs	Ad MSCs
CX3CL1	2.35 ± 0.43	1.60 ± 1.19	5.31 ± 3.21
CX3CR1	0.12 ± 0.01	0.57 ± 0.68	0.41 ± 0.29
XCR1	6.89 ± 6.33	74.70 ± 128.32	0.69 ± 0.91
IL1R1	0.18 ± 0.11	0.04 ± 0.02	1.99 ± 1.00
IL1R2	0.19 ± 0.17	0.10 ± 0.09	25.20 ± 38.61
TNFR1a	0.74 ± 0.49	0.35 ± 0.13	0.89 ± 0.09
TNFR1b	0.48 ± 0.15	0.24 ± 0.08	0.68 ± 0.09
IFNGR1	0.12 ± 0.11	0.15 ± 0.06	0.52 ± 0.49
IFNGR2	0.78 ± 0.56	0.88 ± 0.27	2.71 ± 0.81

It was essential to ensure that MSCs from the three tissues were capable of responding to inflammatory stimuli, for this reason, transcript levels of the receptors for the stimulatory molecules, IL-1β, TNF-α and IFN-ϒ, were assessed. In response to increased activation by a ligand, a cell's sensitivity to that specific ligand commonly transiently reduces by decreasing the expression of the specific receptor interacting with the ligand (Heldin et al., 2016). This is called negative feedback and was used to determine activation of MSCs after inflammatory stimulation.

IL-1β is recognised by IL1R1 and IL1R2. Under resting conditions, Is MSCs expressed the highest levels of IL1R1 (Figure 4-6, D) and IL1R2 (Figure 4-6, E) followed by the BM MSCs and lastly by Ad MSCs. After inflammatory stimulation, MSCs isolated from the BM and Is had downregulated the transcript levels of both receptors; Ad MSCs were slightly higher but the standard deviation was much higher in these samples. TNF-α is recognised by TNFR1a and TNFR1b, and as observed in Figure 4-6, F and G, under resting conditions MSCs from all sources expressed similar levels of transcripts for these receptors. After 24 hours of

inflammatory stimulation *TNFR1a* and *TNFR1b* transcript levels were downregulated in MSCs from every tissue, where Is MSCs had the highest downregulation, followed by BM MSCs and lastly Ad MSCs. Lastly, IFN-ϒ is recognised by IFNGR1 and IFNGR2. Tissue of origin influenced the expression of these receptors as during resting conditions BM MSCs expressed significantly higher levels of IFNGR1 than Ad MSCs and Is MSCs showed higher transcript levels of IFNGR2 than Ad MSCs. After inflammatory stimulus, IFNGR1 transcript levels were downregulated in MSCs isolated from the three tissues while IFNGR2 was only downregulated in cells isolated from the BM and Is. Overall, Ad MSCs downregulated their receptors to a lesser extent than BM or Is MSCs after inflammatory stimulation.

4.3 Analysis of the effect of a double inflammatory stimulus over time on the transcription of chemokines and chemokine receptors

Several reports in the literature suggest that the immunomodulatory activities of MSCs are not spontaneous, that priming by inflammatory cytokines is essential for MSC–mediated immunosuppression, irrespective of the species of origin (Ren et al., 2009). Within a clinical setting, MSCs are usually infused within an inflammatory environment, which provides the required licensing for increasing the immunomodulatory properties of MSCs. When the anti–inflammatory properties of MSCs are studied *in vitro*, this licensing is mimicked in an *in vitro* culture with the addition of pro-inflammatory cytokines; in this specific case with 40 ng/ mL of IFN-ϒ, TNF-α and IL-1β.

As previously discussed, pro-inflammatory cytokine-mediated MSC licensing produced a transcriptional upregulation of chemokines, which could explain why MSCs are more immunosuppressive upon activation. Within clinical settings, it has been reported that pre-treatment of MSCs with inflammatory cytokines prior to administration within animal models of inflammatory diseases boosts the therapeutic effect of MSCs (Duijvestein et al., 2011, Noronha et al., 2019). For this reason, we wondered if pre-licensing the MSCs prior to exposing them to an inflammatory environment would lead to even bigger changes in chemokine

transcriptional levels and therefore, in enhanced therapeutic potential. To study this, cells were pre-licensed for 48 hours, after which cells were washed twice with PBS and stimulated again for another 24 hours. The first stimulation primes the MSCs while the second stimulation would mimic the inflammatory environment MSCs would face when infused into a patient with an inflammatory disorder. Figure 4-7 illustrates the time points at which supplemented medium was added. Culture medium was removed at the time cells were harvested and was kept for analysis.

Two different control conditions were used. In the first one, cells were stimulated for 48 hours, after which cells were washed twice with PBS and fresh culture medium was added; cells were harvested 24 hours later. In the second condition, cells were washed twice with PBS, the culture medium was replaced with fresh medium and the cells were left growing for 48 hours. Cells were then washed twice with PBS, the culture medium was replaced with supplemented medium and the cells were harvested 24 hours later. There is an extensive literature about how cytokine-mediated licensing enhances the potential therapeutic efficacy of MSCs; however, little is known about the role of TLR ligand-mediated activation on the secretion of chemotactic cytokines by MSCs. For this reason, 100 ng/ mL lipopolysaccharide (LPS), 100 ng/mL lipoteichoic acid (LTA) and 4 mg/ ml polyinosinic-polycytidylic acid (poly I:C), as well as the previously described cytokine cocktail, was used for MSC licensing.

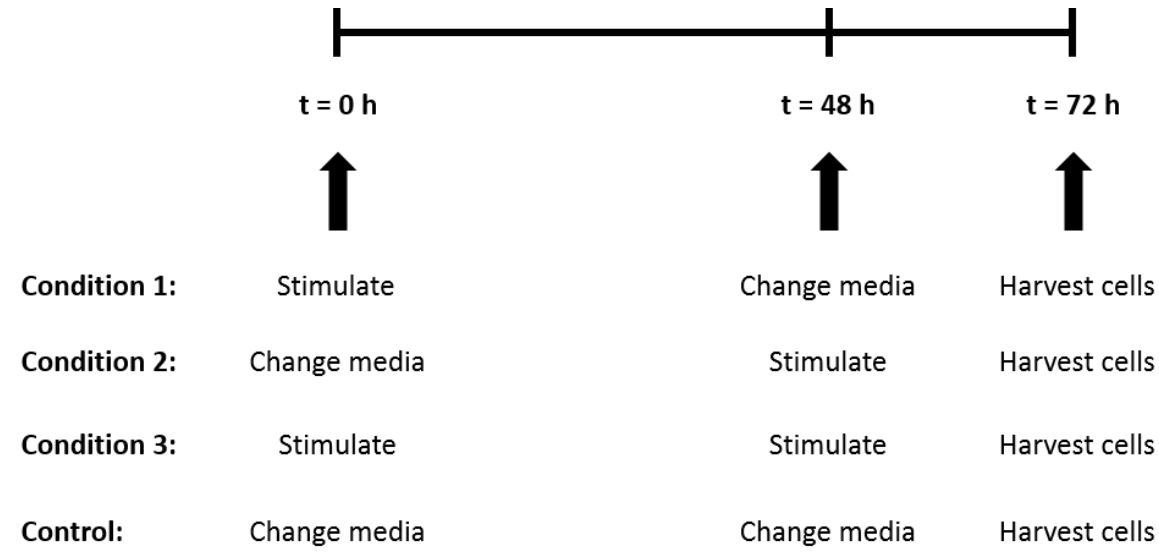


Figure 4-7. Diagrammatic illustration of the time course of the MSC licensing.

On the basis of the results described in Section 4.2, the genes that showed alterations in their transcript levels in a statistically significant manner upon stimulation were selected and their transcriptional levels were assessed after licensing with different inflammatory molecules and different inflammatory conditions.

It is important to bear in mind that in the experiments in Section 4.3, the influence of four different inflammatory agents (cytokine cocktail with TNF-α, IL-1β and IFN-ϒ, LPS, LTA and Poly I:C) at three different time points (72 hours, 24 hours and double stimulation) on 22 different genes was studied, and an untreated sample was used as control. This study was performed in MSCs isolated from three different sources (BM, Is and Ad MSCs), which gave a total of 858 data points per experiment ([4 inflammatory agents × 3 time points + 1 control] × 22 genes × 3 MSC sources).

Among all the licensing molecules tested, the combination of 40 ng/ mL of TNF-α, IL-1β and IFN-ϒ led to the biggest variations in chemokine receptor and ligand expression and among all the conditions tested, harvesting the cells 24 hours after the inflammatory stimulation led to the most significant results. These condition has already been discussed in Section 4.2, for this reason, due to the huge amount of data presented in this chapter, we decided to move the less relevant results to the Appendix. Thus, this results section will only display the fold changes of transcript levels upon licensing of MSCs in tables, while the explanatory text and figures will be displayed in Section 8.1.

4.3.1 Transcription of CC chemokines under resting and inflammatory conditions

As outlined in Section 4.2.1, under resting conditions, BM, Is and Ad MSCs transcribed very few, if any, CC chemokines with the exception of *CCL2*, which was transcribed by all tissue sources of MSCs at similar levels, and *CCL7*, which was very highly expressed by BM MSCs. A pattern of transcriptional upregulation was observed in *CCL2*, *CCL3*, *CCL5*, *CCL7* and *CCL20* genes after licensing in every condition; however, these chemokines were upregulated differentially in MSCs according to their tissue of origin and licensing agent (Section 8.1). CC chemokine transcript levels 24 hours after stimulation (Condition 2) increased in

MSCs from every tissue source; however, this upregulation was not sustained, as cells harvested 72 hours after licensing (Condition 1) showed a decrease in the transcript levels of CC chemokines. A second stimulation 48 hours after the first stimulation (Condition 3) was able to induce the transcription of CC chemokines in MSCs from the three sources; however, the second stimulation was not able to match the transcript levels of CC chemokines found in Condition 2 in MSCs from every source with the exception of Ad MSCs after cytokine-mediated stimulation, where these cells massively increased the transcript levels of CC chemokine ligands. Fold changes of transcriptional regulation upon licensing of MSCs are specified in Table 4-7.

Table 4-7. Fold change in CC chemokine transcript levels of cytokine, LPS, LTA or Poly I:C-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.

MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with either a cocktail of cytokines (40 ng/ mL of IFN-Ɣ, TNF-α and IL-1β), 100 ng/ mL LPS, 100 ng/ mL LTA or 4 µg/ mL Poly I:C. Unstimulated cells were left growing in MSC culture medium as a control. Three different licensing conditions were tested. In the first one, cells were stimulated for 48 hours, after which cells were washed twice with PBS and fresh culture medium was added; cells were harvested 24 hours later. In the second condition, cells were washed twice with PBS, the culture medium was replaced with fresh one and the cells were left growing for 48 hours. Cells were then washed twice with PBS, the culture medium was replaced with supplemented one and the cells were harvested 24 hours later. In the last condition, cells were stimulated for 48 hours, after which cells were washed twice with PBS and were stimulated again for another 24 hours. Figure 4-7 illustrates the time points at which supplemented medium was added. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate CCL transcripts in BM, Is and Ad MSCs under resting and inflammatory conditions. Data are normalised to the housekeeping gene *B2M* and expressed as $2^{(-\Delta CT)}$. Fold change in transcript levels of CC chemokines is represented as mean of fold change ± standard deviation. One Way ANOVA with Tukey's multiple comparisons post-test was performed to compare all MSC sources and the different conditions. Statistically significant differences are marked with a colour code, where $p < 0.05$ is represented by green, $p < 0.01$ is represented by orange, $p < 0.001$ is represented by blue and $p < 0.0001$ is represented by red.

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
CCL2	1	Cyt	1.84 ± 1.43	8.07 ± 1.77	0.51 ± 0.11
		LPS	0.71 ± 0.56	4.58 ± 2.60	1.21 ± 1.04
		LTA	0.75 ± 0.15	2.67 ± 1.79	0.56 ± 0.06
		Poly I:C	0.89 ± 0.33	2.66 ± 0.62	0.41 ± 0.03
	2	Cyt	**4.24 ± 1.14**	**22.93 ± 5.23**	**6.77 ± 1.70**
		LPS	0.98 ± 0.29	**10.01 ± 3.32**	6.71 ± 1.76
		LTA	1.32 ± 0.36	1.02 ± 0.15	3.21 ± 0.60
		Poly I:C	**5.39 ± 1.85**	**10.97 ± 5.25**	2.88 ± 0.56
	3	Cyt	1.38 ± 0.77	5.02 ± 1.31	**202.76 ± 51.81**
		LPS	1.26 ± 0.51	3.19 ± 1.18	11.66 ± 2.49
		LTA	0.82 ± 0.53	0.80 ± 0.09	5.01 ± 1.65
		Poly I:C	2.28 ± 0.79	4.96 ± 1.86	4.47 ± 1.76

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
CCL3	1	Cyt	0.27 ± 0.07	1.02 ± 0.24	1.26 ± 0.45
		LPS	0.50 ± 0.03	1.03 ± 0.75	0.76 ± 0.31
		LTA	0.89 ± 0.14	0.24 ± 0.04	0.73 ± 0.19
		Poly I:C	0.22 ± 0.03	0.27 ± 0.07	0.30 ± 0.10
	2	Cyt	1.23 ± 0.50	3.57 ± 1.01	0.36 ± 0.06
		LPS	0.88 ± 0.16	3.06 ± 0.97	0.56 ± 0.16
		LTA	1.04 ± 0.27	0.45 ± 0.21	0.51 ± 0.08
		Poly I:C	0.61 ± 0.33	0.82 ± 0.25	0.32 ± 0.06
	3	Cyt	0.64 ± 0.35	1.21 ± 0.30	2.88 ± 0.69
		LPS	1.14 ± 0.14	1.18 ± 0.43	0.57 ± 0.15
		LTA	1.16 ± 0.37	1.22 ± 1.37	0.70 ± 0.22
		Poly I:C	1.13 ± 0.13	0.52 ± 0.12	0.84 ± 0.52
CCL5	1	Cyt	11.59 ± 10.85	7.35 ± 2.09	10.95 ± 3.50
		LPS	4.58 ± 4.07	96.57 ± 22.51	2.63 ± 0.41
		LTA	0.25 ± 0.20	0.70 ± 0.30	0.46 ± 0.20
		Poly I:C	107.27 ± 97.50	5.82 ± 1.15	7.86 ± 2.49
	2	Cyt	48.22 ± 45.00	29.01 ± 11.82	9.58 ± 1.87
		LPS	70.79 ± 63.95	29.90 ± 10.46	16.00 ± 5.69
		LTA	1.61 ± 1.53	0.35 ± 0.07	104.45 ± 33.37
		Poly I:C	138.31 ± 134.11	177.83 ± 38.71	52.32 ± 16.60
	3	Cyt	10.25 ± 8.63	5.92 ± 1.49	419.49 ± 93.64
		LPS	10.17 ± 9.13	13.12 ± 6.50	2.83 ± 1.06
		LTA	1.22 ± 1.18	0.64 ± 0.55	0.56 ± 0.26
		Poly I:C	208.49 ± 195.87	117.21 ± 39.74	23.07 ± 23.86
CCL7	1	Cyt	0.39 ± 0.30	1.82 ± 2.02	1.62 ± 0.86
		LPS	0.23 ± 0.14	4.56 ± 5.13	0.90 ± 0.47
		LTA	0.22 ± 0.10	2.30 ± 2.68	0.95 ± 0.46
		Poly I:C	0.33 ± 0.22	0.73 ± 0.49	0.66 ± 0.32
	2	Cyt	1.29 ± 0.70	5.27 ± 5.15	3.77 ± 2.14
		LPS	1.14 ± 0.20	7.54 ± 6.36	6.60 ± 2.51
		LTA	0.87 ± 0.08	1.85 ± 2.26	4.59 ± 2.26
		Poly I:C	0.88 ± 0.68	13.03 ± 17.73	3.38 ± 1.83
	3	Cyt	0.55 ± 0.07	2.87 ± 3.03	30.78 ± 15.81
		LPS	1.72 ± 0.72	6.00 ± 7.25	2.53 ± 1.32
		LTA	0.86 ± 0.67	3.06 ± 3.48	0.92 ± 0.48
		Poly I:C	1.58 ± 0.91	5.10 ± 5.83	0.09 ± 0.04
CCL20	1	Cyt	4.23 ± 0.74	8.74 ± 7.80	0.18 ± 0.14
		LPS	2.45 ± 1.14	28.10 ± 13.76	2.13 ± 1.91
		LTA	1.17 ± 0.63	0.35 ± 0.25	0.02 ± 0.01
		Poly I:C	2.19 ± 0.50	0.68 ± 0.47	0.59 ± 0.57
	2	Cyt	631.34 ± 148.34	19.27 ± 2.37	470.60 ± 222.05
		LPS	42.76 ± 42.62	7.47 ± 2.27	14.68 ± 6.22
		LTA	64.25 ± 66.43	0.57 ± 0.25	13.20 ± 4.91
		Poly I:C	45.35 ± 26.57	8.32 ± 6.09	5.25 ± 2.10
	3	Cyt	27.91 ± 26.21	5.21 ± 0.89	298.33 ± 107.66
		LPS	87.39 ± 80.84	4.40 ± 1.63	5.93 ± 2.20

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
		LTA	8.18 ± 7.03	0.12 ± 0.15	1.01 ± 0.35
		Poly I:C	36.44 ± 30.10	5.21 ± 2.65	0.00 ± 0.00

4.3.2 Transcription of CXC chemokines under resting and inflammatory conditions

As outlined in Section 4.2.2, under resting conditions, BM, Is and Ad MSCs transcribed very few, if any, CXC chemokines with the exception of *CXCL1*, which was transcribed by BM MSCs. A pattern of transcriptional upregulation was observed in *CXCL1*, *CXCL2*, *CXCL5*, *CXCL10* and CXCL16 after licensing in every condition, while stimulation of MSCs led to the downregulation of *CXCL12* (Section 8.1). However, these chemokines were regulated differentially in MSCs according to their tissue of origin and licensing agent. *CXCL1*, *CXCL5*, *CXCL10* and *CXCL16* chemokine transcript levels increased after 24 hours of stimulation (Condition 2) in MSCs from every tissue source; however, this upregulation was not sustained, as cells harvested 72 hours after licensing (Condition 1) showed a decrease in the transcript levels of CC chemokines. *CXCL2* transcript levels increased after 24 hours stimulation (Condition 2) in MSCs from every tissue source; however, while this upregulation was promptly reversed in BM and Is MSCs, Ad MSCs expressed higher amounts of *CXCL2* transcript levels 72 hours after licensing (Condition 1). A second stimulation, 48 hours after the first stimulation (Condition 3), was able to induce the transcription of *CXCL1*, *CXCL2*, *CXCL5*, *CXCL10* and *CXCL16* chemokines in MSCs from the three sources. However, the second stimulation was not able to match the transcript levels of CXC chemokines found in Condition 2 in MSCs from every source except for Ad MSCs after cytokine-mediated stimulation, where these cells massively increased the transcript levels of CXC chemokine ligands. *CXCL12* transcript levels were very dependent on MSC source and licensing agent; cytokine-mediated licensing produced a downregulation of the transcript levels in every condition in BM and Is MSCs, while it produced an upregulation in Ad MSCs. LPS-mediated licensing produced a downregulation of *CXCL12* levels in BM and Ad MSCs, while it was able to induce the transcription levels in Is MSCs after 72 hours stimulation (Condition 1). LTA licensing led to a trend of upregulation in BM MSCs while it produced no statistically significant effect on Is and Ad MSCs. Lastly, Poly I:C stimulation produced a downregulation in BM MSCs, no variation in Is MSCs and a

downregulation in Ad MSCs which was overcome after a double stimulation (Condition 3). Fold changes of transcript levels upon licensing of MSCs are specified in Table 4-8.

Table 4-8. Fold change in CXC chemokine transcript levels of cytokine, LPS, LTA or Poly I:C-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.

MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with either a cocktail of cytokines (40 ng/ mL of IFN-γ, TNF-α and IL-1β), 100 ng/ mL LPS, 100 ng/ mL LTA or 4 µg/ mL Poly I:C. Unstimulated cells were left growing in MSC culture medium as a control. Three different licensing conditions were tested. In the first one, cells were stimulated for 48 hours, after which cells were washed twice with PBS and fresh culture medium was added; cells were harvested 24 hours later. In the second condition, cells were washed twice with PBS, the culture medium was replaced with fresh one and the cells were left growing for 48 hours. Cells were then washed twice with PBS, the culture medium was replaced with supplemented one and the cells were harvested 24 hours later. In the last condition, cells were stimulated for 48 hours, after which cells were washed twice with PBS and were stimulated again for another 24 hours. Figure 4-7 illustrates the time points at which supplemented medium was added. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate CXCL transcripts in BM, Is and Ad MSCs under resting and inflammatory conditions. Data are normalised to the housekeeping gene *B2M* and expressed as $2^{(-\Delta CT)}$. Fold change in transcript levels of CXC chemokines is represented as mean of fold change ± standard deviation. One Way ANOVA with Tukey's multiple comparisons post-test was performed to compare all MSC sources and the different conditions. Statistically significant differences are marked with a colour code, where $p < 0.05$ is represented by green, $p < 0.01$ is represented by orange, $p < 0.001$ is represented by blue and $p < 0.0001$ is represented by red.

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
CXCL1	1	Cyt	3.70 ± 3.41	3.04 ± 3.73	0.09 ± 0.06
		LPS	0.35 ± 0.25	0.78 ± 0.87	0.23 ± 0.06
		LTA	0.09 ± 0.07	1.94 ± 2.28	0.11 ± 0.04
		Poly I:C	0.06 ± 0.05	2.11 ± 2.17	0.13 ± 0.03
	2	Cyt	**13.66 ± 10.35**	**7.77 ± 7.58**	**2.56 ± 0.23**
		LPS	1.78 ± 0.56	5.05 ± 5.82	1.96 ± 0.41
		LTA	0.62 ± 0.10	0.93 ± 0.64	0.95 ± 0.06
		Poly I:C	1.01 ± 0.77	1.21 ± 1.20	0.91 ± 0.12
	3	Cyt	2.49 ± 0.30	3.19 ± 2.58	**57.55 ± 4.31**
		LPS	2.25 ± 1.25	1.20 ± 0.63	1.58 ± 0.09
		LTA	0.18 ± 0.11	2.41 ± 2.56	0.89 ± 0.19
		Poly I:C	0.16 ± 0.08	0.44 ± 0.29	1.45 ± 0.22
CXCL2	1	Cyt	0.95 ± 0.20	0.70 ± 0.41	**64.35 ± 10.64**
		LPS	3.24 ± 0.66	13.12 ± 12.57	0.52 ± 0.17
		LTA	1.01 ± 0.13	0.61 ± 0.22	0.33 ± 0.21
		Poly I:C	0.69 ± 0.28	0.40 ± 0.15	0.19 ± 0.08
	2	Cyt	**7.14 ± 1.15**	**10.04 ± 3.51**	3.18 ± 0.49
		LPS	15.99 ± 4.05	8.70 ± 2.38	3.63 ± 1.65
		LTA	3.03 ± 0.65	0.98 ± 0.71	2.70 ± 0.89
		Poly I:C	2.56 ± 1.19	2.91 ± 0.50	1.40 ± 0.46
	3	Cyt	2.21 ± 0.95	5.49 ± 1.02	**110.74 ± 34.09**
		LPS	12.61 ± 2.99	1.95 ± 0.49	1.34 ± 0.53
		LTA	2.05 ± 1.16	0.52 ± 0.50	0.97 ± 0.42
		Poly I:C	1.20 ± 0.14	1.86 ± 0.44	3.12 ± 1.31

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
CXCL5	1	Cyt	0.41 ± 0.15	27.08 ± 24.87	0.97 ± 0.50
		LPS	0.05 ± 0.01	5.60 ± 4.80	4.38 ± 0.98
		LTA	0.01 ± 0.00	0.40 ± 0.17	0.51 ± 0.09
		Poly I:C	0.01 ± 0.00	0.89 ± 0.62	0.51 ± 0.12
	2	Cyt	**6.86 ± 1.60**	**182.44 ± 101.70**	**26.79 ± 4.95**
		LPS	2.27 ± 2.22	13.58 ± 4.69	**28.05 ± 10.42**
		LTA	1.09 ± 1.06	1.40 ± 1.15	2.96 ± 0.48
		Poly I:C	0.02 ± 0.00	1.46 ± 1.33	2.30 ± 0.46
	3	Cyt	2.22 ± 1.94	**108.75 ± 37.36**	**402.41 ± 86.69**
		LPS	1.85 ± 1.62	14.43 ± 6.41	**19.12 ± 4.38**
		LTA	0.14 ± 0.13	0.10 ± 0.05	0.65 ± 0.20
		Poly I:C	0.13 ± 0.10	1.68 ± 1.53	**26.34 ± 6.16**
CXCL10	1	Cyt	0.11 ± 0.06	0.19 ± 0.15	1.02 ± 0.63
		LPS	0.43 ± 0.31	11.88 ± 15.22	0.31 ± 0.08
		LTA	0.50 ± 0.21	1.42 ± 1.21	0.48 ± 0.18
		Poly I:C	0.69 ± 0.23	0.62 ± 0.50	0.91 ± 0.46
	2	Cyt	10.16 ± 5.26	**19.21 ± 10.64**	**45.62 ± 21.79**
		LPS	1.64 ± 0.46	1.23 ± 1.06	3.65 ± 1.84
		LTA	0.99 ± 0.47	1.14 ± 0.63	12.40 ± 6.16
		Poly I:C	**97.19 ± 36.00**	3.61 ± 2.02	6.92 ± 2.85
	3	Cyt	4.09 ± 2.34	6.51 ± 5.88	**913.91 ± 470.61**
		LPS	1.07 ± 0.69	1.21 ± 0.58	0.64 ± 0.25
		LTA	0.80 ± 0.38	2.73 ± 3.09	1.62 ± 1.12
		Poly I:C	30.72 ± 14.62	2.58 ± 1.92	0.15 ± 0.04
CXCL12	1	Cyt	0.13 ± 0.09	0.87 ± 0.90	2.87 ± 1.41
		LPS	0.27 ± 0.11	**5.88 ± 1.89**	0.66 ± 0.23
		LTA	1.15 ± 0.92	5.47 ± 4.99	1.02 ± 0.46
		Poly I:C	0.17 ± 0.14	2.68 ± 1.59	0.41 ± 0.23
	2	Cyt	0.16 ± 0.12	0.36 ± 0.30	**2.09 ± 0.86**
		LPS	0.39 ± 0.18	1.48 ± 1.62	1.54 ± 0.60
		LTA	1.98 ± 1.13	1.43 ± 0.75	1.14 ± 0.83
		Poly I:C	0.61 ± 0.39	2.69 ± 2.03	1.04 ± 0.57
	3	Cyt	0.22 ± 0.19	0.81 ± 0.59	**5.50 ± 2.08**
		LPS	0.63 ± 0.38	1.70 ± 0.41	0.53 ± 0.27
		LTA	1.88 ± 1.43	0.44 ± 0.38	2.20 ± 1.04
		Poly I:C	0.32 ± 0.23	1.95 ± 1.89	3.01 ± 1.13
CXCL16	1	Cyt	2.95 ± 0.24	0.35 ± 0.23	1.81 ± 0.92
		LPS	**5.07 ± 2.20**	1.15 ± 0.47	0.70 ± 0.31
		LTA	**8.30 ± 2.33**	0.52 ± 0.42	0.81 ± 0.50
		Poly I:C	1.20 ± 0.47	0.31 ± 0.12	0.46 ± 0.23
	2	Cyt	**7.59 ± 1.34**	**5.13 ± 2.00**	**43.11 ± 16.81**
		LPS	4.20 ± 0.62	1.52 ± 0.90	4.96 ± 3.71
		LTA	**10.53 ± 3.08**	0.54 ± 0.31	1.89 ± 0.97
		Poly I:C	3.25 ± 2.57	1.87 ± 1.42	1.57 ± 0.75
	3	Cyt	**5.30 ± 1.62**	**5.01 ± 3.71**	**62.89 ± 24.29**
		LPS	**7.48 ± 2.10**	0.74 ± 0.44	1.57 ± 0.61

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
		LTA	**13.80 ± 1.23**	2.68 ± 3.20	1.29 ± 0.66
		Poly I:C	2.26 ± 0.14	1.04 ± 0.59	2.33 ± 1.11

4.3.3 Transcription of CX3CL1 chemokine and atypical chemokine receptor ACKR4 under resting and inflammatory conditions

As outlined in Section 4.2.6, under resting conditions, BM, Is and Ad MSCs transcribed very little, if any, *CX3CL1* while *ACKR4* was transcribed at higher rates, but in both cases transcript level variations were not observed among the MSCs isolated from the different sources under resting conditions. However, in both cases tissue of origin and licensing agent did have an influence on the transcript levels after inflammatory stimulation (Section 8.1). *CX3CL1* showed a pattern of transcriptional upregulation after licensing while *ACKR4* showed a pattern of transcriptional downregulation. With a few exceptions, *CX3CL1* transcript levels increased after a 24 hours stimulation (Condition 2) in MSCs from every tissue source; however, this upregulation was not sustained, as cells harvested 72 hours after licensing (Condition 1) showed a decrease in the transcript levels of *CX3CL1* compared to Condition 2. A second stimulation 48 hours after the first stimulation (Condition 3) was able to induce transcription of *CX3CL1* in MSCs from the three sources, however, the second stimulation was not able to match the transcript levels observed in Condition 2. Overall, *ACKR4* transcript levels decrease after a 24 hour stimulation (Condition 2) in MSCs from every tissue source; however, this downregulation was not sustained, as even if the downregulation was still notable, cells harvested 72 hours after licensing (Condition 1) showed higher transcript levels than the ones observed 24 hours after licensing. A second stimulation 48 hours after the first stimulation (Condition 3) was able to slow down the reversion of the downregulation in MSCs from the three sources as *ACKR4* transcript levels were like the ones observed 24 hours after licensing. Fold changes of transcriptional regulation upon licensing of MSCs are specified in Table 4-9.

Table 4-9. Fold change in CX3CL1 and ACKR4 transcript levels of cytokine, LPS, LTA or Poly I:C-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.

MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with either a cocktail of cytokines (40 ng/ mL of IFN-Y, TNF-α and IL-1β), 100 ng/ mL LPS, 100 ng/ mL LTA or 4µg/ mL Poly I:C. Unstimulated cells were left growing in MSC culture medium as a control. Three different licensing conditions were tested. In the first one, cells were stimulated for 48 hours, after which cells were washed twice with PBS and fresh culture medium was added; cells were harvested 24 hours later. In the second condition, cells were washed twice with PBS, the culture medium was replaced with fresh one and the cells were left growing for 48 hours. Cells were then washed twice with PBS, the culture medium was replaced with supplemented one and the cells were harvested 24 hours later. In the last condition, cells were stimulated for 48 hours, after which cells were washed twice with PBS and stimulated again for another 24 hours. Figure 4-7 illustrates the time points at which supplemented medium was added. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate *CX3CL1* and *ACKR*4 transcripts in BM, Is and Ad MSCs under resting and inflammatory conditions. Data are normalised to the housekeeping gene *B2M* and expressed as $2^{(-\Delta CT)}$. Fold change in transcript levels of *CX3CL1* and *ACKR4* is represented as mean of fold change ± standard deviation. One Way ANOVA with Tukey's multiple comparisons post-test was performed to compare all MSC sources and the different conditions. Statistically significant differences are marked with a colour code, where $p < 0.05$ is represented by green, $p < 0.01$ is represented by orange, $p < 0.001$ is represented by blue and $p < 0.0001$ is represented by red.

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
CX3CL1	1	Cyt	2.39 ± 0.51	2.34 ± 2.02	2.19 ± 0.73
		LPS	1.40 ± 0.10	**7.44 ± 3.38**	0.29 ± 0.12
		LTA	**5.13 ± 3.48**	2.84 ± 1.46	1.09 ± 0.75
		Poly I:C	0.49 ± 0.32	**5.71 ± 1.25**	0.28 ± 0.14
	2	Cyt	**7.07 ± 3.78**	4.82 ± 4.06	**23.71 ± 5.55**
		LPS	0.61 ± 0.16	1.01 ± 0.88	0.15 ± 0.04
		LTA	2.40 ± 0.65	2.38 ± 2.75	0.24 ± 0.05
		Poly I:C	1.74 ± 1.62	4.29 ± 3.14	0.30 ± 0.09
	3	Cyt	1.24 ± 0.96	1.17 ± 0.80	**12.29 ± 2.38**
		LPS	0.73 ± 0.27	0.77 ± 0.75	0.42 ± 0.11
		LTA	1.20 ± 0.79	4.24 ± 2.95	**5.63 ± 1.68**
		Poly I:C	0.32 ± 0.20	1.92 ± 1.60	1.99 ± 0.43
ACKR4	1	Cyt	**0.02 ± 0.02**	**0.01 ± 0.00**	1.66 ± 1.30
		LPS	**0.07 ± 0.01**	0.18 ± 0.09	0.85 ± 0.30
		LTA	**0.16 ± 0.05**	0.23 ± 0.04	0.80 ± 0.49
		Poly I:C	**0.01 ± 0.00**	0.82 ± 0.57	0.52 ± 0.16
	2	Cyt	**0.02 ± 0.01**	**0.01 ± 0.00**	0.26 ± 0.14
		LPS	**0.05 ± 0.02**	**0.08 ± 0.02**	0.12 ± 0.09
		LTA	**0.26 ± 0.11**	0.47 ± 0.23	0.31 ± 0.18
		Poly I:C	**0.03 ± 0.01**	**0.13 ± 0.03**	0.19 ± 0.05
	3	Cyt	**0.01 ± 0.00**	**0.01 ± 0.00**	0.29 ± 0.12
		LPS	**0.18 ± 0.08**	0.17 ± 0.18	0.18 ± 0.04
		LTA	**0.11 ± 0.04**	0.60 ± 0.11	**3.15 ± 0.66**
		Poly I:C	**0.00 ± 0.00**	**0.13 ± 0.14**	0.50 ± 0.16

4.3.4 Transcription of the receptors for the stimulatory agents under resting and inflammatory conditions

Under resting conditions, BM, Is and Ad MSCs transcribed very little, if any, *IL1R1*, *IL1R2*, *IFNGR2*, *TLR2*, *TLR3* and *TLR4*, while *TNFR1a*, *TNFR1b* and *IFNGR1* were transcribed at higher rates (Section 8.1). In all the cases, transcript level variations were not observed among the MSCs isolated from the different sources under resting conditions. However, tissue of origin did influence the transcriptional regulation upon exposure to the different licensing agents and the different conditions. Fold changes of transcriptional regulation upon licensing of MSCs are summarised in Table 4-10.

Briefly, cytokine-mediated stimulation led to the downregulation of the cytokine receptors (*IL1R1*, *IL1R2*, *TNFR1a*, *TNFR1b*, *IFNGR1* and *IFNGR2*) in BM and Is MSCs under all conditions. Cytokine-mediated stimulation downregulated the cytokine receptors in Ad MSCs too; however, this downregulation was not sustained 72 hours after stimulation and, in some cases, even led to the upregulation of the receptors. LPS and LTA were able to upregulate the cytokine receptor transcript levels in certain conditions in MSCs from the three sources. The effects of Poly I:C in the regulation of the cytokine receptor transcription levels was tissue specific; Poly I:C stimulation of BM MSCs led to the downregulation of all the cytokine receptors but *IFNGR2* in all the conditions tested. Poly I:C was able to downregulate the transcription of all the cytokine receptors but *IFNGR2* in Is MSCs; however, 24 hours after the stimulation the downregulation started getting reversed and the transcript levels started increasing. The effect of Poly I:C stimulation in Ad MSCs was gene dependent and the effect it produced in each of the genes at the established time points will be described later. LPS-mediated stimulation downregulated its receptor, *TLR4*, after 24 hours licensing in BM MSCs; however, 24 hours after the stimulation the downregulation started to reverse, and the transcript levels started increasing. LPS produced no variation in *TLR4* transcript levels after a single stimulation in Is MSCs; however, a second stimulation 48 hours after the first one downregulated the receptor transcript levels.

Overall, LPS-mediated licensing produced no variation in *TLR4* transcript levels in Ad MSCs under any condition. Cytokine and Poly I:C-mediated licensing were

associated with downregulation of the transcription of *TLR4* in BM and Is MSCs; while cytokines upregulated its transcription in every condition and Poly I:C was able to upregulate the transcription of *TLR4* after a double stimulation. LTA-mediated stimulation produced an upregulation of *TLR4* transcript levels in BM MSCs, a downregulation that was reversed after 72 hours in Is MSCs and was only able to upregulate the transcription after a double stimulation in Ad MSCs.

LTA-mediated stimulation upregulated its receptor, *TLR2*, in BM MSCs under every condition, while it produced no variation in Is MSCs and a small downregulation in Ad MSCs after a single stimulation but no variation after a double stimulation. All the licensing agents were able to increase the transcript levels of *TLR2* in BM MSCs. LPS and Poly I:C produced no variation on *TLR2* transcript levels in Is and Ad MSCs while cytokine-mediated licensing produced an upregulation in 24 hours in Is MSCs which was not sustained and Ad MSCs required a double stimulation to upregulate *TLR2* transcript levels.

Poly I:C-mediated stimulation downregulated its receptor, *TLR3*, in MSCs from the three sources under every condition tested. All the licensing agents were able to decrease the transcript levels of *TLR3* in BM MSCs. Cytokine-mediated licensing downregulated *TLR3* transcript levels in Is MSCs under every condition, while it produced an upregulation in Ad MSCs. LPS or LTA-mediated stimulation produced a decrease in the transcript levels 24 hours after the first or second stimulation, while they produced an upregulation 72 hours later in Is MSCs. In contrast, Ad MSCs responded to LPS or LTA licensing by downregulating *TLR3*, but this downregulation was brief as transcript level started increasing after 24 hours.

Table 4-10. Fold change in the receptors of the stimulatory agents' transcript levels of cytokine, LPS, LTA or Poly I:C-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.

MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with either a cocktail of cytokines (40 ng/ mL of IFN-Ɣ, TNF-α and IL-1β), 100 ng/ mL LPS, 100 ng/ mL LTA or 4 μg/ mL Poly I:C. Unstimulated cells were left growing in MSC culture medium as a control. Three different licensing conditions were tested. In the first one, cells were stimulated for 48 hours, after which cells were washed twice with PBS and fresh culture medium was added; cells were harvested 24 hours later. In the second condition, cells were washed twice with PBS, the culture medium was replaced with fresh one and the cells were left growing for 48 hours. Cells were then washed twice with PBS, the culture medium was replaced with supplemented one and the cells were harvested 24 hours later. In the last condition, cells were stimulated for 48 hours, after which cells were washed twice with PBS and were stimulated again for another 24 hours. Figure 4-7 illustrates the time points at which

supplemented medium was added. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate transcript levels of the receptors of the stimulatory agents in BM, Is and Ad MSCs under resting and inflammatory conditions. Data are normalised to the housekeeping gene *B2M* and expressed as $2^{(-\Delta CT)}$. Fold change in transcript levels of the receptors of the stimulatory agents is represented as mean of fold change ± standard deviation. One Way ANOVA with Tukey's multiple comparisons post-test was performed to compare all MSC sources and the different conditions. Statistically significant differences are marked with a colour code, where $p < 0.05$ is represented by green, $p < 0.01$ is represented by orange, $p < 0.001$ is represented by blue and $p < 0.0001$ is represented by red.

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
IL1R1	1	Cyt	0.07 ± 0.04	0.22 ± 0.13	3.20 ± 2.13
		LPS	0.20 ± 0.07	3.47 ± 1.35	3.27 ± 2.77
		LTA	0.25 ± 0.06	3.62 ± 2.31	2.03 ± 1.11
		Poly I:C	0.05 ± 0.02	1.88 ± 1.82	1.04 ± 0.58
	2	Cyt	0.18 ± 0.11	0.04 ± 0.02	1.99 ± 1.00
		LPS	0.48 ± 0.39	0.54 ± 0.25	0.73 ± 0.35
		LTA	1.61 ± 1.53	0.64 ± 0.29	0.73 ± 0.48
		Poly I:C	0.14 ± 0.09	0.56 ± 0.28	0.93 ± 0.39
	3	Cyt	0.19 ± 0.14	0.03 ± 0.01	0.01 ± 0.01
		LPS	0.59 ± 0.41	0.53 ± 0.13	0.83 ± 0.61
		LTA	0.92 ± 1.01	1.38 ± 0.40	5.59 ± 3.74
		Poly I:C	0.05 ± 0.04	0.33 ± 0.26	0.00 ± 0.00
IL1R2	1	Cyt	0.07 ± 0.07	0.86 ± 1.27	13476.79 ± 13682.54
		LPS	2.30 ± 1.21	8.66 ± 12.92	1.72 ± 1.91
		LTA	1.95 ± 1.48	4.04 ± 1.76	5.11 ± 4.66
		Poly I:C	0.52 ± 0.46	3.34 ± 3.13	4.72 ± 7.66
	2	Cyt	0.19 ± 0.17	0.10 ± 0.09	25.20 ± 38.61
		LPS	0.99 ± 1.11	0.71 ± 0.48	1.79 ± 0.79
		LTA	2.88 ± 3.17	2.50 ± 2.97	1.39 ± 1.48
		Poly I:C	0.53 ± 0.28	0.86 ± 0.31	1.70 ± 2.57
	3	Cyt	0.57 ± 0.64	0.47 ± 0.43	17.83 ± 18.07
		LPS	0.96 ± 0.31	0.77 ± 0.76	4.54 ± 6.34
		LTA	0.96 ± 0.46	5.51 ± 6.58	34.53 ± 53.66
		Poly I:C	0.17 ± 0.14	9.71 ± 14.87	2.13 ± 2.64
TNFR1a	1	Cyt	0.69 ± 0.30	0.29 ± 0.18	0.80 ± 0.31
		LPS	0.76 ± 0.10	1.52 ± 0.60	0.47 ± 0.11
		LTA	2.65 ± 1.39	1.53 ± 1.58	0.78 ± 0.24
		Poly I:C	0.39 ± 0.20	0.95 ± 0.65	0.47 ± 0.06
	2	Cyt	0.74 ± 0.49	0.44 ± 0.31	0.89 ± 0.09
		LPS	0.32 ± 0.07	0.84 ± 0.30	0.32 ± 0.06
		LTA	1.71 ± 0.81	0.54 ± 0.54	0.17 ± 0.03
		Poly I:C	0.48 ± 0.08	0.82 ± 0.78	0.32 ± 0.04
	3	Cyt	0.36 ± 0.26	0.08 ± 0.05	5.92 ± 0.68
		LPS	0.62 ± 0.38	0.38 ± 0.14	2.95 ± 0.32
		LTA	1.64 ± 1.36	0.69 ± 0.89	7.05 ± 1.26
		Poly I:C	0.20 ± 0.15	0.17 ± 0.02	2.63 ± 0.50
TNFR1b	1	Cyt	0.32 ± 0.04	0.12 ± 0.08	0.65 ± 0.35
		LPS	0.53 ± 0.11	0.43 ± 0.05	0.45 ± 0.06
		LTA	1.13 ± 0.16	0.69 ± 0.25	0.88 ± 0.19

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
		Poly I:C	0.19 ± 0.05	0.34 ± 0.21	0.44 ± 0.06
	2	Cyt	0.47 ± 0.11	0.24 ± 0.08	0.68 ± 0.09
		LPS	0.71 ± 0.07	0.55 ± 0.04	0.63 ± 0.19
		LTA	1.06 ± 0.25	0.50 ± 0.19	0.28 ± 0.03
		Poly I:C	0.34 ± 0.03	0.30 ± 0.09	0.38 ± 0.09
	3	Cyt	0.41 ± 0.06	0.19 ± 0.01	2.81 ± 0.17
		LPS	0.84 ± 0.16	0.55 ± 0.06	1.01 ± 0.03
		LTA	1.25 ± 0.18	0.67 ± 0.36	0.99 ± 0.19
		Poly I:C	0.20 ± 0.01	0.45 ± 0.04	4.07 ± 0.66
IFNGR1	1	Cyt	0.10 ± 0.10	0.12 ± 0.07	1.18 ± 0.44
		LPS	0.23 ± 0.12	0.68 ± 0.14	0.32 ± 0.28
		LTA	1.01 ± 0.17	1.13 ± 0.12	0.63 ± 0.07
		Poly I:C	0.10 ± 0.01	0.37 ± 0.26	0.42 ± 0.24
	2	Cyt	0.10 ± 0.09	0.15 ± 0.06	0.52 ± 0.49
		LPS	0.10 ± 0.07	0.60 ± 0.19	0.40 ± 0.38
		LTA	0.97 ± 0.22	0.72 ± 0.24	0.38 ± 0.03
		Poly I:C	0.16 ± 0.05	0.48 ± 0.27	0.57 ± 0.10
	3	Cyt	0.06 ± 0.05	0.07 ± 0.03	1.18 ± 0.98
		LPS	0.16 ± 0.10	0.52 ± 0.11	1.04 ± 0.73
		LTA	0.95 ± 0.26	0.88 ± 0.40	2.25 ± 0.40
		Poly I:C	0.07 ± 0.02	0.26 ± 0.12	0.53 ± 0.13
IFNGR2	1	Cyt	0.99 ± 0.27	0.69 ± 0.61	4.84 ± 1.61
		LPS	1.37 ± 0.19	5.73 ± 2.99	1.90 ± 0.70
		LTA	5.81 ± 3.38	6.09 ± 2.63	2.92 ± 0.88
		Poly I:C	0.93 ± 0.63	4.10 ± 2.21	0.85 ± 0.34
	2	Cyt	0.68 ± 0.29	0.84 ± 0.11	2.71 ± 0.81
		LPS	0.59 ± 0.27	2.12 ± 0.90	1.73 ± 0.40
		LTA	2.43 ± 1.26	0.84 ± 0.11	1.11 ± 0.21
		Poly I:C	2.81 ± 1.70	2.38 ± 0.88	1.02 ± 0.21
	3	Cyt	0.39 ± 0.28	0.26 ± 0.29	19.05 ± 5.19
		LPS	0.78 ± 0.22	0.58 ± 0.30	3.51 ± 1.54
		LTA	2.04 ± 1.50	2.08 ± 1.77	10.72 ± 2.57
		Poly I:C	0.28 ± 0.21	2.12 ± 0.90	9.77 ± 2.59
TLR2	1	Cyt	1.44 ± 0.83	0.42 ± 0.19	0.97 ± 0.62
		LPS	4.34 ± 2.29	1.16 ± 0.44	0.41 ± 0.13
		LTA	5.60 ± 2.63	1.56 ± 1.05	0.79 ± 0.21
		Poly I:C	1.30 ± 0.83	0.79 ± 0.19	0.32 ± 0.09
	2	Cyt	4.42 ± 0.83	2.68 ± 0.32	0.32 ± 0.28
		LPS	4.16 ± 2.60	1.41 ± 0.13	0.47 ± 0.10
		LTA	6.85 ± 1.86	1.71 ± 0.84	0.46 ± 0.11
		Poly I:C	5.60 ± 2.41	1.69 ± 0.26	0.20 ± 0.20
	3	Cyt	3.63 ± 1.06	0.82 ± 0.22	22.83 ± 4.59
		LPS	10.22 ± 0.53	2.64 ± 1.42	0.97 ± 0.15
		LTA	8.72 ± 1.01	2.03 ± 0.89	1.13 ± 0.34
		Poly I:C	3.40 ± 0.98	3.07 ± 1.70	1.51 ± 0.14
TLR3	1	Cyt	0.87 ± 0.74	0.45 ± 0.25	5.50 ± 5.82

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
		LPS	1.00 ± 0.38	3.16 ± 2.25	1.40 ± 1.06
		LTA	2.07 ± 0.92	3.61 ± 2.06	2.73 ± 1.04
		Poly I:C	1.14 ± 0.39	2.01 ± 2.33	0.37 ± 0.23
	2	Cyt	2.23 ± 1.21	1.77 ± 0.67	1.01 ± 0.44
		LPS	0.92 ± 0.29	1.07 ± 0.45	0.49 ± 0.31
		LTA	2.09 ± 0.82	1.24 ± 0.47	0.83 ± 0.51
		Poly I:C	1.48 ± 0.95	3.16 ± 1.01	0.82 ± 0.62
	3	Cyt	0.73 ± 0.40	0.41 ± 0.12	**13.84 ± 9.93**
		LPS	0.97 ± 0.45	0.40 ± 0.11	1.01 ± 0.72
		LTA	1.42 ± 0.62	1.22 ± 0.57	1.75 ± 0.93
		Poly I:C	1.70 ± 0.99	1.36 ± 1.44	2.19 ± 1.65
TLR4	1	Cyt	0.53 ± 0.22	0.20 ± 0.16	**2.31 ± 0.61**
		LPS	0.73 ± 0.20	**2.20 ± 0.77**	1.19 ± 0.21
		LTA	**2.01 ± 0.81**	**3.11 ± 1.21**	1.27 ± 0.43
		Poly I:C	0.51 ± 0.22	0.98 ± 0.44	0.60 ± 0.12
	2	Cyt	0.80 ± 0.43	0.52 ± 0.25	1.17 ± 0.26
		LPS	0.43 ± 0.14	0.82 ± 0.41	0.91 ± 0.38
		LTA	**2.23 ± 1.29**	0.52 ± 0.25	0.44 ± 0.08
		Poly I:C	0.49 ± 0.08	1.19 ± 0.30	0.60 ± 0.20
	3	Cyt	0.60 ± 0.36	0.13 ± 0.06	**5.64 ± 1.31**
		LPS	**0.76 ± 0.25**	0.36 ± 0.15	1.12 ± 0.26
		LTA	**1.86 ± 1.15**	0.82 ± 0.26	**3.54 ± 1.36**
		Poly I:C	0.29 ± 0.18	0.37 ± 0.20	**2.82 ± 1.17**

4.4 Analysis of the chemokine secretion profile by MSCs under resting and inflammatory conditions

4.4.1 Analysis of CC chemokine secretion under resting and inflammatory conditions

Under resting conditions, BM, Is and Ad MSCs secreted less than 0.35 pg of CC chemokines per mg of total protein (Figure 4-8). A pattern of upregulation was observed in CCL2 (A), CCL5 (B) and CCL7 (C) upon licensing. However, these chemokines were upregulated differentially in MSCs according to their tissue of origin. Analysis of the CC chemokine protein levels 24 hours after stimulation (Condition 2) resulted in upregulated secretion in MSCs from every tissue source; however, this upregulation was not sustained as supernatant harvested 72 hours after licensing (Condition 1) showed a decrease in the secretion of CC chemokines. A second stimulation 48 hours after the first stimulation (Condition 3) was able to induce the production of CC chemokines in MSCs from the three

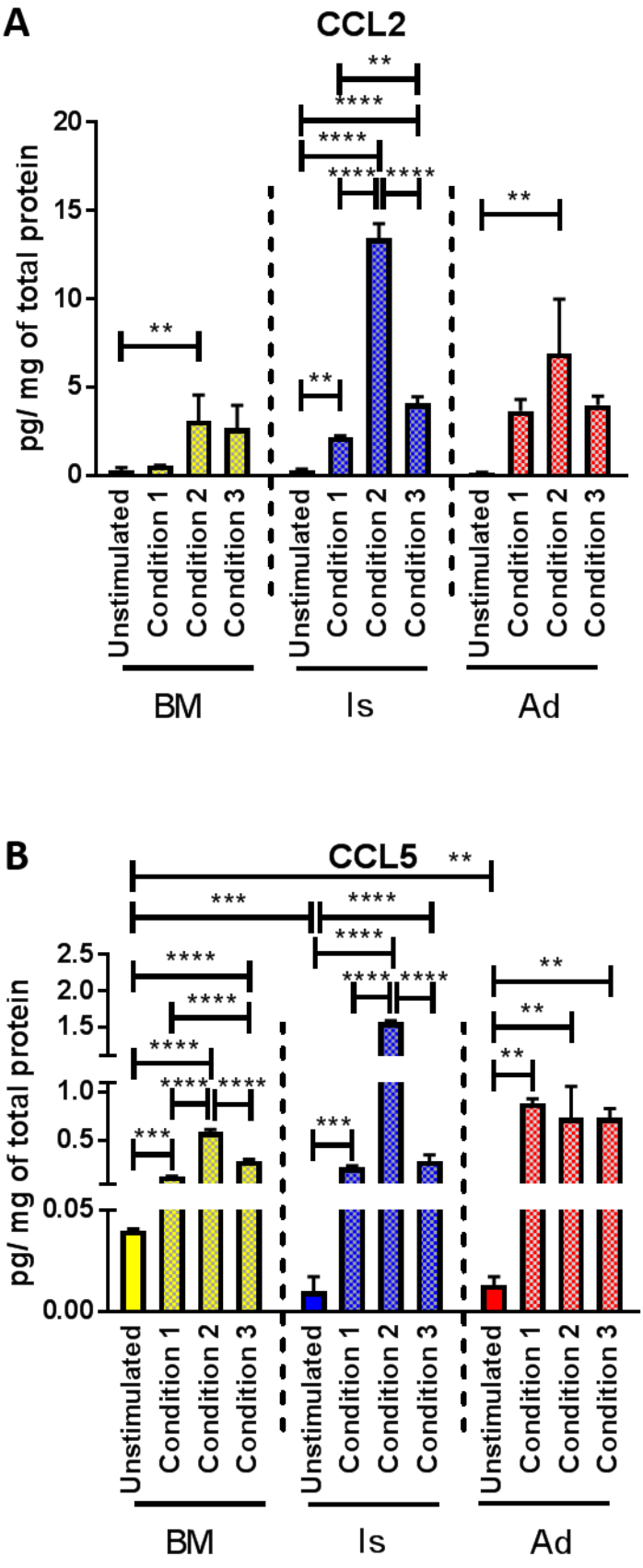
sources; however, this second stimulation was not able to mimic the secretion level of CC chemokines found in Condition 2. Fold changes in CC chemokine secretion upon cytokine-mediated licensing of MSCs are shown in Table 4-11.

MSCs isolated from the three tissues were producing CCL2 under resting conditions but Is MSCs secreted the highest levels of CCL2 (BM = 0.29 pg/ mg; Is = 0.32 pg/ mg; Ad = 0.16 pg/ mg) (Figure 4-8, A). Cytokine-mediated licensing produced an upregulation of the secretion in all the conditions tested in MSCs of the three tissue sources. After 72 hours of stimulation (Condition 1), CCL2 secretion was upregulated in MSCs from the three tissues but was only statistically significant in Is MSCs. However, 24 hours of stimulation (Condition 2) produced a statistically significant upregulation of CCL2 secretion in BM, Is and Ad MSCs, where Is MSCs were secreting 13 pg of CCL2 per mg of total protein. A second cytokine-mediated stimulation 48 hours after the first one (Condition 3) led to a higher secretion of CCL2 compared to unstimulated MSCs or Condition 1 in MSCs from every tissue source, suggesting that cells were responsive to the second stimulation. However, CCL2 levels were lower than 24 hours after a single stimulation. The lower limit of quantification for CCL2 in the Mouse Magnetic Luminex Assay was 210.58 pg/ mL and all the analysed samples were above this value.

Secretion of CCL5 under resting conditions was low in every source of MSCs; however, BM MSCs secreted statistically significant higher amounts of CCL5 than Is and Ad MSCs (BM = 0.039 pg/ mg; Is = 0.010 pg/ mg; Ad = 0.013 pg/ mg) (Figure 4-8, B). Cytokine-mediated licensing produced an upregulation of the secretion of CCL5 in all the conditions tested in MSCs from the three tissue sources. After 72 hours of stimulation (Condition 1), CCL5 secretion was significantly upregulated in MSCs from the three tissues; however, despite the highest secretion under resting conditions, BM MSCs had secreted the least CCL5 and Ad MSCs the most. 24 hours after stimulation (Condition 2) BM and Is MSCs had secreted statistically significantly higher amounts of CCL5 compared to control or Condition 2; however, these levels were much higher in Is MSCs as they had secreted 1.56 pg of CCL5 per mg of total protein. After 24 hours of licensing, Ad MSCs had significantly upregulated the secretion of CCL5 compared to unstimulated conditions but no variations were observed when compared with

Condition 1, suggesting that Ad MSC licensing had a more prolonged effect than the one generated on BM or Is MSCs. A second cytokine-mediated stimulation 48 hours after the first one (Condition 3) led to a higher secretion of CCL2 compared to unstimulated MSCs or Condition 1 in BM and Is MSCs, suggesting that cells were responsive to the second stimulation but not able to replicate the effect achieved after 24 hours of licensing; however, despite the upregulation in the production of CCL5 compared to the control, no variations were observed in the production of CCL5 by Ad MSCs compared to Condition 1 or 2. The lower limit of quantification for CCL5 in the Mouse Magnetic Luminex Assay was 73.42 pg/ mL and all the analysed samples were above this value.

MSCs isolated from the three tissues were producing CCL7 under resting conditions but Is MSCs secreted the highest levels of CCL7 (BM = 0.13 pg/ mg; Is = 0.14 pg/ mg; Ad = 0.08 pg/ mg) (Figure 4-8, C). Cytokine-mediated licensing produced an upregulation in the secretion in all the conditions tested in MSCs of the three tissue sources. After 72 hours of stimulation (Condition 1), CCL7 secretion was upregulated in the MSCs from the three tissues but was only statistically significant in Is and Ad MSCs. 24 hours of stimulation (Condition 2) produced an upregulation of CCL7 secretion in BM, Is and Ad MSCs; however, this upregulation was only statistically significant in Is and Ad MSCs. A second cytokine-mediated stimulation 48 hours after the first one (Condition 3) led to a higher secretion of CCL2 compared to unstimulated MSCs or Condition 1 in Is and Ad MSCs from every tissue source, suggesting that cells were responsive to the second stimulation; however, CCL2 levels were lower than 24 hours after a single stimulation. BM MSCs had the highest CCL7 protein levels in Condition 3; however, the amount of protein secreted was not statistically significantly different to the levels of secreted protein in Conditions 1 or 2. The lower limit of quantification for CCL7 in the Mouse Magnetic Luminex Assay was 5.6 pg/mL and all the analysed samples were above this value.


A
CCL2
pg/ mg of total protein
20
15
10
5
0
**
**
****
****
****
****
**
Unstimulated
Condition 1
Condition 2
Condition 3
BM
Is
Ad
B
CCL5
pg/ mg of total protein
2.5
2.0
1.5
1.0
0.5
0.05
0.00
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Unstimulated
Condition 1
Condition 2
Condition 3
BM
Is
Ad

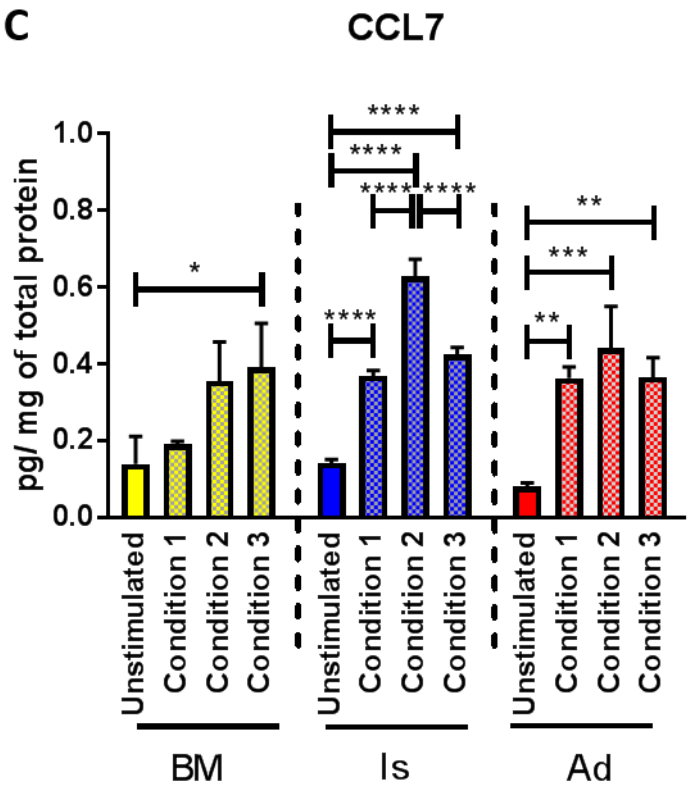


Figure 4-8. Cytokine-mediated stimulation, repetitive stimulus and MSC tissue origin impacts CC chemokine secretion in MSCs.
MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with a cocktail of cytokines (40 ng/ mL of IFN-ϒ, TNF-α and IL-1β). Unstimulated cells were left growing in MSC culture medium as a control. Three different licensing conditions were tested. In the first one, cells were stimulated for 48 hours, after which cells were washed twice with PBS and fresh culture medium was added; cells were harvested 24 hours later. In the second condition, cells were washed twice with PBS, the culture medium was replaced with fresh one and the cells were left growing for 48 hours. Cells were then washed twice with PBS, the culture medium was replaced with supplemented one and the cells were harvested 24 hours later. In the last condition, cells were stimulated for 48 hours, after which cells were washed twice with PBS and were stimulated again for another 24 hours. Figure 4-7 illustrates the time points at which supplemented medium was added. Luminex was performed to evaluate protein secretion in BM, Is and Ad MSCs under resting and inflammatory conditions. Each bar represents an n of 3 independent experiments and is graphed as mean ± SEM. Data are normalised to total amount of protein in medium and expressed as picograms of protein of interest per mg of total protein. Appropriate statistical analysis was performed and includes Students paired T test between one MSC tissue source (Resting vs Inflammatory Conditions) and One Way ANOVA with Tukey's multiple comparisons post-test to compare all MSC sources. Statistically significant differences are marked with the appropriate number of asterisks. p = 0.05 was considered the limit for statistical significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Table 4-11. Fold change in CC chemokine secretion of cytokine-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.
Following the experimental set up explained in Figure 4-8, fold change in CC chemokine protein levels is represented as mean of fold change ± standard deviation. Statistically significant differences are marked with a colour code, where $p < 0.05$ is represented by green, $p < 0.01$ is represented by orange, $p < 0.001$ is represented by blue and $p < 0.0001$ is represented by red.

Protein	Condition	BM MSCs	Is MSCs	Ad MSCs
CCL2	1	3.00 ± 2.10	6.72 ± 0.79	22.91 ± 5.90
	2	18.38 ± 16.43	41.47 ± 4.27	44.54 ± 19.65
	3	15.84 ± 14.63	12.63 ± 1.52	24.39 ± 2.55
CCL5	1	3.20 ± 0.04	28.80 ± 11.61	73.27 ± 22.14
	2	14.69 ± 0.64	206.50 ± 86.46	65.53 ± 37.30
	3	7.19 ± 0.39	39.83 ± 19.56	59.72 ± 15.70
CCL7	1	1.84 ± 1.14	2.61 ± 0.12	4.56 ± 0.70
	2	3.60 ± 2.50	4.45 ± 0.48	5.57 ± 1.43
	3	3.99 ± 2.95	3.01 ± 0.23	4.52 ± 0.09

4.4.2 Analysis of CXC chemokine secretion under resting and inflammatory conditions

Under resting conditions, BM, Is and Ad MSCs secreted less than 0.5 pg of CXC chemokines per mg of total protein (Figure 4-9). A pattern of upregulation was observed in CXCL1 (A), CXCL2 (B) and CXCL10 (C), while CXCL12 (D) presented a pattern of downregulation after licensing. However, these chemokines were regulated differentially in MSCs according to their tissue of origin. Analysis of the CXC chemokine protein levels 24 hours after stimulation (Condition 2) demonstrated a marked upregulation of the secretion of CXCL1, CXCL2 and CXCL10 in MSCs from every tissue source; however, this upregulation was not sustained as supernatant harvested 72 hours after licensing (Condition 1) showed a decrease in the secretion of CXC chemokines. A second stimulation 48 hours after the first stimulation (Condition 3) was able to induce the production of CXC chemokines in MSCs from the three sources, however, the second stimulation was not able to mimic the secretion level of CC chemokines found in Condition 2. Regarding CXCL12, 24 hours of stimulation (Condition 2) produced no effect in BM and Ad MSCs while it produced a downregulation in Is MSCs. Condition 1 and 3 produced a downregulation in the secretion of CXCL12 from MSCs of every source. Fold changes in CXC chemokine secretion upon cytokine-mediated licensing of MSCs are specified in Table 4-12.

MSCs isolated from the three tissues were producing very little CXCL1 under resting conditions but BM MSCs secreted the highest levels of CXCL1 (BM = 0.07 pg/ mg; Is = 0.02 pg/ mg; Ad = 0.01 pg/ mg) (Figure 4-9, A). Cytokine-mediated licensing produced an upregulation of the secretion in all the conditions tested in MSCs of the three tissue sources. After 72 hours of stimulation (Condition 1),

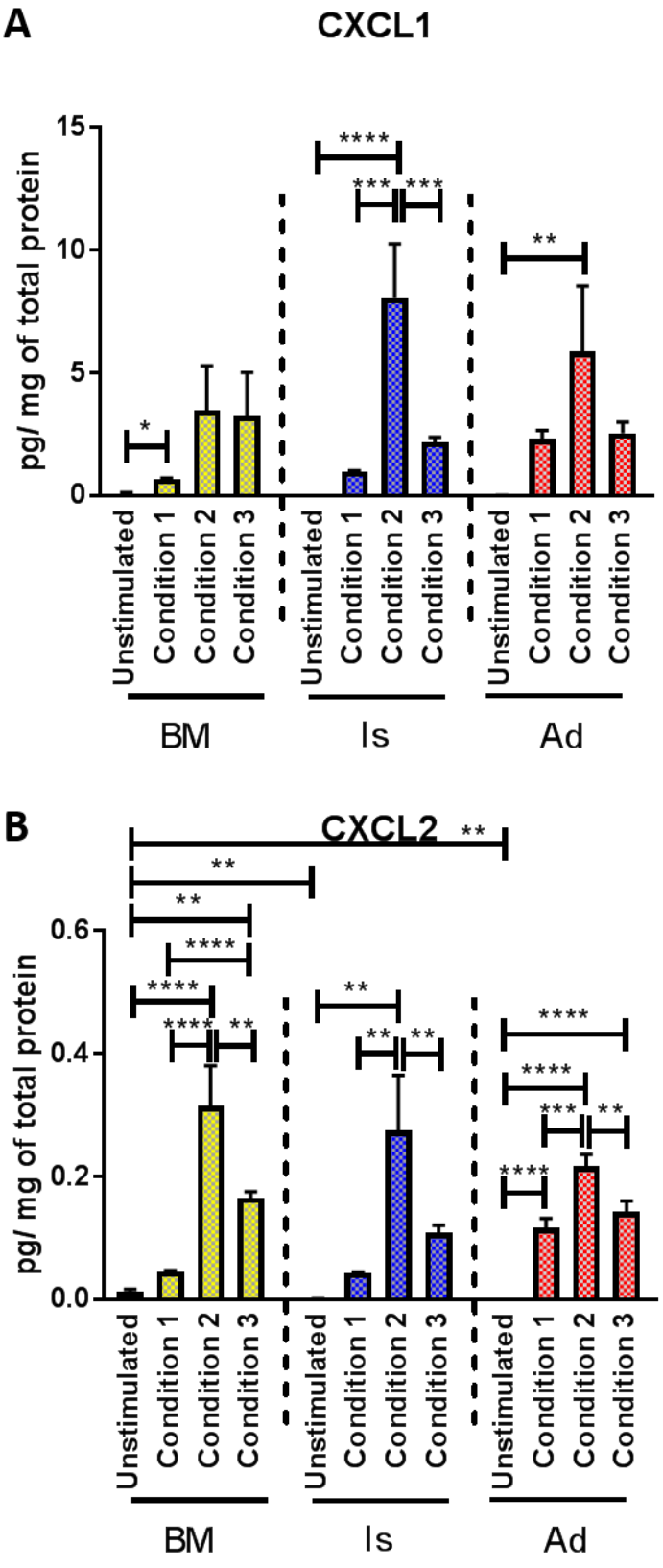
CXCL1 secretion was upregulated in MSCs from the three tissues but was only statistically significant in BM MSCs. However, 24 hours of stimulation (Condition 2) produced a statistically significant upregulation of CXCL1 secretion in Is and Ad MSCs, where Is MSCs were secreting 8 pg of CXCL1 per mg of total protein. A second cytokine-mediated stimulation 48 hours after the first one (Condition 3) led to a statistically significant higher secretion of CXCL1 compared to unstimulated MSCs or Condition 1 in Is and Ad MSCs, suggesting that cells were responsive to the second stimulation; however, CXCL1 levels were lower than 24 hours after a single stimulation. The lower limit of quantification for CXCL1 in the Mouse Magnetic Luminex Assay was 33.83 pg/ mL and all the analysed samples were above this value.

MSCs isolated from the three tissues were producing very little CXCL2 under resting conditions but BM MSCs secreted the highest levels of CXCL2 (BM = 0.012 pg/ mg; Is = 0.0007 pg/ mg; Ad = 0.0001 pg/ mg) (Figure 4-9, B). Cytokine-mediated licensing produced an upregulation of the secretion in all the conditions tested in MSCs of the three tissue sources. After 72 hours of stimulation (Condition 1), CXCL2 secretion was upregulated in MSCs from the three tissues but was only statistically significant in Ad MSCs. However, 24 hours of stimulation (Condition 2) produced a statistically significant upregulation of CXCL2 secretion in MSCs from every source, with BM MSCs secreting 0.31 pg of CXCL2 per mg of total protein. A second cytokine-mediated stimulation 48 hours after the first one (Condition 3) led to a statistically significant higher secretion of CXCL2 compared to unstimulated MSCs or Condition 1 in MSCs from every source, suggesting that cells are responsive to the second stimulation; however, CXCL2 levels were lower than 24 hours after a single stimulation. The lower limit of quantification for CXCL2 in the Mouse Magnetic Luminex Assay was 5.14 pg/ mL and all the analysed samples were above this value.

MSCs isolated from the three tissues were producing very little CXCL10 under resting conditions but BM MSCs secreted the highest levels of CXCL10 (BM = 0.011 pg/ mg; Is = 0.010 pg/ mg; Ad = 0.009 pg/ mg) (Figure 4-9, C). Cytokine-mediated licensing produced an upregulation of the secretion in all the conditions tested in MSCs of the three tissue sources. After 72 hours of stimulation (Condition 1), CXCL10 secretion was upregulated in a statistically

significant manner in MSCs from the three tissues. However, 24 hours of stimulation (Condition 2) produced an even greater upregulation of CXCL10 secretion in MSCs from every source, with Is MSCs secreting 2.4 pg of CXCL10 per mg of total protein. A second cytokine-mediated stimulation 48 hours after the first one (Condition 3) led to a statistically significant higher secretion of CXCL10 compared to unstimulated MSCs or Condition 1 in all sources of MSCs, suggesting that cells were responsive to the second stimulation; however, CXCL10 levels were lower than 24 hours after a single stimulation. The lower limit of quantification for CXCL10 in the Mouse Magnetic Luminex Assay was 59.51 pg/ mL and all the analysed samples were above this value.

MSCs isolated from the three tissues were producing CXCL12 under resting conditions but BM MSCs secreted the highest levels of this CXC chemokine (BM = 0.56 pg/ mg; Is = 0.25 pg/ mg; Ad = 0.16 pg/ mg) (Figure 4-9, D). Cytokine-mediated licensing produced no statistically significant effect on the secretion of CXCL12 in any of the conditions tested in BM and Ad MSCs, while it produced a downregulation in the secretion of CXCL12 by Is MSCs. After 72 hours of stimulation (Condition 1), CXCL12 secretion was downregulated in MSCs from the three tissues, even if it was only statistically significant in Is MSCs. 24 hours of stimulation (Condition 2) produced a downregulation of the same level as Condition 1 in Is MSCs, while it produced no effect in BM and Ad MSCs. A second cytokine-mediated stimulation 48 hours after the first one (Condition 3) led to a greater downregulation in the secretion of CXCL12 by Is MSCs compared to unstimulated MSCs or Condition 1 and 2. Condition 3 led to a statistically non-significant downregulation of CXCL12 secretion by BM and Ad MSCs. The lower limit of quantification for CXCL12 in the Mouse Magnetic Luminex Assay was 319.88 pg/ mL and all the analysed samples were above this value.


A
CXCL1
pg/ mg of total protein
15
10
5
0
*
****
***
***
**
Unstimulated
Condition 1
Condition 2
Condition 3
BM
Is
Ad
B
CXCL2
pg/ mg of total protein
0.6
0.4
0.2
0.0
**
****
***

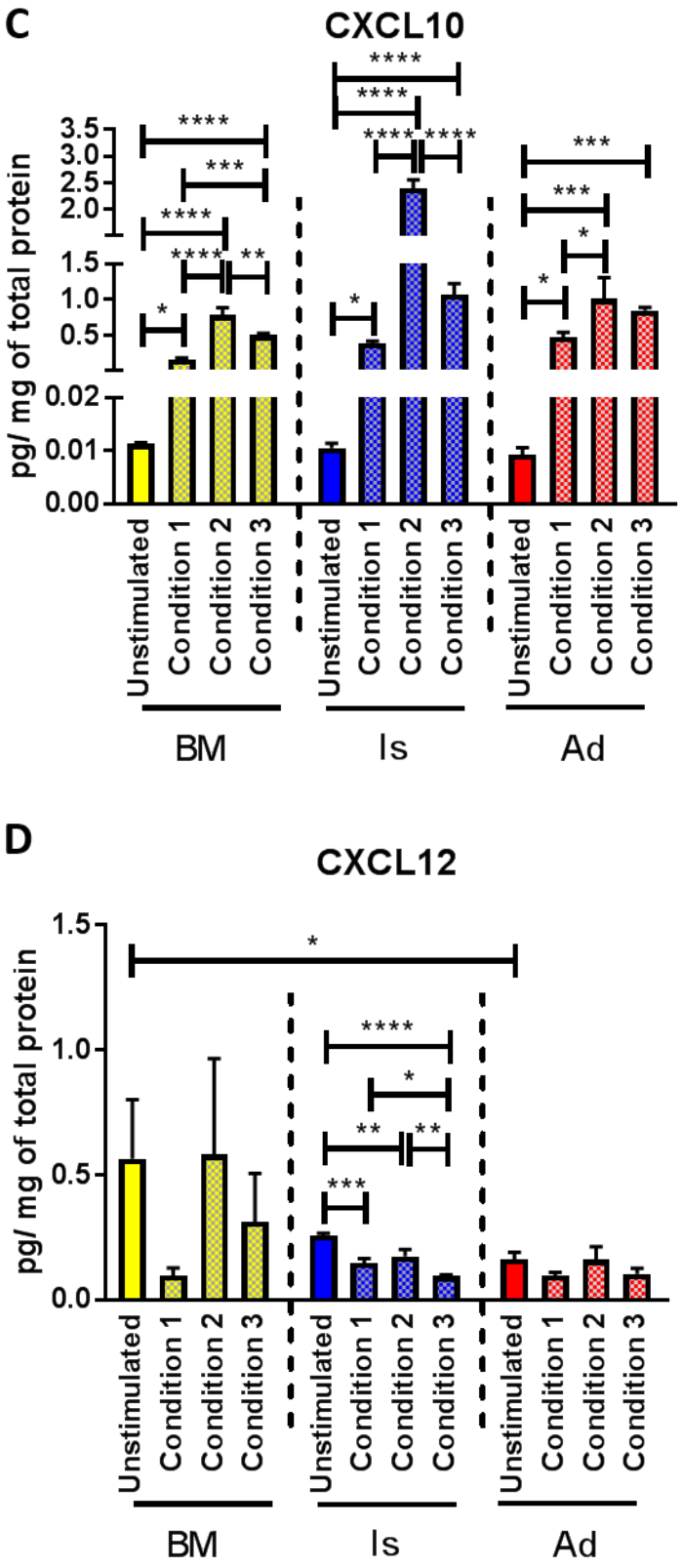


Figure 4-9. Cytokine-mediated stimulation, repetitive stimulus and MSC tissue origin impacts CXC chemokine secretion in MSCs.
MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with a cocktail of cytokines (40 ng/ mL of IFN-ϒ, TNF-α and IL-1β). Unstimulated cells were left growing in MSC culture medium as a control. Three different licensing conditions were tested. In the first one, cells were stimulated for 48 hours, after which cells were washed twice with PBS and fresh culture medium was added; cells were harvested 24 hours later. In the second condition, cells were washed twice with PBS, the culture medium was replaced with fresh one and the cells were left growing for 48 hours. Cells were then

washed twice with PBS, the culture medium was replaced with supplemented one and the cells were harvested 24 hours later. In the last condition, cells were stimulated for 48 hours, after which cells were washed twice with PBS and were stimulated again for another 24 hours. Figure 4-7 illustrates the time points at which supplemented medium was added. Luminex was performed to evaluate protein secretion in BM, Is and Ad MSCs under resting and inflammatory conditions. Each bar represents an n of 3 independent experiments and is graphed as mean ± SEM. Data are normalised total amount of protein in medium and expressed as picograms of protein of interest per mg of total protein. Appropriate statistical analysis was performed and includes Students paired T test between one MSC tissue source (Resting vs Inflammatory Conditions) and One Way ANOVA with Tukey's multiple comparisons post-test to compare all MSC sources. Statistically significant differences are marked with the appropriate number of asterisks. p = 0.05 was considered the limit for statistical significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Table 4-12. Fold change in CXC chemokine secretion of cytokine-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.
Following the experimental set up explained in Figure 4-8, fold change in CXC chemokine protein levels is represented as mean of fold change ± standard deviation. Statistically significant differences are marked with a colour code, where $p < 0.05$ is represented by green, $p < 0.01$ is represented by orange, $p < 0.001$ is represented by blue and $p < 0.0001$ is represented by red.

Protein	Condition	BM MSCs	Is MSCs	Ad MSCs
CXCL1	1	17.30 ± 15.64	47.59 ± 8.98	178.92 ± 35.55
	2	112.58 ± 124.64	401.24 ± 151.04	451.60 ± 161.68
	3	0.31 ± 0.16	106.12 ± 22.97	194.01 ± 20.36
CXCL2	1	3.96 ± 1.38	67.94 ± 26.72	961.84 ± 195.65
	2	28.06 ± 11.58	384.46 ± 78.04	1792.17 ± 262.76
	3	0.31 ± 0.16	175.15 ± 74.87	1184.16 ± 222.43
CXCL10	1	15.24 ± 1.38	37.43 ± 4.38	52.76 ± 11.24
	2	69.18 ± 8.45	229.39 ± 26.36	111.16 ± 31.80
	3	44.58 ± 2.40	103.07 ± 17.41	92.26 ± 9.59
CXCL12	1	0.22 ± 0.14	0.57 ± 0.06	0.61 ± 0.03
	2	1.34 ± 0.91	0.68 ± 0.10	1.03 ± 0.35
	3	0.31 ± 0.16	0.37 ± 0.02	0.64 ± 0.02

4.5 Discussion and conclusions

The establishment of standardised MSC isolation and culture protocols described in Chapter 3 enabled an objective comparison of the transcriptional and protein profile of BM, Is and Ad MSCs. For this reason, the aim of this chapter was to study the transcriptional profile of chemokines and chemokines receptors by BM, Is and Ad MSCs under resting and inflammatory conditions, which is summarised in Figure 4-10. Moreover, careful analysis of the transcript data enabled the identification of genes of interest to be targeted for protein assays. The chemokine secretion of BM, Is and Ad MSCs under resting and inflammatory conditions is summarised in Figure 4-11.

The use of different inflammatory agents, as well as the different conditions in which MSCs were stimulated with the same inflammatory agent, did not show a clear trend of up or down regulation of genes that could suggest a better inflammatory agent for MSC licensing. For these reasons, instead of discussing the results per condition, we have decided to discuss the results by gene of interest, focusing on the results obtained harvesting the cells 24 hours after licensing with 40 ng/ml of TNF-α, IL-1β and IFN-γ (Condition 2). In this manner, it is possible to compare the expression of all the genes analysed in this study in Section 4.2, which is summarised in Figure 4-10. More importantly, careful analysis of the transcript data enabled the identification of genes of interest to be targeted for protein assays; the secretion of chemokines by BM, Is and Ad MSCs under resting and inflammatory conditions is summarised in Figure 4-11. Moreover, the role of the chemokines and their receptors will be discussed, and the clinical implications of the expression and regulation upon inflammatory stimulus of these molecules will be examined.

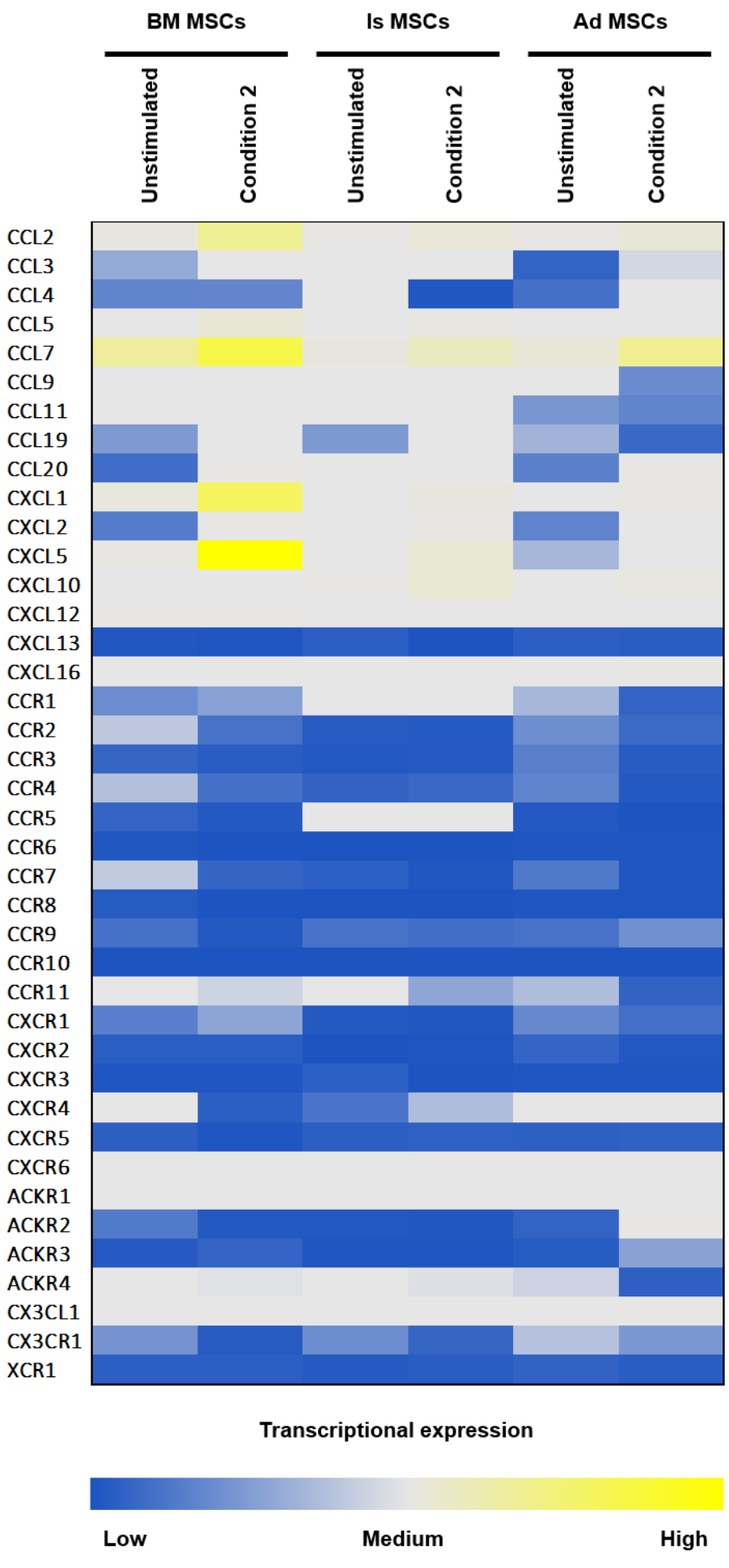

BM MSCs
Is MSCs
Ad MSCs
Unstimulated
Condition 2
Unstimulated
Condition 2
Unstimulated
Condition 2
CCL2
CCL3
CCL4
CCL5
CCL7
CCL9
CCL11
CCL19
CCL20
CXCL1
CXCL2
CXCL5
CXCL10
CXCL12
CXCL13
CXCL16
CCR1
CCR2
CCR3
CCR4
CCR5
CCR6
CCR7
CCR8
CCR9
CCR10
CCR11
CXCR1
CXCR2
CXCR3
CXCR4
CXCR5
CXCR6
ACKR1
ACKR2
ACKR3
ACKR4
CX3CL1
CX3CR1
XCR1
Transcriptional expression
Low
Medium
High

Figure 4-10. Heat map representing the transcriptional expression of CC and CXC chemokine ligands and receptors by MSCs from different sources under resting and inflammatory conditions.
Data from Figure 4-1, Figure 4-2, Figure 4-3, Figure 4-4, Figure 4-5 and Figure 4-6 are combined and presented as a heat map to illustrate the chemokine and chemokine receptor transcriptional profile of MSCs maintained under resting and inflammatory conditions. The heat map summarises each tissue source of MSC highest and lowest transcribed genes under resting conditions and inflammatory stimulation. Genes with low $2^{(-\Delta CT)}$ values are highlighted in blue, genes with intermediate $2^{(-\Delta CT)}$ values are highlighted in grey and genes with high $2^{(-\Delta CT)}$ values are highlighted in yellow.

4.5.1 Chemokine receptor expression by MSCs

As previously described, one of the most critical aspects of MSC use as cellular therapeutics is related to MSC homing and engraftment within the target tissue following their administration. MSC homing encompasses both non–systemic, where MSCs are transplanted locally at the target tissue and are then guided to the site of injury, and systemic homing, where MSCs are administered intra-venously or intra-arterially and the cells must exit the circulation and migrate to the site of injury (Nitzsche et al., 2017). In both cases, chemokine receptors have been described to be relevant for this process, in combination with other molecules as selectins and integrins (Xiao Ling et al., 2016, Liu et al., 2018a).

Under resting conditions, BM, Is and Ad MSCs were most likely not transcribing CC chemokine receptors, except for *CCR1* (Figure 4-3, A) and *CCR5* (Figure 4-3, E), CXC chemokine receptors, except for *CXCR6* (Figure 4-4, F) and atypical chemokine receptors, except for *ACKR1* (Figure 4-5, A) and *ACKR4* (Figure 4-5, D). These results correlate with the literature as BM MSCs have been reported to have essentially undetectable chemokine receptor transcripts and low levels of these transcripts have been observed in Ad MSCs. However, mRNA levels do not always correlate with protein levels, and in this specific case, with surface chemokine receptor expression and functionality (Ahmadian Kia et al., 2011, Bidkhori et al., 2016). Under resting conditions, little variation was observed in the transcription of these receptors among MSC sources. BM MSCs transcribed significantly higher levels of *CCR7*, *CXCR6* and *ACKR4*; Is MSCs transcribed significantly higher levels of *CCR1*, *CCR10* and *CXCR3*, while Ad MSCs transcribed significantly higher levels of *CCR3*. Altogether, these findings suggest that chemokine receptor expression by MSCs depends on MSC tissue of origin. Chemokine receptors are G protein-coupled receptors and they all share a very

similar structure which makes it very difficult to generate specific antibodies towards them that do not generate high non–specific background. For this reason, due to the lack of specific mouse antibodies, analysis of protein expression of chemokine receptors could not be performed.

Differential surface expression of chemokine receptors by MSCs could lead to MSCs migrating towards different organs. The expression of CCR1 in macrophages and neutrophils led to kidney infiltration in renal ischemia-reperfusion injury (Furuichi et al., 2008); CCR5 directs $CD8^+$ T cells towards the brain (Martin-Blondel et al., 2016); CCR7 programs naïve T cells and B cells to migrate to the spleen and lymph nodes (Bjorkdahl et al., 2003); CCR3, CCR4 and CCR10 are highly expressed by T cells in skin (Fujimoto et al., 2008, Ma et al., 2002); CXCR3 mediates T cell recruitment into the kidney (Panzer et al., 2007); CXCR4 expression is increased on the surface of extravascular neutrophils in the lung and the bone marrow (30); CXCR6 is highly expressed by liver-infiltrating $CD8^+$ T cells (Sato et al., 2005b). If specific chemokines can target immune cells into specific tissues, the expression of these chemokines should be able to target MSCs into those tissues. In fact, CCR7 targets MSCs to secondary lymphoid organs (Li et al., 2014, Ma et al., 2016), CXCR3-deficient MSCs fail to infiltrate into the nephritic kidney (Lee et al., 2018) and CXCR4 receptor overexpression in MSCs improves treatment of acute lung injury (Yang et al., 2015). Taking all this into account, we could hypothesise that CCR7 and CXCR6 expression would direct BM MSCs towards lymphoid organs as the spleen, the lymph nodes and the liver; Is MSCs would migrate towards the kidneys due to their CCR1 and CXCR3 expression; and Ad MSCs could have the potential to migrate towards the skin and bone marrow.

Unlike conventional chemokine receptors, ACKRs interact with chemokines without inducing cell migration but rather regulate chemokine gradients by interacting with chemokines from the environment. ACKR1 can interact, with high affinity, with both CC and CXC chemokines and it is not believed to have ligand-scavenging properties, just ligand presentation on the cell surface (Pruenster et al., 2009); ACKR2 binds, internalises and degrades CC chemokines (Fra et al., 2003); ACKR3 interacts and scavenges two chemokines, CXCL12, the ligand of CXCR4, and CXCL11, one of the ligands of CXCR3 (Burns et al., 2006);

and ACKR4 binds the homeostatic chemokines CCL19, CCL21, CCL25, and CXCL13. As ACKRs do not induce cell migration and there is little literature on MSCs and ACKRs, it is hard to determine the clinical relevance of the expression of these molecules by MSCs, however it suggests that ACKRs in MSCs would be involved in regulating the availability of chemokine ligands. MSCs from the three sources would have the potential to regulate CC and CXC chemokines due to their similar transcript levels of ACKR1; however, BM MSCs transcribed significantly higher *ACKR4* transcript levels, suggesting a better regulation of the homeostatic chemokines CCL19, CCL21, CCL25 and CXCL13 (Hughes and Nibbs, 2018).

The degree of difficulty understanding the role of chemokine receptors in MSCs increases when we consider the effect of inflammatory stimulation, as it produced a downregulation of the transcription of the receptors which was tissue specific. Cytokine-mediated stimulation decreased the transcription of *CCR7*, *CXCR6* and *ACKR4* in BM MSCs, which would make the cells less prone to migrate towards lymphoid organs (Ma et al., 2016) and would reduce the scavenging potential for homeostatic chemokines, which could help to dampen inflammation in a more efficient manner. Cytokine-mediated licensing decreased *CXCR3* transcript levels, making it more difficult for these cells to migrate towards the kidney, even if *CCR1* expression was not altered. Lastly, cytokine-mediated stimulation decreased *CCR3* transcript levels in Ad MSCs, which could stop the migration of MSCs towards the skin or epithelial tissues.

Altogether, BM, Is and Ad MSCs expressed low levels of chemokine receptor transcripts and few significant differences between MSC populations were observed; *CCR7*, *CXCR6* and *ACKR4* were specific to BM MSCs; *CCR1*, *CCR10* and *CXCR3* were specific to Is MSCs; while *CCR3* was specific to Ad MSCs, suggesting that the tissue of origin of MSCs influences the chemokine receptor expression and therefore, could affect their *in vivo* migratory capacity. Overall, Ad MSCs downregulated their receptors to a lesser extent than BM or Is MSCs after inflammatory stimulation, which is likely explained by the higher resistance of Ad MSCs to stress (El-Badawy et al., 2016). These standardised comparison of MSCs isolated from different sources provides evidence to support further investigation into the expression and function of chemokine receptors by MSCs

from different sources to analyse the impact tissue of origin has on the *in vivo* migratory capacity and therefore, on the clinical outcome.

4.5.2 Chemokine secretion by MSCs

As previously described, *in vitro* cultured MSCs have been shown to secrete a wide range of chemokines, including CCL2, CCL3, CCL4, CCL5, CCL7, CCL20, CXCL1, CXCL2, CXCL5, CXCL10, CXCL12 and CX3CL1 (Chen et al., 2008, Ren et al., 2008, Meirelles Lda et al., 2009). However, the lack of standardised isolation and culture methods has led to very few studies regarding the comparison of chemokines secreted by MSCs isolated from different tissues in human (Thirlwell, 2018) and none for mouse MSCs. The aforementioned chemokines have the ability to recruit towards MSCs cells from the immune system including macrophages, neutrophils, monocytes, eosinophils, DCs, T Cells, B cells and NK cells (Deshmane et al., 2009, De Filippo et al., 2013, Dwinell et al., 2001). For this reason, we consider that understanding MSC chemokine secretion will enable prediction of the interaction of these cells with the immune system and therefore, their immunomodulatory potential in an *in vivo* setting (Chen et al., 2008, Ren et al., 2008, Meirelles Lda et al., 2009). Therefore, standardised comparison of the chemokine secretion of MSCs isolated from different sources could lead to the identification of MSCs that could be more relevant in specific clinical settings. The chemokine secretion of BM, Is and Ad MSCs under resting and inflammatory conditions is summarised in Figure 4-11.

In the current study, under resting conditions, BM, Is and Ad MSCs transcribed and secreted low levels of CC chemokines, with the exception of CCL2 and CCL7 (Figure 4-8, A and C), which were both expressed at similar levels in MSCs from every source; and CXC chemokines, with the exception of CXCL1 and CXCL12, which were all secreted by all tissue source MSCs at similar levels (Figure 4-9, A and D). These chemokines are strong chemoattractants for monocytes (CCL2 and CCL7), neutrophils (CXCL1) and lymphocytes (CXCL12). Under inflammatory stimulation, BM, Is and Ad MSCs upregulated the transcription and secretion of the previously mentioned chemokines, whilst also inducing the transcription and secretion of CCL5, CXCL2 and CXCL10. Inflammatory stimulation produced no effect in the transcription and secretion of CXCL12. After inflammatory stimulation, Is MSCs were able to secrete the highest amounts of every

chemokine aforementioned. Even if the transcriptional level of many chemokines has been analysed under different conditions, this discussion section is going to focus on the chemokines that were secreted at high level under resting and inflammatory conditions (CCL2, CCL5, CCL7, CXCL1, CXCL2, CXCL10 and CXCL12) and the specific immune cells that MSCs could attract due to the secretion of those chemokines. It is important to mention that the secretion of CCL2, CCL5, CCL7, CXCL1, CXCL10 and CXCL12 by MSCs has previously been reported (Kyurkchiev et al., 2014, Thirlwell, 2018, Lee et al., 2012, Kimura et al., 2014); but to my knowledge, differences in chemokine transcription, secretion and leukocyte recruitment by murine MSCs isolated from different tissue sources has not been documented.

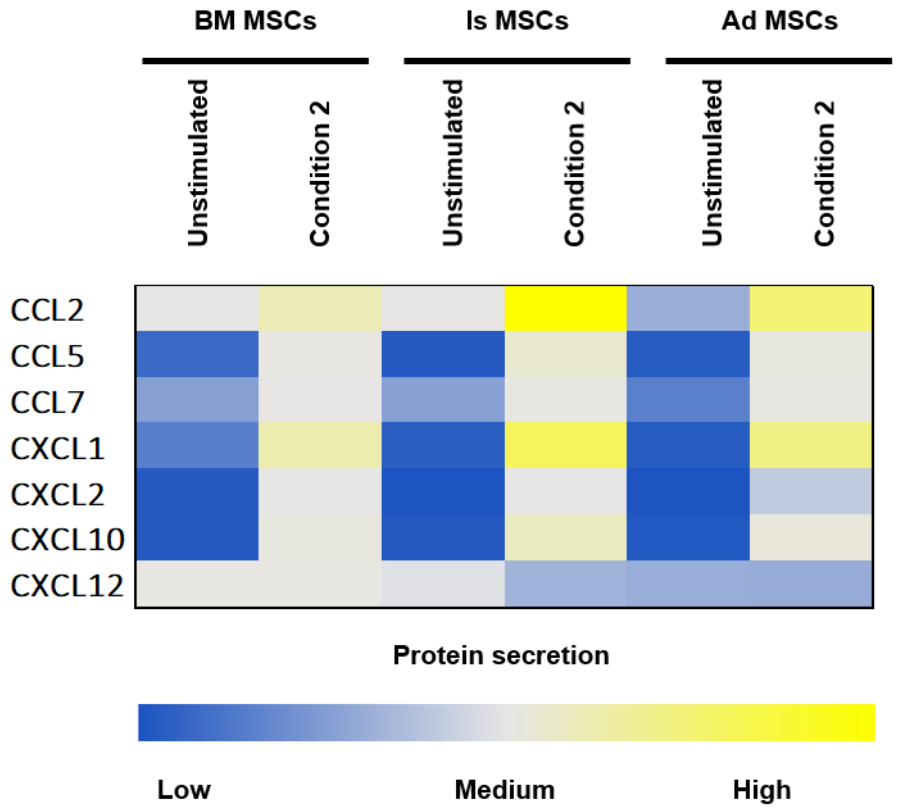


Figure 4-11. Heat map representing the secretion of CC and CXC chemokine ligands by MSCs from different sources under resting and inflammatory conditions.
Data from Figure 4-8 and Figure 4-9 are combined and presented as a heat map to illustrate the chemokine secretion profile of MSCs maintained under resting and inflammatory conditions. The heat map summarises each tissue source of MSC highest and lowest secreted chemokines under resting conditions and inflammatory stimulation. Proteins with low secretion are highlighted in blue, proteins with intermediate secretion are highlighted in grey and proteins with high secretion are highlighted in yellow.

CCL2, also known as the monocyte chemoattractant protein 1 (MCP–1), is secreted by many cell types including endothelial cells, fibroblasts, epithelial cells and smooth muscle cells, but monocytes and macrophages have been identified as the major source of CCL2 (Deshmane et al., 2009, Yoshimura et al., 1989). On the one hand, CCL2 is involved in the regulation of the migration and infiltration of monocytes, memory T cells and NK cells and is involved in the development of inflammatory disorders including rheumatoid arthritis (Hayashida et al., 2001), multiple sclerosis (Sørensen et al., 2004) and insulin-resistant diabetes (Sartipy and Loskutoff, 2003). On the other hand, CCL2 is involved in angiogenesis and neovascularisation (Daly and Rollins, 2003, Rose et al., 2003). BM, Is and Ad MSCs transcribed (Figure 4-1, A) and secreted (Figure 4-8, A) substantial levels of CCL2 under resting conditions. These transcriptional and protein levels were significantly upregulated after inflammatory stimulation by MSCs isolated from all tissue sources. BM MSCs had the highest *CCL2* expression levels while, at protein level, Is MSCs secreted the highest levels of CCL2 under cytokine-mediated inflammatory stimulation, suggesting the involvement of post-transcriptional mechanisms in the expression of CCL2. Moreover, Is MSCs could have the potential to attract more monocytes than BM and Ad MSCs. Secretion of CCL2 by human BM, Is and Ad MSCs has previously been reported (Thirlwell, 2018) but to my knowledge, differences in CCL2 transcription, secretion and monocyte and T cell chemoattraction by mouse MSCs isolated from different tissue sources have not been documented.

CCL5, also known as “regulated upon activation normal T cell expressed and secreted” (RANTES), is expressed by T lymphocytes, macrophages, platelets, synovial fibroblasts, tubular epithelium, and certain types of tumour cells and is involved in the recruitment of T cells, eosinophils and basophils predominantly but also NK cells, DCs and MCs (Marques et al., 2013). CCL5 is a key pro-inflammatory chemokine involved in viral (Glass et al., 2003) and helminth (Souza et al., 2011) infections and enhancing inflammation in diseases such as asthma (Lukacs, 2001) or atherosclerosis; however, CCL5 is also involved in angiogenesis (Suffee et al., 2011). BM, Is and Ad MSCs transcribed (Figure 4-1, D) and secreted (Figure 4-8, B) little CCL5 under resting conditions. However, inflammatory stimulation significantly upregulated CCL5 transcript and protein levels in MSCs isolated from all tissue sources. At the transcriptional level, BM

MSC were expressing the highest *CCL5* transcript levels, while at protein level, Is MSCs secreted the most CCL5 after cytokine-mediated inflammatory stimulation, suggesting the involvement of post-transcriptional mechanisms in the expression and secretion of CCL5. Moreover, under inflammatory conditions Is MSCs could have the potential to recruit the most leukocytes.

CCL7, previously known as monocyte chemoattractant protein 3 (MCP3), is expressed in fibroblasts, epithelial cells, and endothelial cells and it specifically attracts monocytes, eosinophils, basophils, DCs, NK cells and activated T lymphocytes (Liu et al., 2018b). CCL7 is involved in antiviral, antibacterial and antifungal immunity due to the monocyte mobilization from the bone marrow towards sites of inflammation; moreover, CCL7 is the only member of the CC chemokine family that can induce the migration of neutrophils (Fioretti et al., 1998). However, an excess of CCL7 is involved in several diseases including psoriasis (Brunner et al., 2015), acquired immunodeficiency syndrome (Atluri et al., 2016) and acute neutrophilic lung inflammation and pulmonary fibrosis (Mercer et al., 2014). BM, Is and Ad MSCs transcribed (Figure 4-1, E) and secreted (Figure 4-8, C) little CCL7 under resting conditions. However, inflammatory stimulation significantly upregulated CCL7 transcript and protein levels in MSCs isolated from all tissue sources. At transcriptional level, BM MSC were expressing the highest *CCL7* transcript levels, while at protein level, Is MSCs secreted the most CCL7 after cytokine-mediated inflammatory stimulation, suggesting the involvement of post-transcriptional mechanisms in the expression and secretion of CCL7. Moreover, under inflammatory conditions Is MSCs could have the potential to recruit more monocytes than BM and Ad MSCs.

CXCL1, also known as keratinocyte chemoattractant (KC), and CXCL2, also known as macrophage inflammatory protein 2 (MIP2), are the functional homologs of human CXCL8 (Hol et al., 2010). CXCL1 is expressed by keratinocytes, endothelial cells, monocytes and macrophages while CXCL2 is expressed by endothelial cells and megakaryocytes (Shea-Donohue et al., 2008). Despite CXCL1 and 2 not being the direct homologues of CXCL8, they both belong to the same major cluster of CXC chemokines and they are both involved in neutrophil recruitment via CXCR2 (Zlotnik and Yoshie, 2000b), which is why they are going to be discussed together. However, their role is not redundant;

CXCL1 is critical for luminal and subendothelial cell neutrophil crawling, while CXCL2 is involved in the correct breaching of endothelial junctions (Girbl et al., 2018), but they are both involved in angiogenesis, tumorigenesis, wound healing and inflammation (Gillitzer and Goebeler, 2001, Kolaczkowska and Kubes, 2013, De Filippo et al., 2013). However, they are also involved in the development and maintenance of the inflammation in autoimmune diseases as rheumatoid arthritis (Udalova et al., 2016), multiple sclerosis (Grist et al., 2018) and psoriasis (Lowes et al., 2014) among others. BM, Is and Ad MSCs transcribed (Figure 4-2, A and B) and secreted (Figure 4-9, A and B) little CXCL1 and CXCL2 under resting conditions. However, inflammatory stimulation significantly upregulated CXCL1 and CXCL2 transcriptional and protein levels in MSCs isolated from all tissue sources. At the transcript level, BM and Is MSCs expressed the highest *CXCL1* and *CXCL2* transcript levels respectively, while at the protein level, Is MSCs secreted the most CXCL1 and CXCL2 after cytokine-mediated inflammatory stimulation, suggesting the involvement of post-transcriptional mechanisms in the expression and secretion of CXCL1 and CXCL2. Moreover, under inflammatory conditions Is MSCs could have the potential to recruit more neutrophils than BM and Ad MSCs.

CXCL10, also known as interferon–γ-inducible protein 10 (IP-10), is produced by a wide range of cell types including monocytes, neutrophils, endothelial cells, keratinocytes, fibroblasts, MSCs, DCs, hepatocytes and astrocytes (Vazirinejad et al., 2014). The CXCL10/ CXCR3 axis is involved in the chemoattraction of macrophages, monocytes and activated T and NK cells (Crow et al., 2001). Moreover, CXCL10 is involved in the modulation of T cell development and function as well as being an inhibitor of neovascularization, even in a tumoral environment (Vazirinejad et al., 2014). Due to the pro-inflammatory role of CXCL10, this CXC chemokine is involved in several pathologies including multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus and idiopathic inflammatory myopathy among others (Lee et al., 2009). BM, Is and Ad MSCs transcribed (Figure 4-2, D) and secreted (Figure 4-9, C) little CXCL10 under resting conditions. However, inflammatory stimulation significantly upregulated CXCL10 transcript and protein levels in MSCs isolated from all tissue sources. Under cytokine-mediated inflammatory stimulation, Is MSCs transcribed and

secreted the most CXCL10, suggesting that Is MSCs could attract more monocytes than BM and Ad MSCs.

CXCL12, also known as stromal-derived factor 1α (SDF-1α), is constitutively secreted by stromal cells in the bone marrow, lymph nodes, liver and lungs (Ieranò et al., 2019). CXCL12 induces the migration of haematopoietic progenitor and stem cells as well as being a very strong chemoattractant for endothelial cells and several leukocytes including pre-B cells, T cells and monocytes (Askari et al., 2003, Janssens et al., 2018). CXCL12 interacts with its receptors CXCR4 and ACKR3 to fulfil extremely important physiological processes, including embryogenesis, haematopoiesis, angiogenesis and inflammation (Janssens et al., 2018). Despite its homeostatic functions, increased expression of CXCL12 is involved in cancer progression, inflammatory bowel disease, rheumatoid arthritis, asthma and amyotrophic lateral sclerosis among others (Mousavi, 2020). BM, Is and Ad MSCs transcribed (Figure 4-2, E) and secreted (Figure 4-9, C) little CXCL12 under resting conditions. Opposite to the other mentioned chemokines, inflammatory stimulation did not lead to an upregulation of CXCL10 transcript and protein levels in MSCs isolated from any of the tissue sources. Under homeostatic or cytokine-mediated inflammatory stimulation, BM MSCs transcribed and secreted the most CXCL12, suggesting that BM MSCs could attract more progenitor and stem cells than Is and Ad MSCs.

Altogether, inflammatory stimulation led to the secretion of substantial levels of CCL2, CCL5, CCL7, CXCL1, CXCL2, CXCL10 and CXCL12, which could potentially induce the recruitment of all sorts of leukocytes but more predominantly, neutrophils, monocytes/ macrophages, NK cells and T cells (Deshmane et al., 2009, De Filippo et al., 2013, Dwinell et al., 2001). The recruitment of these cells by these chemokines could be considered detrimental within a clinical setting due to their role in triggering alloimmunity and graft rejection by recognizing allogenic nonself antigens (Lakkis and Li, 2018, Oberbarnscheidt et al., 2014, Choi et al., 2007). However, it is essential to bear in mind that MSCs are well known for their immunomodulatory potential producing a switch from pro-inflammatory environments towards anti-inflammatory ones.

The CCL2/ CCR2 axis promotes the differentiation of monocytes towards anti-inflammatory phenotypes, which are involved in the regulation of the resolution

phase of inflammation and the repair of damaged tissues and would therefore promote graft survival (Sierra-Filardi et al., 2014). In addition, secreted CCL2 by MSCs is not only relevant for monocyte attraction, but for T cells too as the CCL2/ CCR2 axis is crucial for the suppression of autoreactive T cells by MSCs (Lee et al., 2017a). Autoreactive T cells are present in pancreatic islets of T1DM patients and are able to mediate cross-reactive alloreactivity in T1DM that receive pancreas transplant (Burrack et al., 2018).

CCL5 expression has been proven to supress the anti–tumour immunity in triple negative breast cancer due to the chemoattraction of T cells, NK cells and macrophages (Araujo et al., 2018). The CCL5/ CCR1 and CXCL10/ CXCR3 axis have been described to modulate allogeneic T cell responses contributing to the development of GVHD following allogeneic stem cell transplantation (Choi et al., 2007, Piper et al., 2007); however, GVHD can be treated by infusion of MSCs, which leads to long–term graft function (Le Blanc et al., 2008). Little is known about the role of CCL7 in transplantation as CCL7 can induce chemotaxis of both pro-inflammatory and anti–inflammatory macrophages (Xuan et al., 2015). Galleu et al. described the essential role of cytotoxic cells to initiate MSC–mediated immunosuppression in a murine model of GvHD; MSCs need to be targeted by cytotoxic T cells to undergo apoptosis so they can then be engulfed by macrophages and produce indoleamine 2,3 dioxygenase (IDO) (Galleu et al., 2017). In addition, impaired expression of CCL5 in Ad MSCs leads to delayed repair of the vasculature of ischemic regions (Kimura et al., 2014). Moreover, CCL5 mediated recruitment of NK cells could be essential for alloimmunity and tolerance as NK cells can produce IL–10 and other anti–inflammatory cytokines (Benichou et al., 2011).

Regarding neutrophil recruitment, CXCL1 mediated neutrophil infiltration has been shown to be essential for the recruitment of allospecific $CD4^+$ T cells into the graft and is therefore associated with low survival rates of the allografts (Amescua et al., 2008). CXCL1 and CXCL2 mediated neutrophil infiltrates are involved in the ischemia and reperfusion injury after transplantation that leads to the onset and persistence of acute rejection in cardiac allografts (Shimizu and Mitchell, 2008). However, neutrophils could also inhibit graft inflammation and its subsequent rejection by enhancing wound and tissue repair coupled with

neovascularization (Christoffersson et al., 2012). Moreover, apoptosis of neutrophils is anti–inflammatory and pro–resolution of the inflammation process itself as it reduces the secretion of pro–inflammatory molecules by phagocytic cells (Byrne and Reen, 2002).

Lastly, the presence of CXCL12 within a graft leads to progenitor cell and stem cell recruitment, angiogenesis and tissue regeneration after lung transplantation (Gomperts et al., 2006). Moreover, the addition of CXCL12 has been shown to increase the long-term survival of microencapsulated auto-, allo- and xenogeneic islets in murine models of diabetes without systemic immune suppression (Sremac et al., 2019). For these reasons, the overexpression of CXCL12 in MSCs would be therapeutically beneficial within a clinical setting.

4.5.3 Conclusions

To summarise and conclude, this chapter aimed to determine if murine MSCs isolated from different tissues had a specific pattern of chemokine and chemokine receptor expression at transcript, and most importantly, protein level. BM, Is and Ad MSCs all had very low transcript levels of chemokine receptors under resting and stimulatory chemokines, which suggests that homing into specific tissues is unlikely but if transplanted locally at the target tissue, MSCs would be unlikely to migrate away from the graft site. Due to the lack of specific antibodies towards murine chemokine receptors, protein analysis could not be performed. For this reason, to understand the functionality of receptors expressed by MSC, migration assays towards chemokines would have to be performed. Moreover, this chapter aimed to assay chemokine secretion by BM, Is and Ad MSCs and to understand if tissue of origin of MSCs led to a differential chemokine secretion under resting and inflammatory conditions. MSCs were able to produce large quantities of CCL2, CCL5, CXCL1 and CXCL10 at differential levels depending on MSC source of origin under inflammatory conditions, with Is MSCs secreting the most, which could induce the migration of large amounts of monocytes/ macrophages and neutrophils and NK cells and T cells in a smaller amount. This chapter showed differential chemoattraction potential of MSCs depending on their tissue of origin and suggests that MSCs isolated from various

tissues would act differentially *in vivo*. For this reason, determining the immune cell attraction *in vivo* would be essential to have a better understanding of the potential behaviour of MSCs from different sources within a clinical setting.

Chapter 5

Defining the immune cell recruitment profile of MSCs *in vivo*

5 Defining the immune cell recruitment profile of MSCs *in vivo*

5.1 Introduction and aims

In Chapter 4 we determined that MSCs isolated from the bone marrow, islets of Langerhans and adipose tissue expressed overall low levels of chemokine receptor transcripts and that tissue of origin determines a specific pattern of chemokine transcription. BM, Is and Ad MSCs secreted CCL2, CCL7, CXCL1 and CXCL12 under resting conditions at similar levels, suggesting that MSCs have the potential to attract monocytes, neutrophils and lymphocytes. In addition, inflammatory licensing led to a substantial upregulation in the secretion of the aforementioned chemokines, whilst also inducing the transcription and secretion of CCL5, CXCL2 and CXCL10 by MSCs from all sources. Therefore, we hypothesised that inflammatory stimulation would lead to an increased recruitment of leukocytes by MSCs, with a predominance of neutrophils and monocytes/ macrophages.

MSCs have been described to have stronger immunomodulatory and anti-inflammatory properties after licensing with TNF-α, IL-1ß and IFN-γ (Krampera et al., 2006, Krampera et al., 2007, Di Nicola et al., 2002, Beyth et al., 2005); which may be at odds with an increased secretion of chemokines with the ability to recruit leukocytes. It is essential to determine the immune recruitment potential of MSCs and its outcome, as infusion of MSCs into a patient with an existing inflammatory condition could lead to the recruitment of leukocytes and exacerbate inflammation. Monocytes and neutrophils are essential cell populations in early stages of acute inflammation and may be regarded as monofunctional pro-inflammatory cells. However, there is a relatively new body of literature surrounding the existence of pro-inflammatory and anti-inflammatory or pro-angiogenic subsets of monocytes/ macrophages and neutrophils. Neutrophils are a heterogenic population and distinct neutrophil subsets with different roles have been identified based on their cell surface receptor expression. As an example, $CD11b^{+}$/ $Gr\text{-}1^{+}$ neutrophils are recruited to grafts where they are involved in the re-vascularization of the transplanted tissue (Christoffersson et al., 2012). More importantly, this subset of neutrophils

is CXCR4 high and would therefore be able to respond to the CXCL12 secretion by MSCs.

Macrophages can be categorised in two major subsets, pro-inflammatory macrophages also known as M1 macrophages, and anti-inflammatory macrophages, known as M2 macrophages. The interaction between apoptotic neutrophils and macrophages promotes type 2 macrophage polarization (Marwick et al., 2018), leading to an anti-inflammatory environment. In the same way, MSC infusions induce a switch from pro-inflammatory to anti-inflammatory macrophages, driving alleviation of myocardial injury and restoration of cardiac function (Jin et al., 2019). Moreover, CXCL12 production has been described to modulate the differentiation of monocytes towards a distinctive pro-angiogenic and immunosuppressive phenotype (Sánchez-Martín et al., 2011). Taking all this into account, increased secretion of chemokine ligands and the subsequent recruitment of immune cells by licensed MSCs could have the potential to exacerbate inflammation or, in contrast, promote an anti-inflammatory milieu after infusion into a patient. Thus, the phenotype of the recruited immune cells would determine if MSCs could be beneficial or detrimental to tissue regeneration in a clinical setting.

Having have showed in the previous chapter differential chemoattraction potential of MSCs depending on their tissue of origin, determining the immune cell attraction *in vivo* was the next logical step in this study to allow a better understanding of the potential behaviour of MSCs from different sources within a clinical setting. To do this, the murine air pouch model was used, which is a well-established model to study immune infiltration into an artificially created air pouch on the back of mice (Dyer et al., 2019). This model has already been used to assess the immune cell infiltration in response to licensed human Is MSC infusion into the air pouch of mice (Thirlwell, 2018, Dyer et al., 2019). For this reason, the air pouch was used as an *in vivo* environment to study the immune cell infiltration in response to resting and licensed BM, Is and Ad MSCs. Licensing of MSCs was carried out with 40 ng/ ml of TNF-α, IL-1β and IFN-ϒ for 24 hours. MSCs were thoroughly washed prior to infusion into the air pouch to avoid non-specific immune cell attraction by the inflammatory cytokines.

Therefore, this chapter aimed to assess; i) the *in vivo* immune cell attraction profile of resting and licensed mouse MSCs, ii) the phenotype of infiltrated immune cells and iii) the fate of MSCs after infusion.

Results

5.2 Flow cytometry gating strategies

Subcutaneous injection of sterile air into the intracapsular area of the mouse creates a hollow pocket of air, where MSCs are infused, surrounded by a membrane. Flow cytometry was used to analyse the immune cell infiltration into the air pouch fluid and membrane of C57BL/6 female mice 24 hours after MSC infusion into the air pouch.

An array of cell markers (CD45, CD11b, Ly6g, F480, Siglec F, Ly6c and CD11c) coupled with a specific gating strategy **(**Figure 5-1**)** was used to identify neutrophils (Figure 5-1, D.i), macrophages (Figure 5-1, E.i), eosinophils (Figure 5-1, F.i), monocytes (Figure 5-1**,** G.i) and CD11b high $CD11c^+$ myeloid cells (Figure 5-1**,** I). CD11b and CD11c are co-expressed in myeloid-lineage DCs, while they are absent in lymphoid-lineage plasmacytoid DCs (Musumeci et al., 2019, Donaghy et al., 2001). CD11b is expressed on a variety of leukocytes, while CD11c is usually expressed by DCs. However, the expression of these markers can be upregulated on activated cells irrespective of their naïve expression status, which makes it impossible to confirm that the CD11b high $CD11c^+$ population are DCs without the addition of other DC markers such as CD24 or MHC Class II.

An additional array of mouse cell markers was used to analyse the infiltration of NK cells (Figure 5-2, D.i), $CD8^+$ (Figure 5-2, E.i) and $CD4^+$ T cells (Figure 5-2, E.ii) and B cells (Figure 5-2, F.i) into the air pouch fluid and membrane (Figure 5-2). CD73 was used to enumerate infused MSCs.

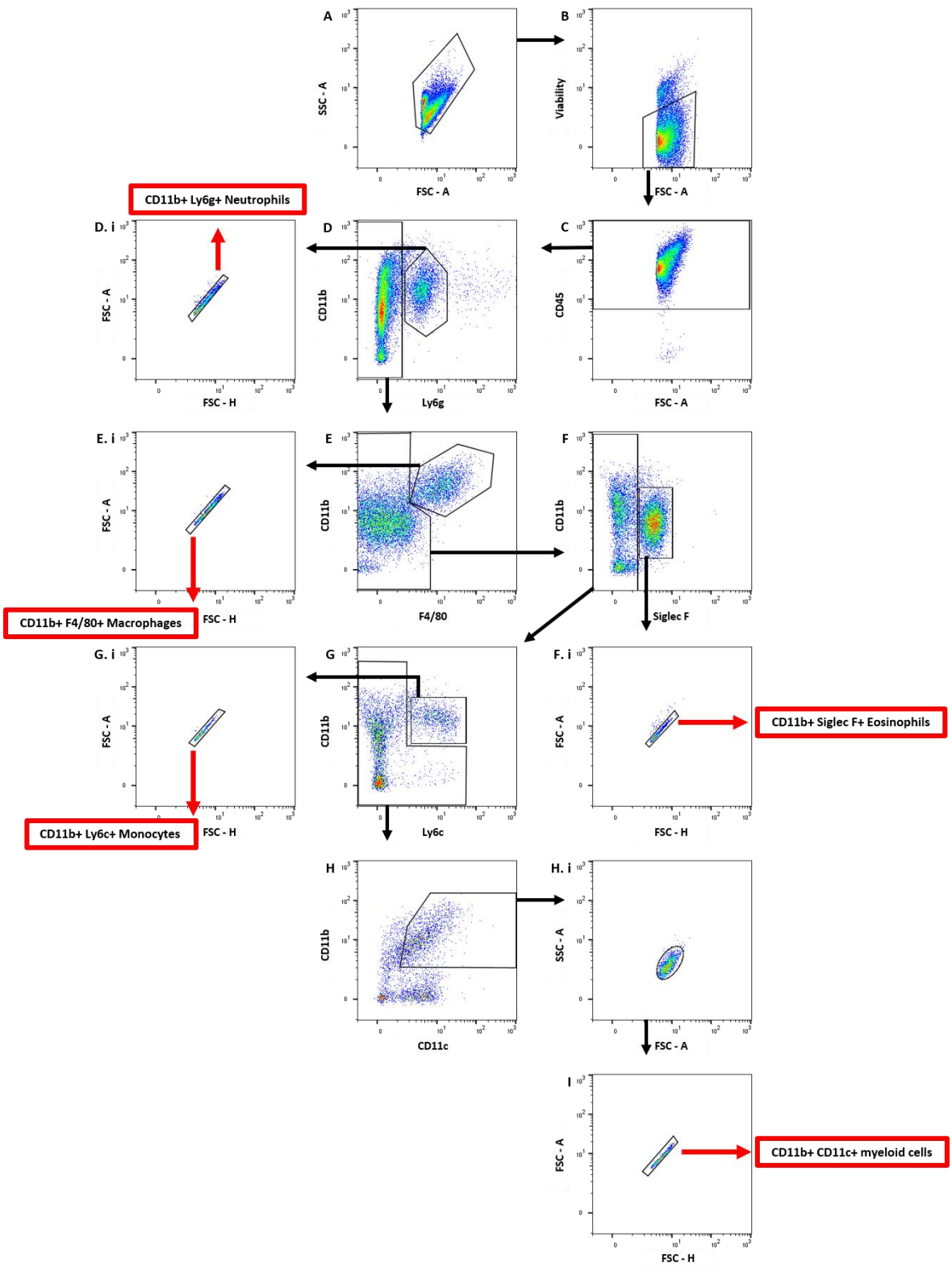


Figure 5-1. Flow cytometry gating strategy to identify various mouse innate immune cells in the air pouch of C57BL/6 mice.

Flow cytometry was used to assess and identify the immune cell infiltration into the air pouches of C57BL/6 mice. Black arrows highlight the gating pathway and red arrows highlight gated cell populations. Cells were selected based on forward versus side scatter (**A**) and viable cells were selected (**B**). $CD45^+$ cells were selected **(C)** and $CD11b^+$, $Ly6g^+$ cells were considered neutrophils after doublet exclusion **(D.i).** Remaining cells were assessed for their expression of F480. $F480^+$, $CD11b^+$ cells were considered macrophages after doublet exclusion **(E.i).** The remaining population of cells were assessed for Siglec F expression. Siglec F^+ cells were considered eosinophils after doublet exclusion **(F.i).** $CD11b^+$ $Ly6c^+$ cells were considered monocytes after doublet exclusion **(G.i).** The remaining cells were assessed for the expression of CD11c. CD11b

high CD11c^{+} were classified as CD11b^{+} CD11c^{+} myeloid cells after doublets exclusion and ensuring they were a single population **(I).**

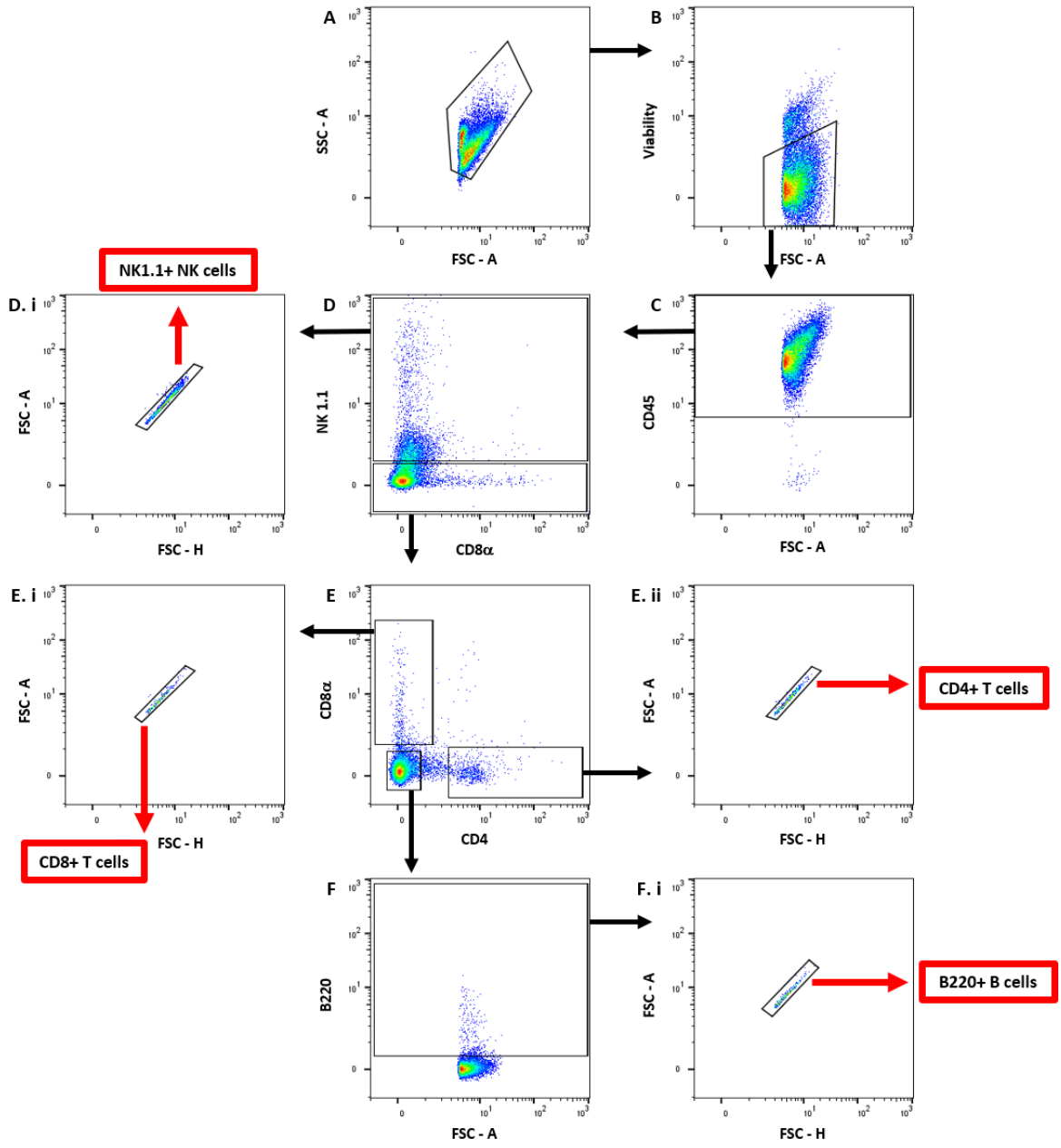


Figure 5-2. Flow cytometry gating strategy to identify various mouse adaptive immune cells in the air pouch of C57BL/6 mice.

Flow cytometry was used to assess and identify the immune cell infiltration into the air pouches of C57BL/6 mice. Black arrows highlight the gating pathway. Red arrows highlight the gated cell population. Cells were selected based on forward versus side scatter (**A**) and viable cells were selected (**B**). CD45^{+} cells were selected **(C)** and assessed for their expression of NK1.1, CD8, CD4 and B220. NK1.1 positive cells were considered NK cells after doublet exclusion **(D.i)** and the remaining cells were assessed for the expression of CD8 and CD4 **(E)** and considered CD8^{+} T cells (**E.i**) or CD4^{+} T cells (**E.ii**), respectively after doublet exclusion. Cells negative for CD4 and CD8 were assessed for their expression of B220 and positive cells were considered B cells **(F.i).**

5.3 Analysis of the cellular infiltration into the air pouches

Using flow cytometry, the total number of CD45 positive cells that had infiltrated into the air pouches was assessed. All the data are expressed as number of cells per mL of fluid extracted from the air pouch.

5.3.1 PBS only Controls

First, the immune cell infiltration produced by the generation of the air pouch itself and injection of PBS was assessed (Figure 5-3). Minimal migration of $CD45^+$ cells into control air pouches was observed, which showed that the creation of the air pouch, or PBS injection, did not lead to the generation of an inflammatory site with subsequent immune cell infiltration. Macrophages and NK cells were the predominant immune cells present in the air pouches of control mice, followed by smaller numbers of neutrophils, monocytes and eosinophils.

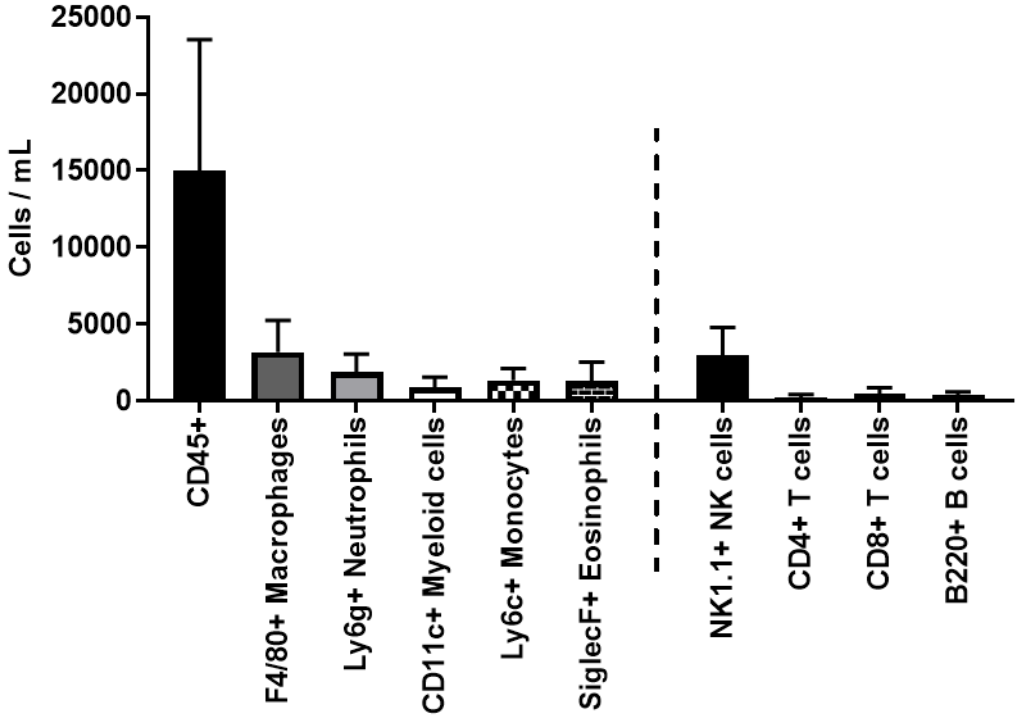


Figure 5-3. Generation of the air pouch on C57BL/6 mice and injection of PBS into the air pouch led to minimal migration of $CD45^+$ cells.
Air pouches were created on the dorsal of 8-week C57BL/6 female mice as explained in Section 2.4.2.1. The sixth day mice received a sterile PBS injection and mice were sacrificed, and tissues were harvested 24 hours later. Flow cytometry was used to assess the immune cell infiltration into the air pouches of mice, and it is expressed as number of cells per mL of fluid extracted from the air pouch. The total number of $CD45^+$, $F480^+$ macrophages, $Ly6g^+$ neutrophils, $CD11b^+$ $CD11c^+$

myeloid cells, Ly6c$^+$ monocytes and Siglec F$^+$ eosinophils that had infiltrated into the air pouches of mice was determined and graphed, as well as the total number of NK1.1$^+$ NK cells, CD8$^+$ T cells, CD4$^+$ T cells and B220$^+$ B cells that had infiltrated into the air pouches of mice. Each bar group represents 5 mice ± SEM.

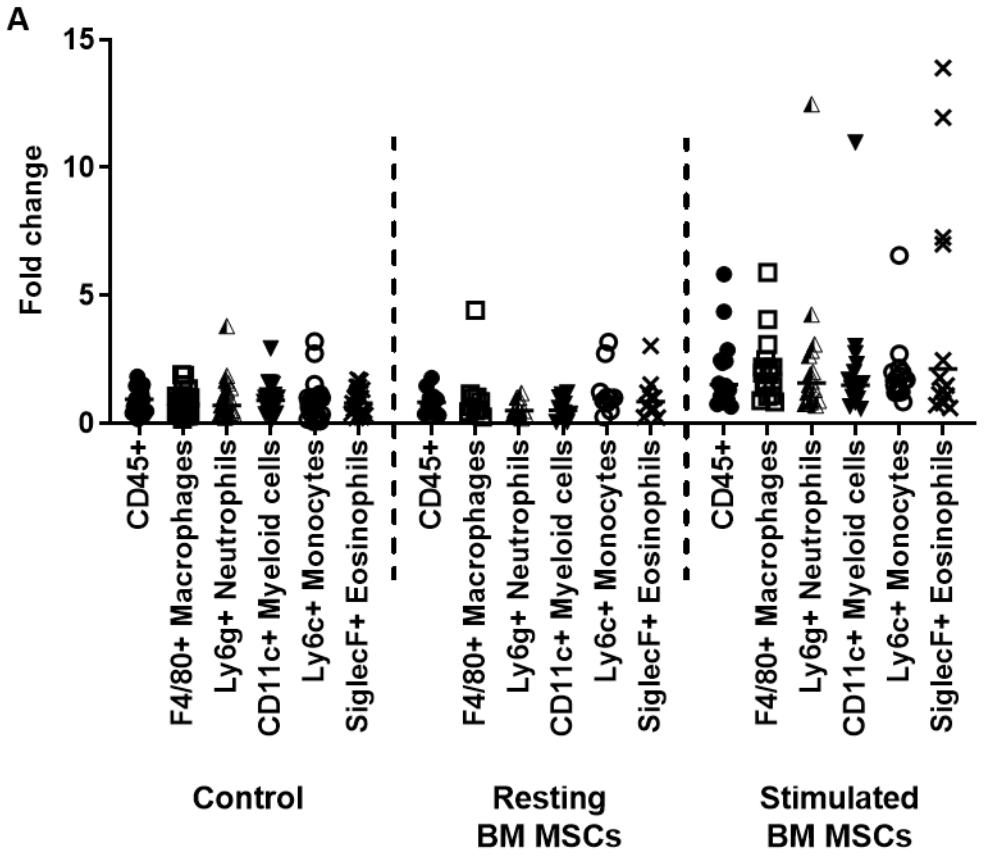
Next, immune cell infiltration needed to be tested after the infusion of MSCs from three different sources, BM, Is and Ad MSCs, in two different conditions, resting and after licensing, which meant a large number of mice to work with. For this reason, this experiment was performed for MSCs from each tissue independently. Data were therefore normalised to the PBS control infiltrates in each individual set of experiments in order to be able to compare the data from different experiments and replicates.

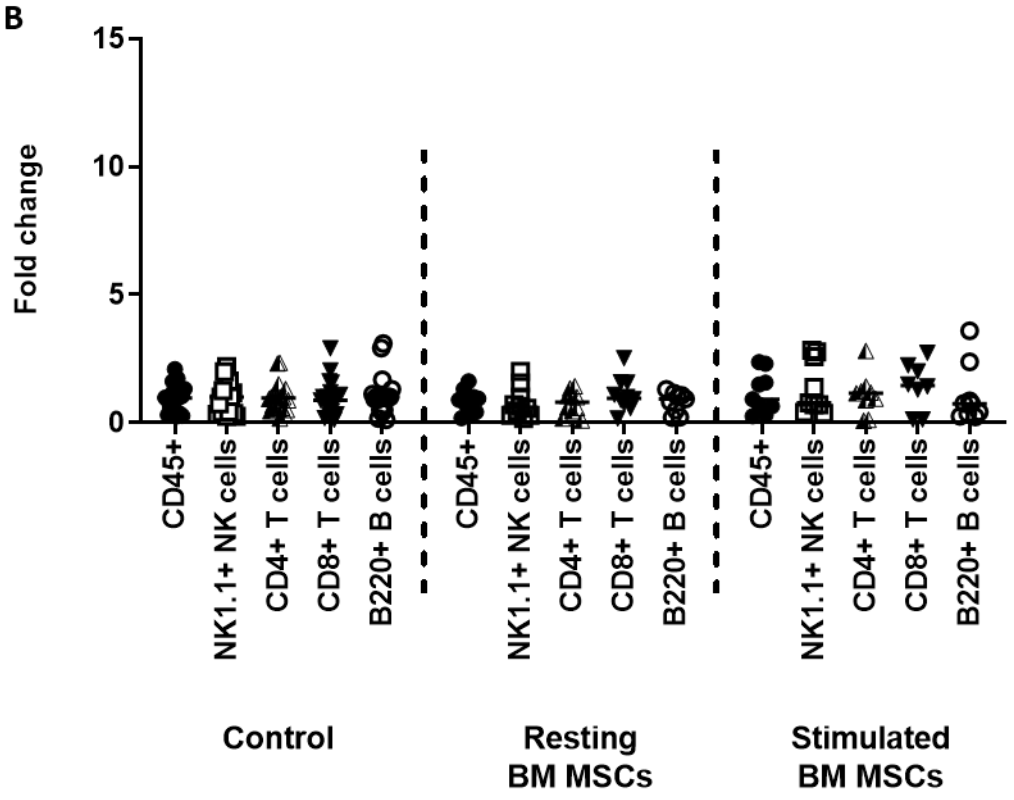
5.3.2 Administration of BM, Is and Ad MSCs

As shown in Figure 5-4 and Figure 5-5, migration of innate or adaptive immune cells towards resting BM (A and B), Is (C and D) and Ad MSCs (E and F) was observed and there was no statistically significant variation in the number of CD45$^+$ cells, F4/80$^+$ macrophages, Ly6g$^+$ neutrophils, CD11b$^+$ CD11c$^+$ myeloid cells, Ly6c$^+$ monocytes, SiglecF$^+$ eosinophils, NK1.1$^+$ NK cells, CD4$^+$ T cells, CD8$^+$ T cells and B220$^+$ B cells recruited per mL of fluid extracted when compared to the control PBS mice.

Conversely, infusion of licensed MSCs into the air pouch of mice resulted in an at least 2-fold increase in infiltrating CD45$^+$ cells compared to PBS control mice regardless of the MSCs tissue of origin. However, the immune cells recruited and the numbers of these cells within the air pouch was MSC source of origin dependent. Stimulated MSCs from every source were able to produce a statistically significant increased recruitment of F4/80$^+$ macrophages compared to resting MSCs, with Ad MSCs recruiting the most. Regarding Ly6g$^+$ neutrophils, stimulation of MSCs led to a statistically significant increase in the recruitment of these cells; however, BM MSCs did not recruit as many Ly6g$^+$ neutrophils as Is and Ad MSCs. The recruitment of CD11b$^+$ CD11c$^+$ myeloid cells was statistically significantly higher in stimulated MSCs from every source compared to resting MSCs, and all the licensed MSCs recruited CD11b$^+$ CD11c$^+$ myeloid cells at similar levels. Stimulated MSCs from every source were able to produce a statistically

significant increased recruitment of Ly6c$^+$ monocytes compared to resting MSCs, with Ad MSCs recruiting the most. The recruitment of SiglecF$^+$ eosinophils was statistically significantly higher in stimulated MSCs from every source compared to resting MSCs, and all the licensed MSCs recruited SiglecF$^+$ eosinophils at similar levels. Stimulated Ad were the only MSCs able to produce a statistically significant increase in the recruitment of NK1.1$^+$ NK cells and CD4$^+$ T cells compared to resting MSCs. Stimulated Is and Ad MSCs were both able to produce a statistically significant increase in the recruitment of CD8$^+$ T cells compared to resting conditions, while the number of CD8$^+$ T cells recruited from stimulated BM MSCs did not differ with respect to the number of CD8$^+$ T cells recruited by unstimulated BM MSCs; stimulated Ad MSCs were able to recruit significantly more CD8$^+$ T cells than stimulated Is MSCs. Regarding B220$^+$ B cells, no statistically significant differences in the number of recruited B220$^+$ B cells were found between stimulated MSCs and resting MSCs regardless of the tissue of origin.

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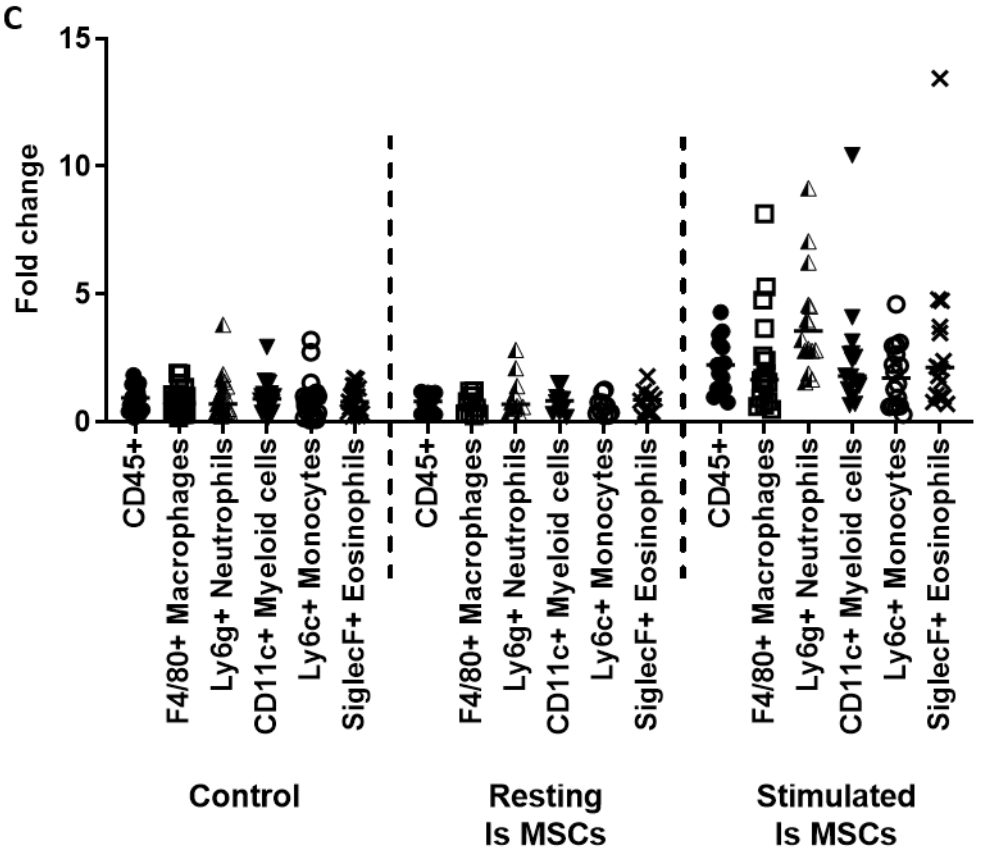
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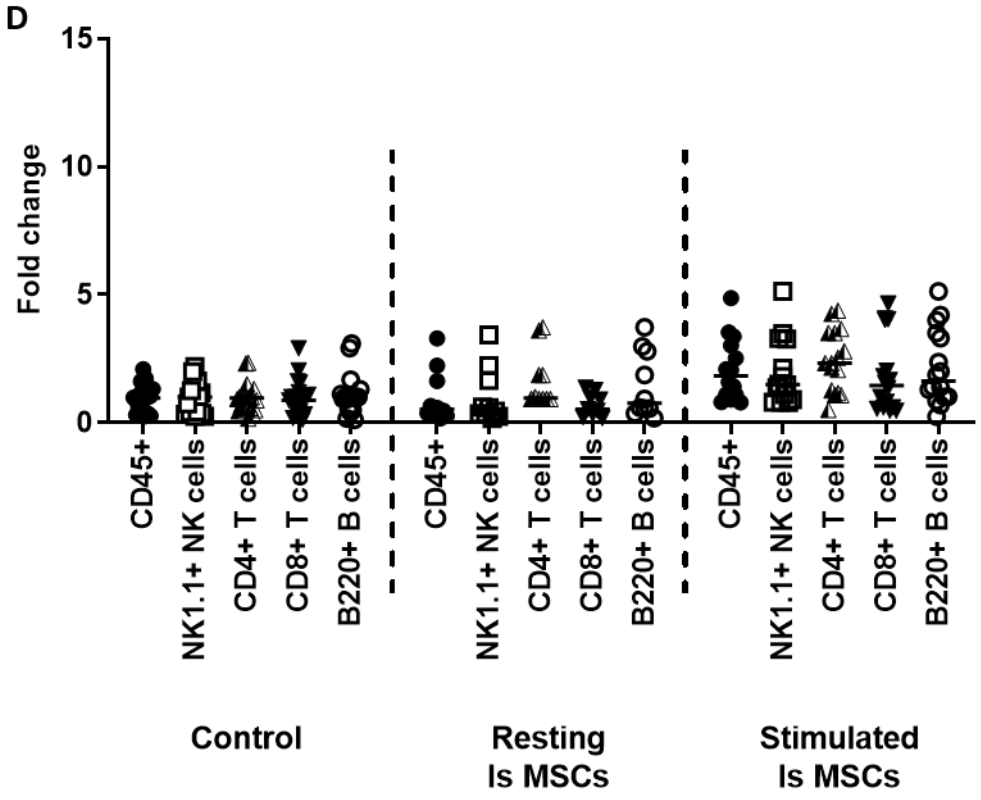
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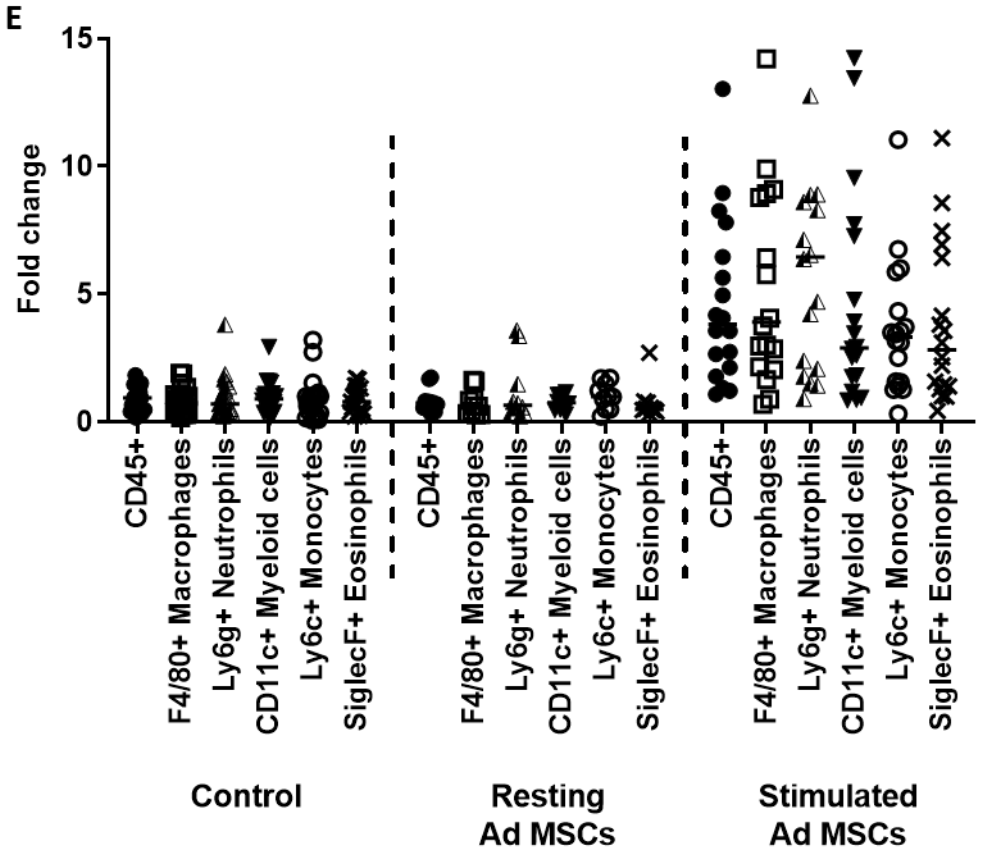
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Ad MSCs

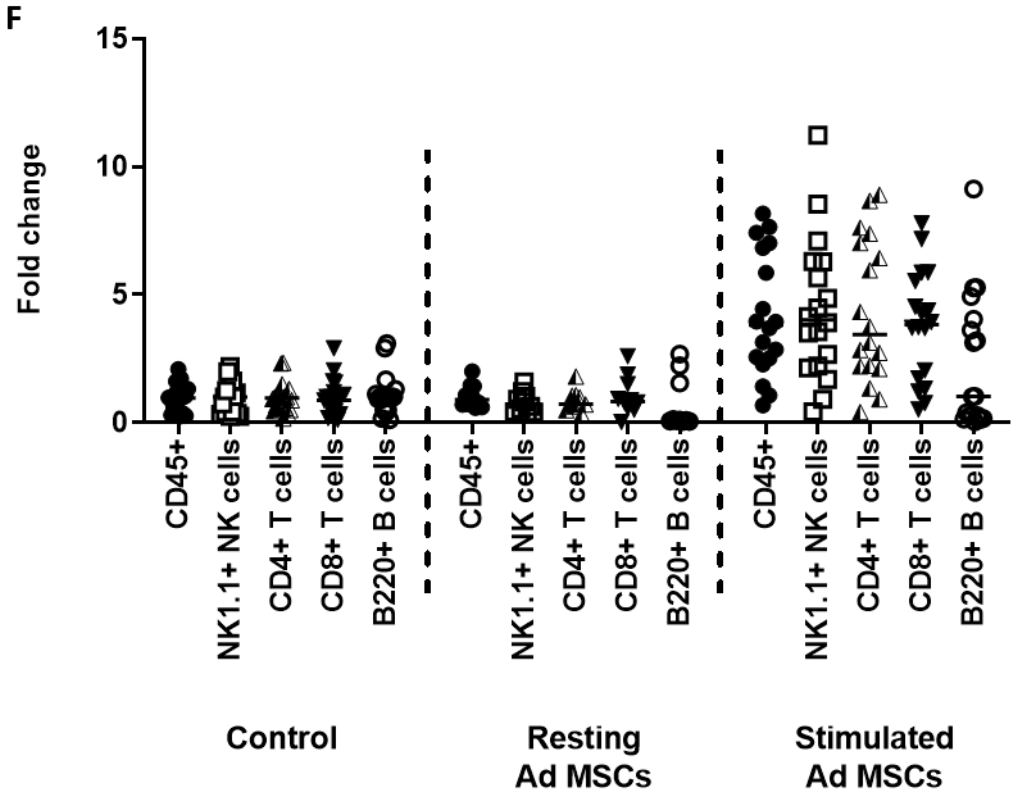


Figure 5-4. Immune infiltration into the air pouch of C57BL/6 female mice.
Air pouches were created on the dorsal of 8-week C57BL/6 female mice as explained in Section 2.4.2.1. The sixth day mice received an injection of either $1x10^6$ resting or stimulated MSCs in 1 mL of sterile PBS or sterile PBS alone after which mice were sacrificed, and tissues were harvested 24 hours later. Flow cytometry was used to assess the immune cell infiltration into the air pouches of mice, and it is expressed as number of cells per mL of fluid extracted from the air pouch. Data was then normalised to the PBS control. The total number of $CD45^+$, $F480^+$ macrophages, $Ly6g^+$ neutrophils, $CD11b^+$ $CD11c^+$ myeloid cells, $Ly6c^+$ monocytes and Siglec F^+ eosinophils that had infiltrated into air pouches of mice was determined and are graphed in **A**, **C** and **E**. The total number of $NK1.1^+$ NK cells, $CD8^+$ T cells, $CD4^+$ T cells and $B220^+$ B cells that had infiltrated into the air pouches of mice are graphed in **B**, **D** and **F**. To assess if MSC tissue of origin influenced the immune cell infiltrate, BM (**A** and **B**), Is (**C** and **D**) and Ad MSCs (**E** and **F**) were injected into the air pouches of mice. One Way ANOVA with Tukey's multiple comparisons was used to compare the immune cell infiltration among PBS control, resting and stimulated MSCs. Statistically significant differences are marked with the appropriate number of asterisks in Table 5-1.

Table 5-1. Statistical analysis of the immune infiltration produced by PBS, resting and licensed MSCs into the air pouch of C57BL/6 female mice.
Following the experimental set up explained in Figure 5-4, One Way ANOVA with Tukey's multiple comparisons was used to compare the immune cell infiltrate among PBS control, resting and stimulated MSCs. Significant differences are marked with the appropriate number of asterisks. p = 0.05 was considered the limit for statistical significance; * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.

	Immune cell	PBS Control vs Resting MSCs	PBS Control vs Stimulated MSCs	Resting MSCs vs Stimulated MSCs
BM MSCs	**$CD45^+$**	ns	**	***
	$F4/80^+$ Macrophages	ns	ns	*
	$Ly6g^+$ Neutrophils	ns	**	***
	$CD11c^+$ Myeloid cells	ns	*	**
	$Ly6c^+$ Monocytes	ns	ns	*
	$SiglecF^+$ Eosinophils	ns	**	**
	$NK1.1^+$ NK cells	ns	ns	ns
	$CD4^+$ T cells	ns	ns	ns
	$CD8^+$ T cells	ns	ns	ns
	$B220^+$ B cells	ns	ns	ns
Is MSCs	**$CD45^+$**	ns	****	****
	$F4/80^+$ Macrophages	ns	**	**
	$Ly6g^+$ Neutrophils	ns	****	****
	$CD11c^+$ Myeloid cells	ns	**	*
	$Ly6c^+$ Monocytes	ns	***	**
	$SiglecF^+$ Eosinophils	ns	****	***
	$NK1.1^+$ NK cells	ns	*	ns
	$CD4^+$ T cells	ns	***	ns
	$CD8^+$ T cells	ns	*	*
	$B220^+$ B cells	ns	ns	ns
Ad MSCs	**$CD45^+$**	ns	**	***
	$F4/80^+$ Macrophages	ns	ns	*
	$Ly6g^+$ Neutrophils	ns	**	***
	$CD11c^+$ Myeloid cells	ns	**	*
	$Ly6c^+$ Monocytes	ns	ns	*
	$SiglecF^+$ Eosinophils	ns	**	**
	$NK1.1^+$ NK cells	ns	****	****
	$CD4^+$ T cells	ns	****	****
	$CD8^+$ T cells	ns	****	****
	$B220^+$ B cells	ns	ns	ns

It is important to examine if MSCs from different sources not only recruited different types of immune cells, but also recruited them in statistically significant different amounts. For this reason, for an easier comparison of the immune cell recruitment produced by each source of MSCs, fold changes in the recruitment of immune cells by resting and stimulated BM, Is and Ad MSCs is

represented in Figure 5-5 and the significant differences in the recruitment by these cells is detailed in Table 5-2.

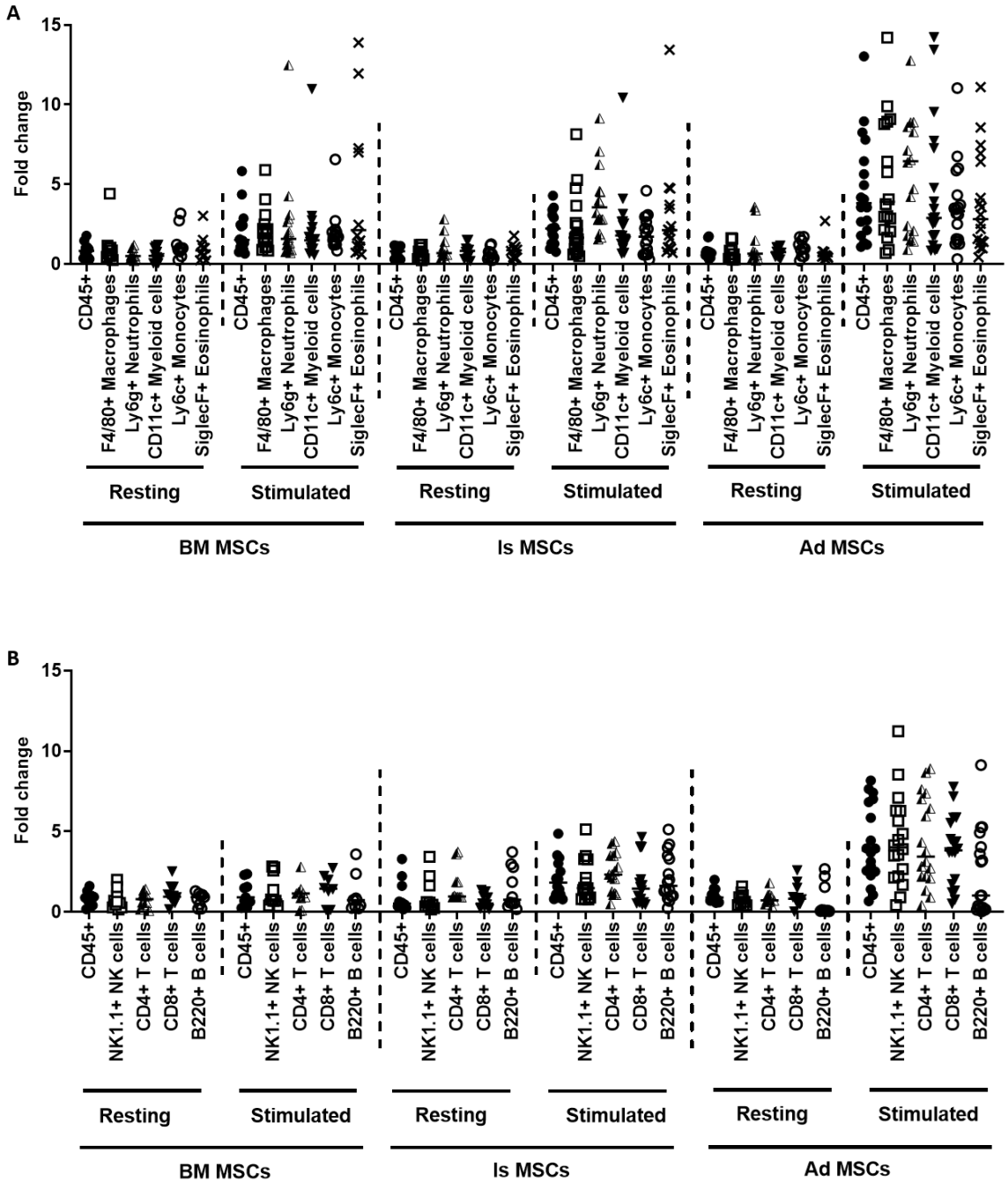


Figure 5-5. Comparison of the immune attraction profile among MSCs from different sources under resting and stimulated conditions in the air pouch.
Following the experimental set up explained in Figure 5-4, PBS control mice were used as a control to normalise all the data and the infiltration of immune cells in the air pouches (**A** and **B**) was compared among MSCs from different sources. One Way ANOVA with Tukey's multiple comparisons was used to compare the immune cell infiltrate originated by resting and stimulated MSCs from the different sources. Statistically significant differences are marked with the appropriate number of asterisks in Table 5-2.

Table 5-2. Statistical analysis of the immunoreactivity of resting and stimulated BM, Is and Ad MSCs into the air pouches and air pouch membranes of C57BL/6 female mice.
Following the experimental set up explained in Figure 5-5, One Way ANOVA with Tukey's multiple comparisons was used to compare the immune cell infiltrate among resting and stimulated BM, Is and Ad MSCs. Statistically significant differences are marked with the appropriate number of asterisks. p = 0.05 was considered the limit for statistical significance; * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.

Tissue	Immune cell	Stim. BM MSCs vs Stim. Is MSCs	Stim. BM MSCs vs Stim. Ad MSCs	Stim. Is MSCs vs Stim. Ad MSCs
AIR POUCH	**$CD45^+$**	ns	**	**
	$F4/80^+$ Macrophages	ns	**	**
	$Ly6g^+$ Neutrophils	ns	***	ns
	$CD11c^+$ Myeloid cells	ns	ns	ns
	$Ly6c^+$ Monocytes	ns	*	*
	$SiglecF^+$ Eosinophils	ns	ns	ns
	$NK1.1^+$ NK cells	ns	**	**
	$CD4^+$ T cells	ns	***	*
	$CD8^+$ T cells	ns	**	**
	$B220^+$ B cells	ns	ns	ns

+++ No statistically significant differences were found among BM, Is and Ad resting MSCs.

5.4 Analysis of the cellular infiltration into the air pouch membrane

Using flow cytometry, the total numbers of CD45 positive cells that had infiltrated into the membrane of the air pouches was assessed. All the data are expressed as number of cells per mg of membrane.

5.4.1 PBS only Controls

First, the immune cell infiltration produced by the generation of the air pouch itself and introduction of PBS was assessed (Figure 5-6). Minimal migration of $CD45^+$ cells into control air pouch membranes was observed, which proved that the creation of the air pouch or PBS injection itself, did not lead to the generation of an inflammatory site with subsequent immune cell infiltration. Macrophages and NK cells were the predominant immune cell present in the membranes of air pouches of control mice, followed by smaller numbers of neutrophils, monocytes and eosinophils.

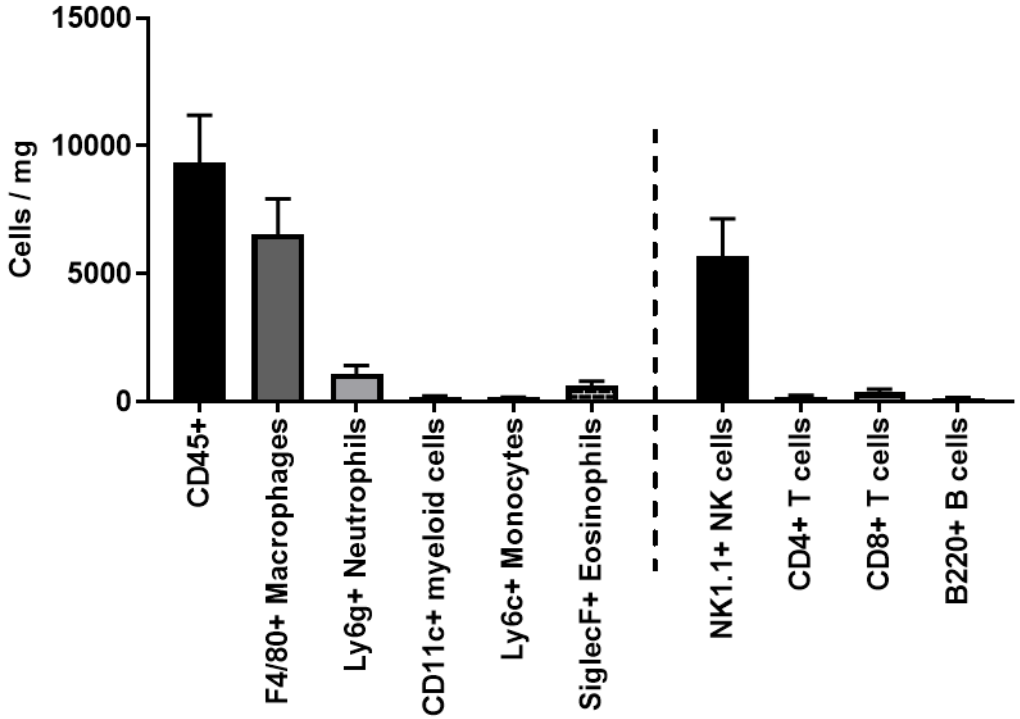


Figure 5-6. Generation of the air pouch on C57BL/6 mice and injection of PBS into the air pouch led to minimal migration of CD45$^+$ cells towards the air pouch membrane.
Air pouches were created on the dorsal of 8-week C57BL/6 female mice as explained in Section 2.4.2.1. The sixth day mice received a sterile PBS injection and mice were sacrificed, and tissues were harvested 24 hours later. Flow cytometry was used to assess the immune cell infiltration into the membrane of the air pouches of mice and it is expressed as number of cells per mg of membrane. The total number of CD45$^+$, F480$^+$ macrophages, Ly6g$^+$ neutrophils, CD11b$^+$ CD11c$^+$ myeloid cells, Ly6c$^+$ monocytes and Siglec F$^+$ eosinophils that had infiltrated into the membrane of the air pouches of mice was determined and graphed, as well as the total number of NK1.1$^+$ NK cells, CD8$^+$ T cells, CD4$^+$ T cells and B220$^+$ B cells that had infiltrated into the membrane of the air pouches of mice. Each bar group represents 5 mice ± SEM.

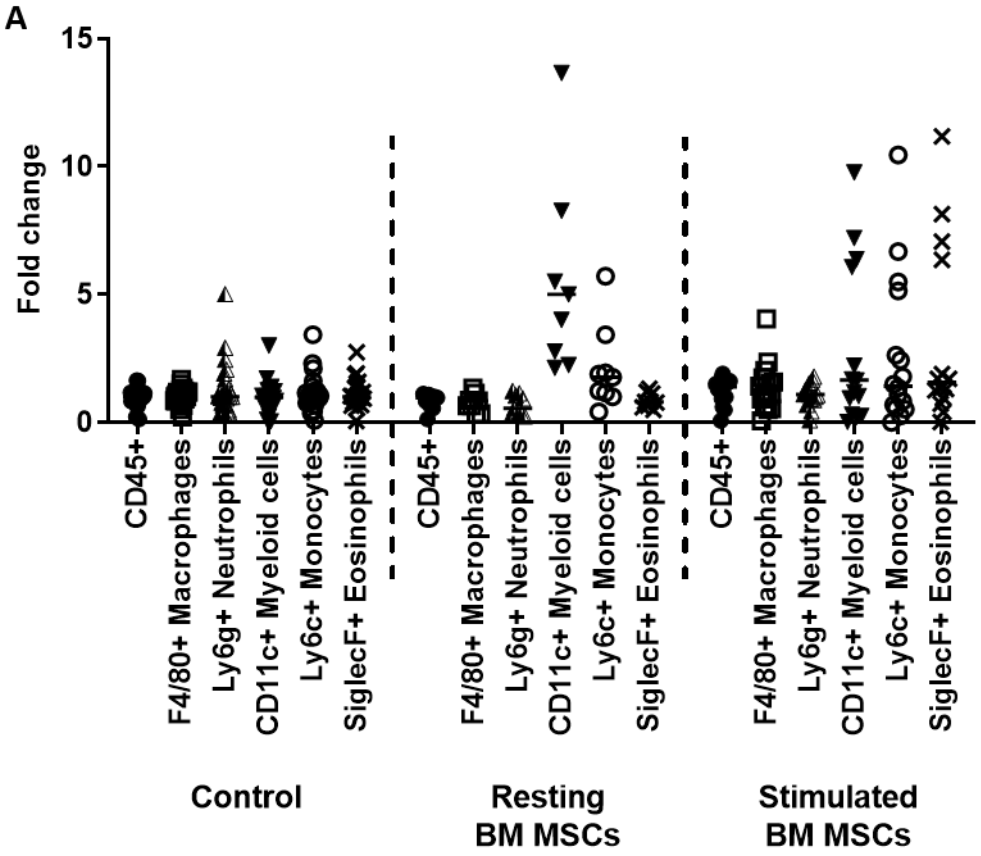
Next, immune cell infiltration needed to be tested after the infusion of MSCs from the three different sources, BM, Is and Ad MSCs, in two different conditions, resting and after licensing, which meant a large number of mice to work with. For this reason, this experiment was performed for MSCs from each tissue independently. Data were therefore normalised to the PBS control infiltrates in each individual set of experiments in order to be able to compare the data from the different experiments and replicates.

5.4.2 Administration of BM, Is and Ad MSCs

As shown in Figure 5-7, under resting conditions, minimal infiltration of innate or adaptive immune cells was observed in the air pouch membranes of the mice

infused with BM (A and B), Is (C and D) and Ad MSCs (E and F) and no statistically significant variation was seen in the number of $CD45^+$ cells, $F4/80^+$ macrophages, $Ly6g^+$ neutrophils, $CD11b^+$ $CD11c^+$ myeloid cells, $Ly6c^+$ monocytes, $SiglecF^+$ eosinophils, $NK1.1^+$ NK cells, $CD4^+$ T cells, $CD8^+$ T cells and $B220^+$ B cells recruited per mg of membrane harvested when compared to the control PBS mice was observed.

Infusion of licensed MSCs into the air pouch resulted in a small increase in the number of $CD45^+$ cells infiltrates compared to PBS control mice; however, this increase in the number of $CD45^+$ cells was only statistically significant in BM MSCs. Despite the small variation in the overall number of $CD45^+$ cells, the infusion of stimulated MSCs from each source led to some statistically significant variations in the population analysed using flow cytometry. Infusion of stimulated MSCs from any source produced no effect on the number of infiltrated $F4/80^+$ macrophages, $SiglecF^+$ eosinophils and $CD4^+$ T cells in the membrane of the air pouch. The number of $Ly6g^+$ neutrophils was increased in a statistically significant manner after the infusion of stimulated Is MSCs, while stimulated BM and Ad MSCs did not produce an infiltration of these cells in the membrane of the air pouch compared to the infusion of unstimulated MSCs or the PBS control mice. Resting BM MSCs were the only MSCs able to trigger the infiltration of $CD11b^+$ $CD11c^+$ myeloid cells in the membrane of the air pouch after infusion and this recruitment by BM MSCs was maintained after stimulation of the cells prior to infusion into the air pouch. This increased recruitment of $CD11b^+$ $CD11c^+$ myeloid cells was not statistically significant when compared to the PBS control mice; however, stimulated BM MSCs recruited statistically significantly more $CD11b^+$ $CD11c^+$ myeloid cells into the membrane of the air pouch than stimulated Is and Ad MSCs. Stimulated BM MSCs were the only MSCs able to trigger the infiltration of $Ly6c^+$ monocytes into the membrane of the air pouch after infusion. The infiltration of $NK1.1^+$ NK cells was statistically significantly higher in stimulated BM and Is MSCs compared to resting MSCs or PBS control mice, while licensing of Ad MSCs produced no change in the number of $NK1.1^+$ NK cells recruited. Stimulated Is MSCs were the only MSCs able to produce a statistically significant increase in the infiltration of $CD8^+$ T cells and $B220^+$ B cells in the membrane of the air pouch.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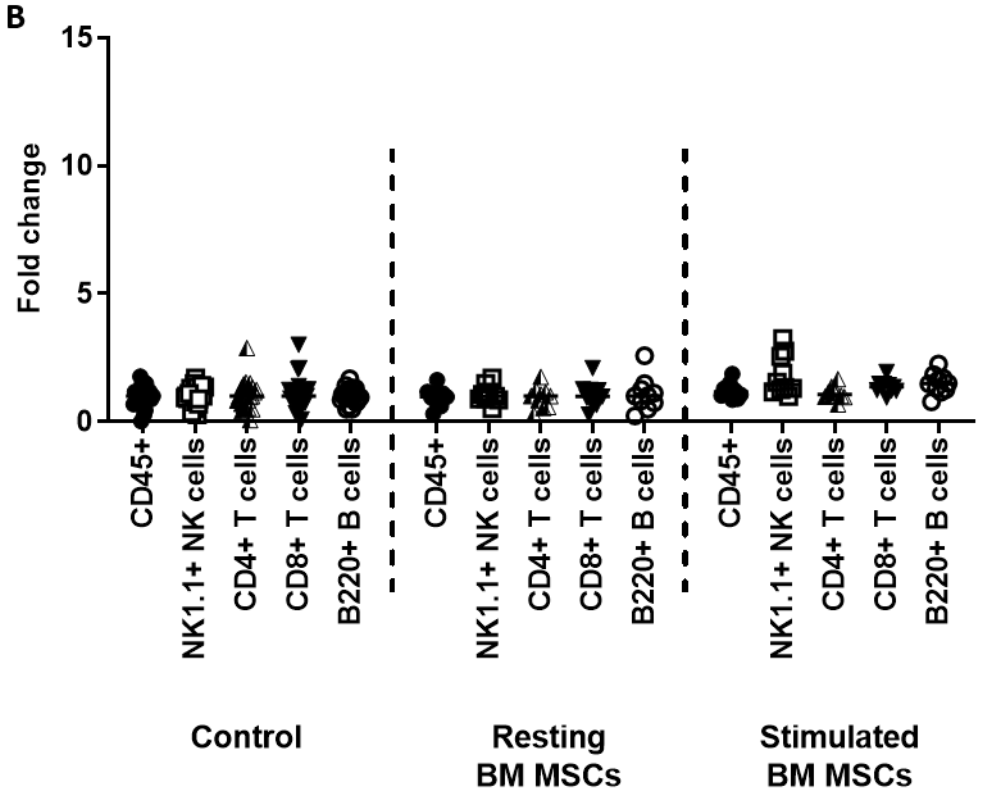
SiglecF+ Eosinophils
Control
Resting BM MSCs
Stimulated BM MSCs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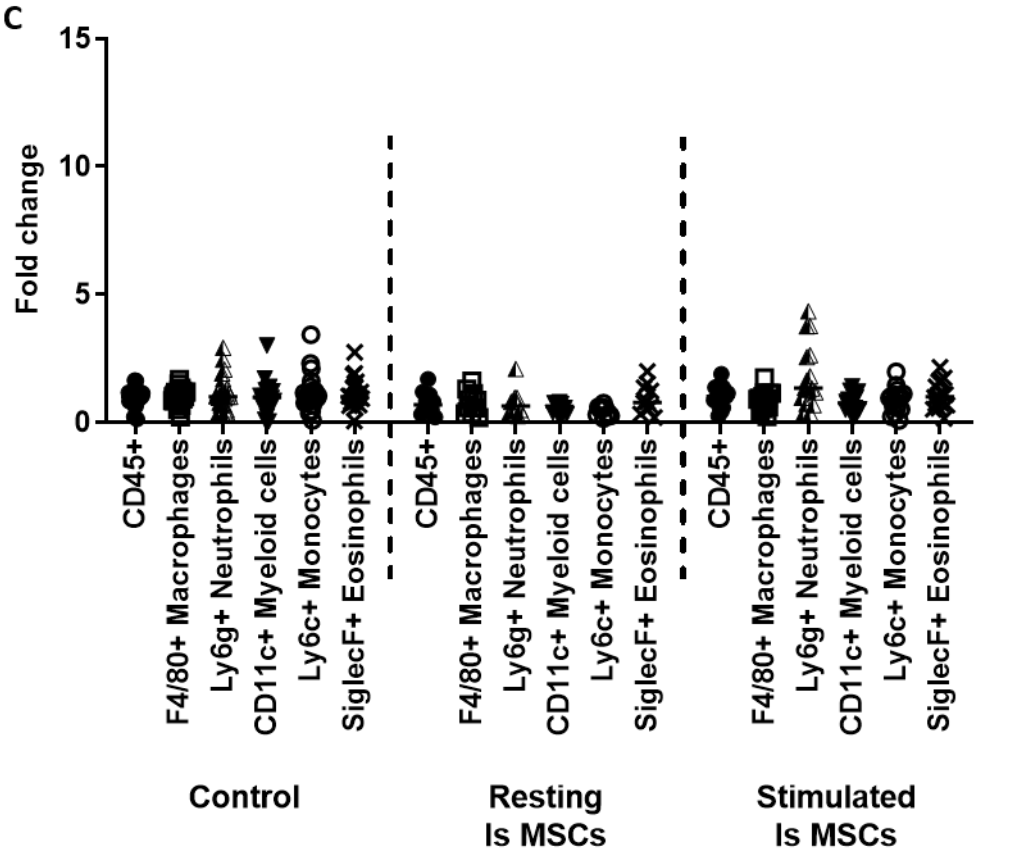
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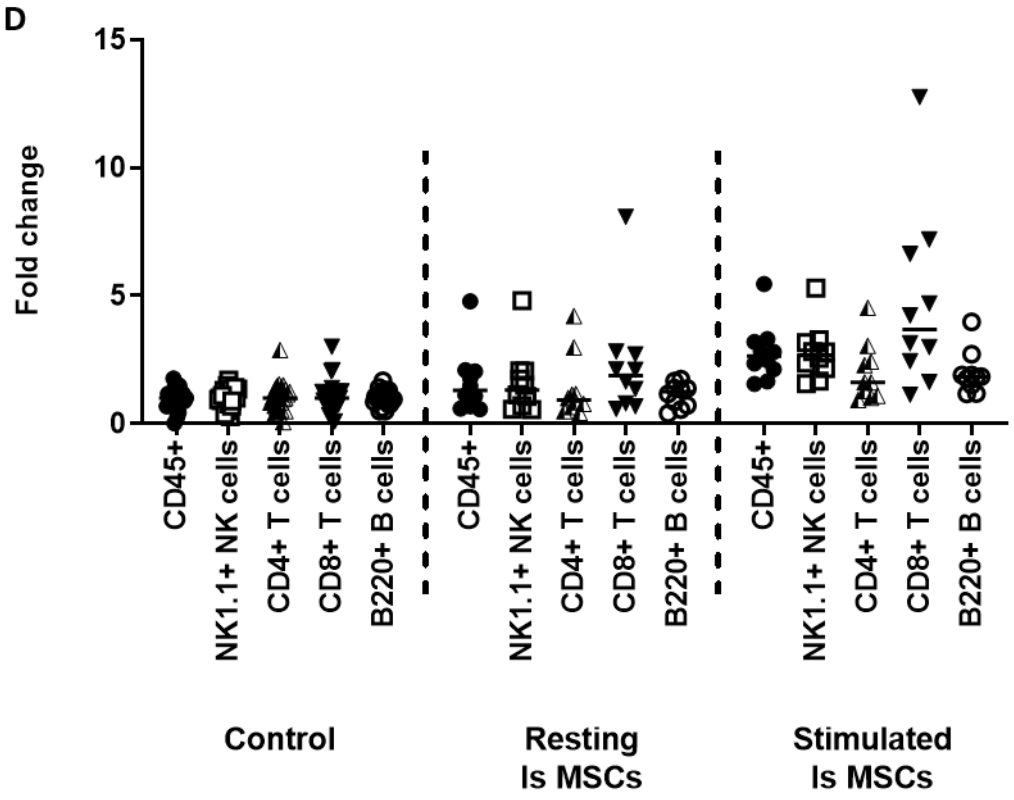
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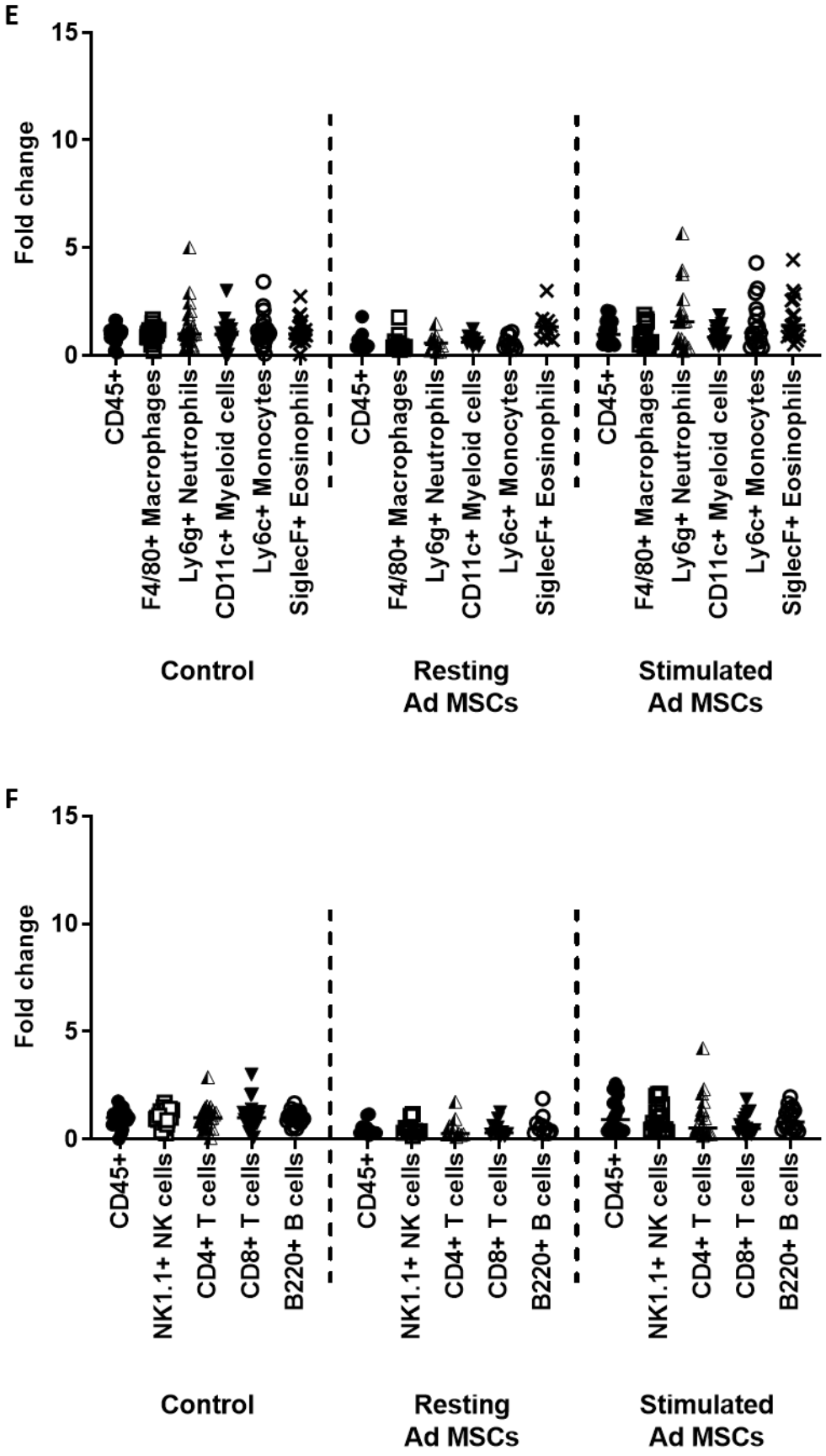


Figure 5-7. Immune infiltration into the air pouch membrane of C57BL/6 female mice.
Air pouches were created on the dorsal of 8-week C57BL/6 female mice as explained in Section 2.4.2.1. The sixth day mice received an injection of either $1x10^6$ resting or stimulated MSCs in 1 mL of sterile PBS or sterile PBS alone and mice were sacrificed, and tissues were harvested 24 hours later. Flow cytometry was used to assess the immune cell infiltration into the membrane of the air

pouches of mice and it is expressed as number of cells per mg of membrane. Data was then normalised to the PBS control. The total number of $CD45^+$, $F480^+$ macrophages, $Ly6g^+$ neutrophils, $CD11b^+$ $CD11c^+$ myeloid cells, $Ly6c^+$ monocytes and Siglec F^+ eosinophils that had infiltrated into the membrane of air pouches of mice was determined and are graphed in **A**, **C** and **E**. The total number of $NK1.1^+$ NK cells, $CD8^+$ T cells, $CD4^+$ T cells and $B220^+$ B cells that had infiltrated into the membrane of the air pouches of mice are graphed in **B**, **D** and **F**. To assess if MSC tissue of origin influenced the immune cell infiltrate, BM (**A** and **B**), Is (**C** and **D**) and Ad MSCs (**E** and **F**) were injected into the air pouches of mice. One Way ANOVA with Tukey's multiple comparisons was used to compare the immune cell infiltrate among PBS control, resting and stimulated MSCs. Statistically significant differences are marked with the appropriate number of asterisks in Table 5-3.

Table 5-3. Statistical analysis of the immune infiltration produced by PBS, resting and licensed MSCs into the membrane of the air pouch of C57BL/6 female mice.
Following the experimental set up explained in Figure 5-7, One Way ANOVA with Tukey's multiple comparisons was used to compare the immune cell infiltrate among PBS control, resting and stimulated MSCs. Statistically significant differences are marked with the appropriate number of asterisks. p = 0.05 was considered the limit for statistical significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

	Immune cell	PBS Control vs Resting MSCs	PBS Control vs Stimulated MSCs	Resting MSCs vs Stimulated MSCs
BM MSCs	**$CD45^+$**	ns	*	*
	$F4/80^+$ Macrophages	ns	ns	ns
	$Ly6g^+$ Neutrophils	ns	ns	ns
	$CD11c^+$ Myeloid cells	ns	ns	ns
	$Ly6c^+$ Monocytes	ns	***	ns
	$SiglecF^+$ Eosinophils	ns	ns	ns
	$NK1.1^+$ NK cells	ns	***	**
	$CD4^+$ T cells	ns	ns	ns
	$CD8^+$ T cells	ns	ns	ns
	$B220^+$ B cells	ns	*	ns
Is MSCs	**$CD45^+$**	ns	ns	ns
	$F4/80^+$ Macrophages	ns	ns	ns
	$Ly6g^+$ Neutrophils	ns	***	***
	$CD11c^+$ Myeloid cells	ns	ns	ns
	$Ly6c^+$ Monocytes	ns	ns	*
	$SiglecF^+$ Eosinophils	ns	ns	ns
	$NK1.1^+$ NK cells	ns	****	**
	$CD4^+$ T cells	ns	*	ns
	$CD8^+$ T cells	ns	****	*
	$B220^+$ B cells	ns	****	**
Ad MSCs	**$CD45^+$**	ns	ns	ns
	$F4/80^+$ Macrophages	ns	ns	ns
	$Ly6g^+$ Neutrophils	ns	ns	ns
	$CD11c^+$ Myeloid cells	ns	ns	ns
	$Ly6c^+$ Monocytes	ns	ns	*
	$SiglecF^+$ Eosinophils	ns	ns	ns
	$NK1.1^+$ NK cells	ns	ns	*
	$CD4^+$ T cells	ns	ns	ns
	$CD8^+$ T cells	ns	ns	ns
	$B220^+$ B cells	ns	ns	ns

It is important to examine if MSCs from different sources not only recruited different types of immune cells, but also recruited them in statistically significant different amounts. For this reason, for an easier comparison of the immune cell recruitment produced by each source of MSCs, fold changes in the recruitment of immune cells by resting and stimulated BM, Is and Ad MSCs is represented in Figure 5-8 and the significant differences in the recruitment by these cells is detailed in Table 5-4.

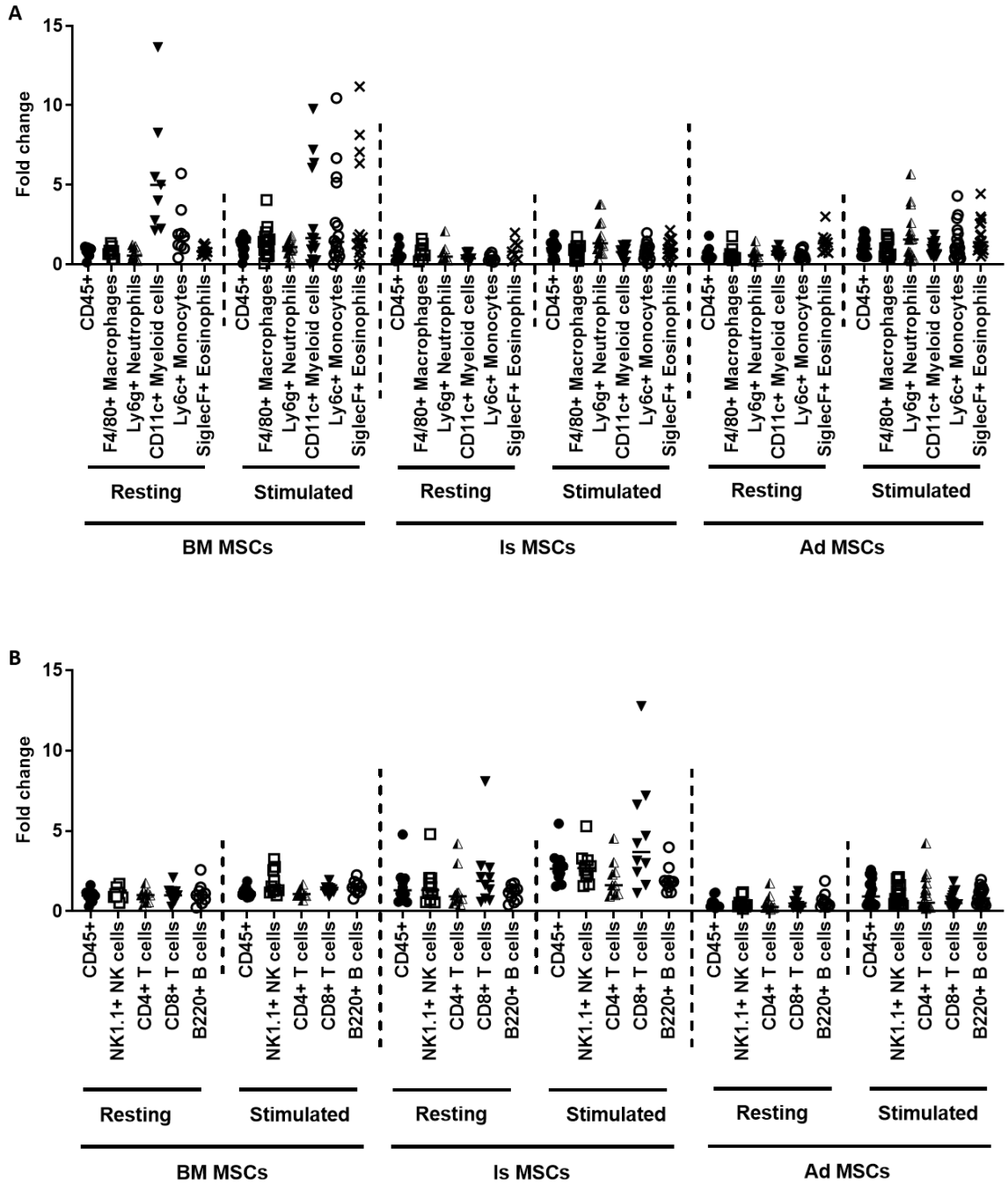


Figure 5-8. Comparison of the immune attraction profile among MSCs from different sources under resting and stimulated conditions in the air pouch membrane.

Following the experimental set up explained in Figure 5-7, PBS control mice were used as a control to normalise all the data and the infiltration of immune cells in the air pouches (**A** and **B**) was compared among MSCs from different sources. One Way ANOVA with Tukey's multiple comparisons was used to compare the immune cell infiltrate originated by resting and stimulated MSCs from the different sources. Statistically significant differences are marked with the appropriate number of asterisks in Table 5-4.

Table 5-4. Statistical analysis of the immunoreactivity of resting and stimulated BM, Is and Ad MSCs into the air pouches and air pouch membranes of C57BL/6 female mice.
Following the experimental set up explained in Figure 5-8, One Way ANOVA with Tukey's multiple comparisons was used to compare the immune cell infiltrate among resting and stimulated BM, Is and Ad MSCs. Significant differences are marked with the appropriate number of asterisks. p = 0.05 was considered the limit for statistical significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Tissue	Immune cell	Stim. BM MSCs vs Stim. Is MSCs	Stim. BM MSCs vs Stim. Ad MSCs	Stim. Is MSCs vs Stim. Ad MSCs
MEMBRANE	**$CD45^+$**	ns	ns	ns
	$F4/80^+$ Macrophages	ns	ns	ns
	$Ly6g^+$ Neutrophils	ns	ns	ns
	$CD11c^+$ Myeloid cells	*	*	ns
	$Ly6c^+$ Monocytes	***	**	ns
	$SiglecF^+$ Eosinophils	ns	ns	ns
	$NK1.1^+$ NK cells	*	*	****
	$CD4^+$ T cells	ns	ns	ns
	$CD8^+$ T cells	***	ns	****
	$B220^+$ B cells	ns	ns	***

+++ No statistically significant differences were found among BM, Is and Ad resting MSCs.

5.5 Distribution of MSCs within the air pouch

To determine the fate of the MSCs injected into the air pouch, an MSC marker was included into the antibody cocktail for easy and quantifiable detection of MSCs. As shown in Chapter 3, BM, Is and Ad MSCs all express very high levels of CD73, therefore, an anti-CD73 antibody was included into the flow cytometry panel, which in combination with the $CD45^-$ expression of MSCs provided an easy strategy to identify MSCs (Figure 5-9).

Firstly, the CD45 negative population was gated in both the air pouches (Figure 5-9, A) and membranes of mice (B). CD45 negative cells were then assessed for their CD73 expression in both the air pouch (D) and the membrane (F). CD73 FMOs were used for the identification of CD73 positive expression (C and E). MSCs were not found in the air pouches or membranes of the mice. To rule out the possibility of the cells being dead, the dead cell gate was removed and both

the air pouches (G) and the membranes (H) were assessed for the presence of CD73 positive cells. Live or dead CD73^{+} cells were not detected in the air pouches or membrane of mice.

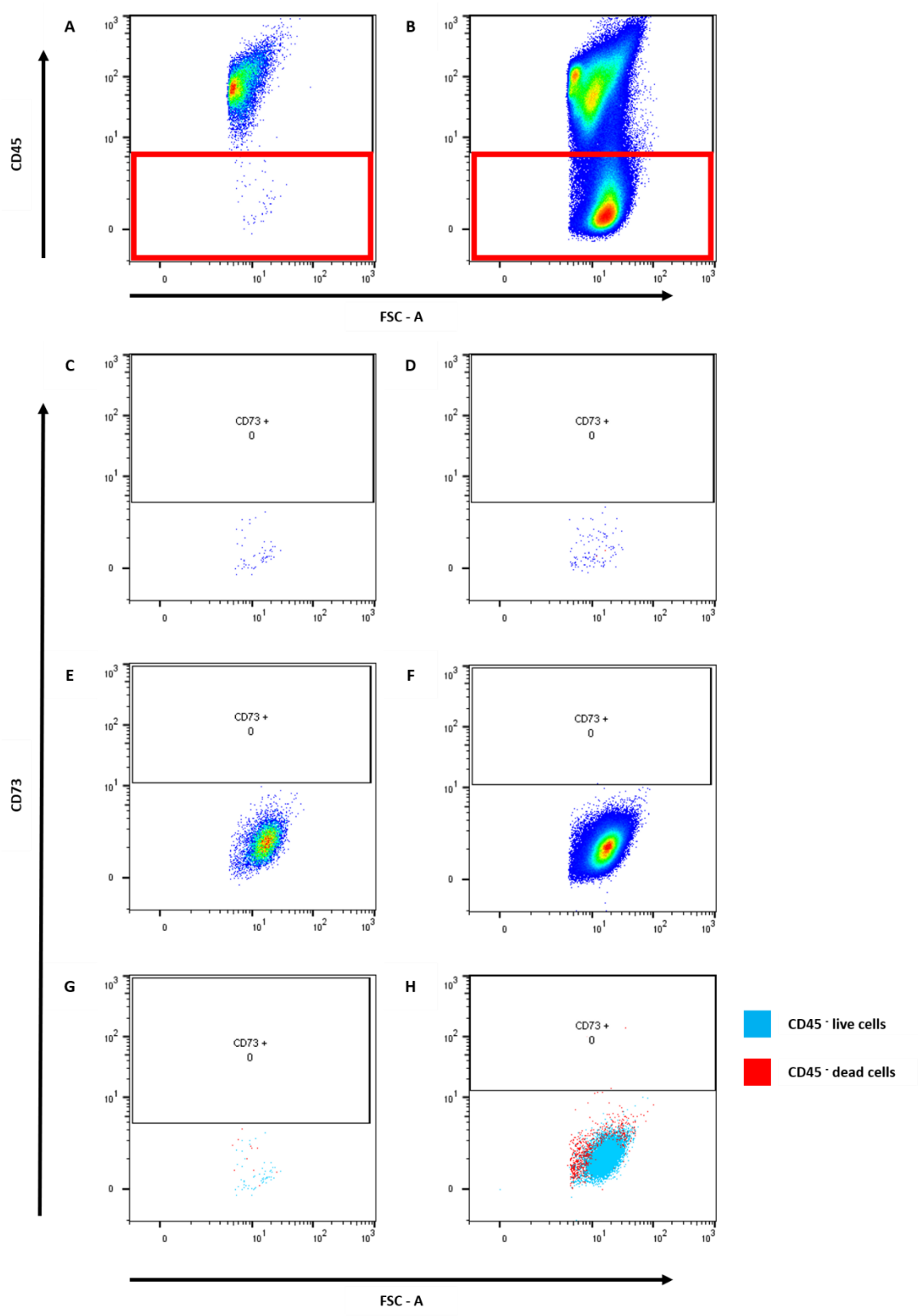


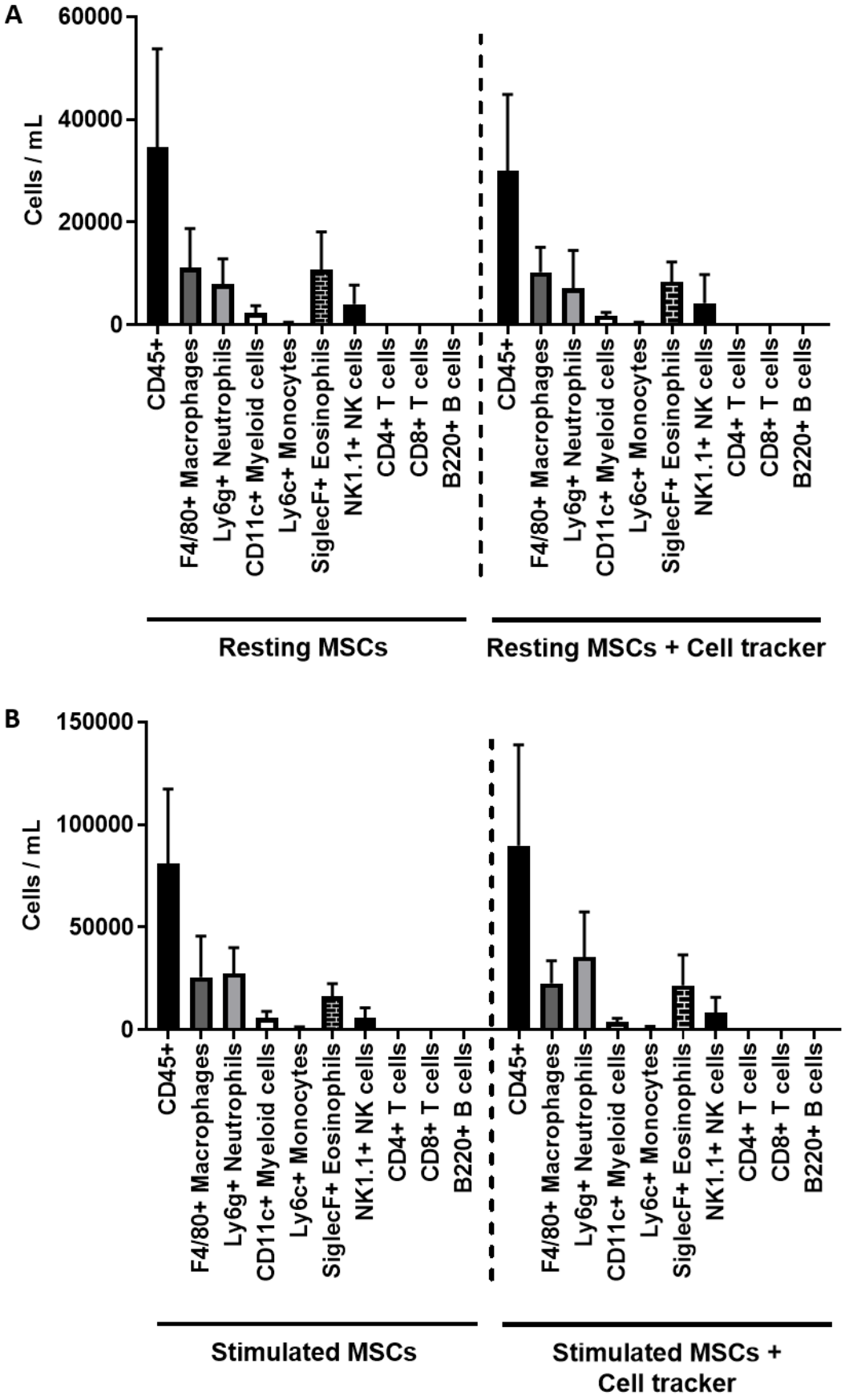
Figure 5-9. Assessment of the CD45 negative infiltration into the air pouches and membranes of C57BL/6 female mice.
Air pouches were created on the dorsal of 8-week C57BL/6 female mice as explained in Section 2.4.2.1. The sixth day mice received an injection of either $1x10^6$ resting or stimulated MSCs in 1 mL of sterile PBS or sterile PBS alone and mice were sacrificed, and tissues were harvested 24 hours later. Flow cytometry was used to assess de presence of CD73 positive cells. First, CD45 negative population was gated in the air pouches (**A**) and membranes (**B**) of mice. CD45 negative cells were then assessed for their CD73 expression in both the air pouch (**D**) and the membrane (**F**). CD73 FMOs were used for the identification of CD73 positive expression (**C** and **E**). Dead cell gate was removed and both the air pouches (**G**) and the membranes (**H**) were assessed for the presence of CD73 positive cells.

5.6 Assessment and validation of the immune-reactivity of the Cell-Tracker Green CMFDA

Necrotic and apoptotic cell death involves the disruption of the plasma membrane, which could explain the lack of detection of the CD73 protein on the surface of cells. In addition, MSCs could have undergone an engulfment process by the recruited phagocytic cells and therefore the anti-CD73 would not be able to interact with the CD73 surface protein. For these reasons, due to the lack of detection of CD73 positive cells within the air pouches and membranes of C57BL/6 female mice, we decided to use a cell tracker to determine the fate of the injected MSCs.

CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate) is a fluorescent dye that passes through the cell membrane, where it remains as cell membrane-impermeant reaction products. It is retained in living cells and it is transferred to daughter cells, but not to close cells in a population, which would therefore enable the detection of the cell tracker if the cell has divided or differentiated. Despite its low toxicity, it was essential to determine that the cell tracker would not affect the immune-reactivity of the MSCs stained with it (Figure 5-10). Staining of resting MSCs with the cell tracker lead to no variation in the immune cell attraction profile of MSCs as no statistically significant differences were found in the number of cells found in the air pouches (Figure 5-10, A) and membranes (B) of mice injected with the Green CMFDA stained MSCs compared to the control unstained MSCs. Similarly, the staining of stimulated MSCs with the cell tracker produced no variation in the immune cell attraction profile of MSCs, proved by the lack of statistically significant differences between the number of cells found in the air pouches (C) and

membranes (D) of mice injected with the Green CMFDA stained stimulated MSCs compared to the control unstained stimulated MSCs.



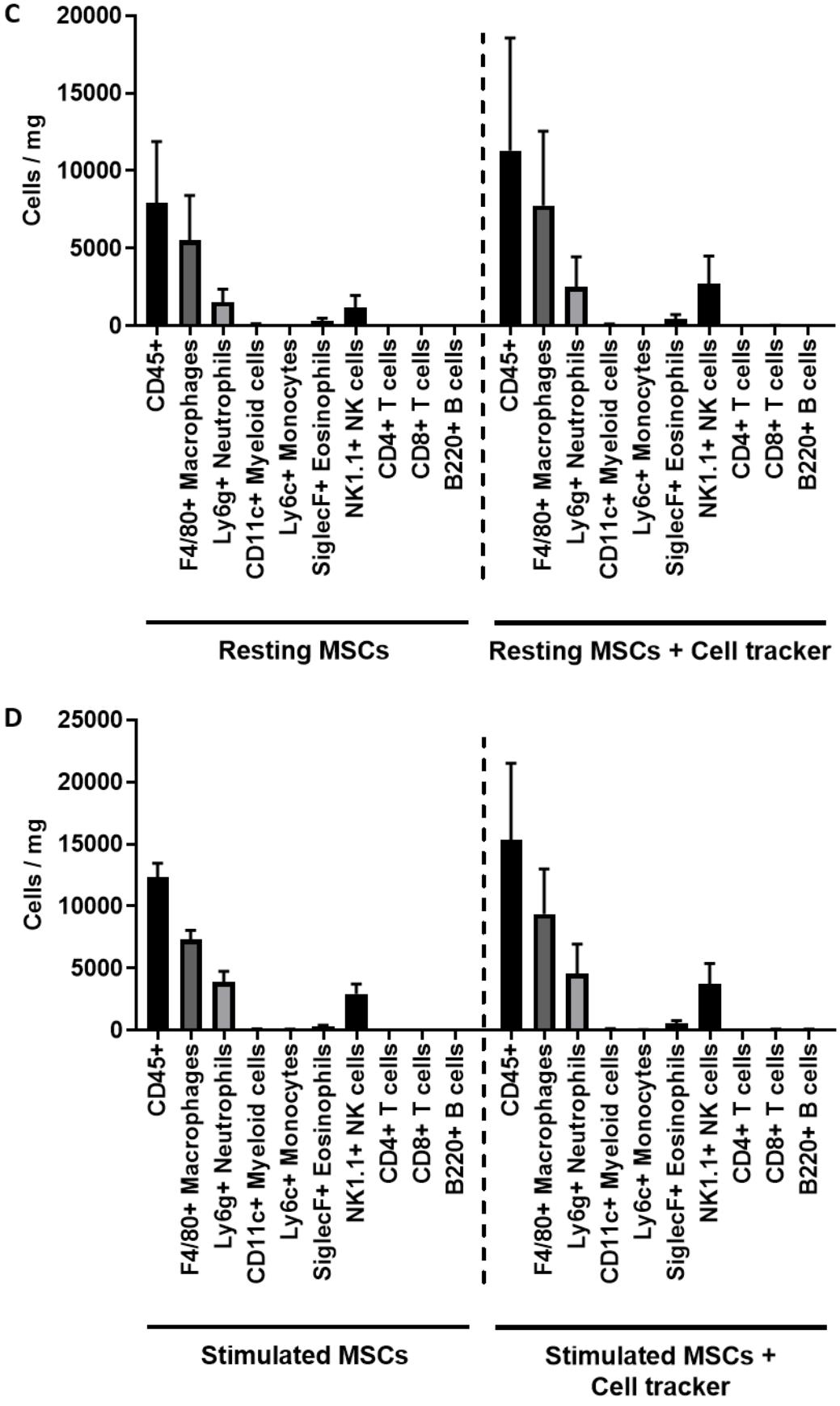


Figure 5-10. Staining with the Cell-tracker Green CMFDA did not produce a variation on the immune attraction profile of MSCs injected into C57BL/6 female mice.
Air pouches were created on the dorsal of 8-week C57BL/6 female mice as explained in Section 2.4.2.1. The sixth day mice received an injection of either resting or stimulated MSCs in 1 mL of

sterile PBS or resting or stimulated MSCs pre-stained with the Cell-tracker Green CMFDA in 1 mL of sterile PBS. Mice were sacrificed and tissues were harvested 24 hours later. Flow cytometry was used to assess the immune cell infiltration into the air pouches of mice, and it is expressed as number of cells per mL of fluid extracted from the air pouch (**A** and **B**) or per mg of membrane (**C** and **D**). Each bar group represents 5 mice ± SEM. Students (unpaired) T test was used to assess statistical differences between mice injected with unstained and Green CMFDA-stained MSCs.

Despite the previous lack of CD73 positive staining, we looked for the double staining of cell tracker and CD73. Once again, the CD45 negative population was gated out (Figure 5-11, A) and cells were gated for Green CMFDA and CD73. Cells from control mice injected with PBS were used as a negative control for CD73 and Green CMFDA due to the lack of infused MSCs on those samples (B). As observed in Figure 5-11, C, there was a small detection of Green CMFDA stained cells but those cells lacked the CD73 positive staining observed in cultured MSCs stained with the Green CMFDA cell tracker (D).

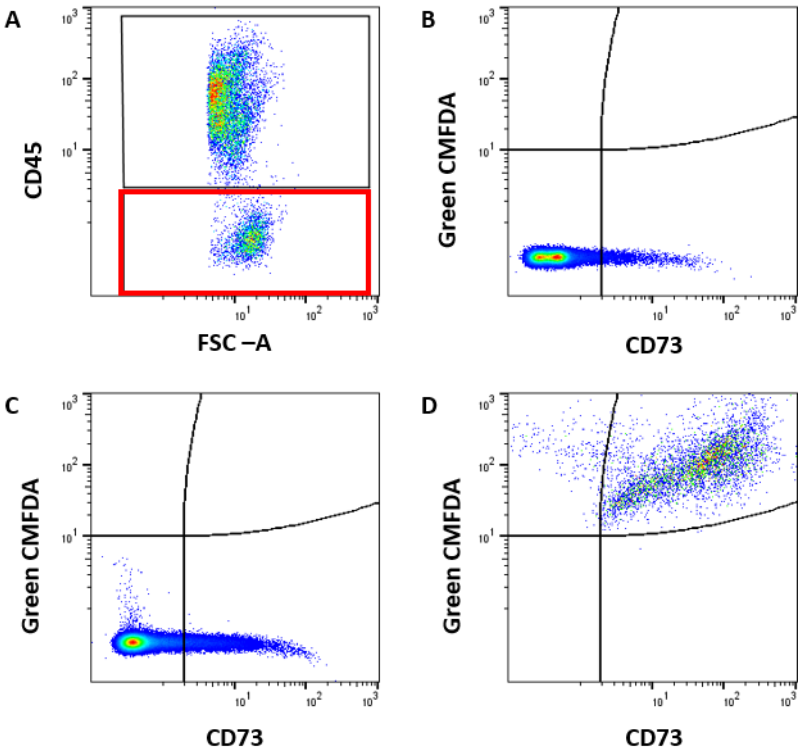


Figure 5-11. Lack of CD73 and cell tracker Green CMFDA double positive expression on the air pouches and membranes of the mice injected with Green CMFDA stained MSCs.
Air pouches were created on the dorsal of 8-week C57BL/6 female mice as explained in Section 2.4.2.1. The sixth day mice received an injection of either resting or stimulated MSCs in 1 mL of sterile PBS, resting or stimulated MSCs pre-stained with the Cell-tracker Green CMFDA in 1 mL of sterile PBS or sterile PBS alone. Mice were sacrificed, and tissues were harvested 24 hours later. Flow cytometry was used to assess de presence of CD73 and Green CMFDA positive cells. First, the CD45 negative population was gated in (**A**). CD45 negative cells were then assessed for their CD73 and Green CMFDA expression. Due to the lack of mouse MSCs on those samples, cells from control mice injected with PBS were used as a negative double stain for MSCs (**B**). Cells from

mice injected with Green CMFDA stained MSCs were analysed for their CD73 and Green CMFDA levels (**C**). Cultured MSCs stained with the cell tracker were used as a positive control of Green CMFDA and CD73 staining (**D**).

As there was no positive signal for the MSC marker CD73, we then focused on searching Green CMFDA signal within the air pouch and membrane samples. When we removed the dead gate within the CD45 negative population we were able to detect Green CMFDA signal in both dead and live cells (Figure 5-12, A), however, as shown in Figure 5-11, these cells were not MSCs. Due to the presence of Green CMFDA signal within the death gate, we wanted to see if we could find Green CMFDA signal in immune cells, which would suggest an interaction between the membrane of MSCs and the immune cells. Green CMFDA positive signal was found in $F4/80^+$ macrophages, $NK1.1^+$ NK cells and $CD8^+$ T cells (Figure 5-12, B).

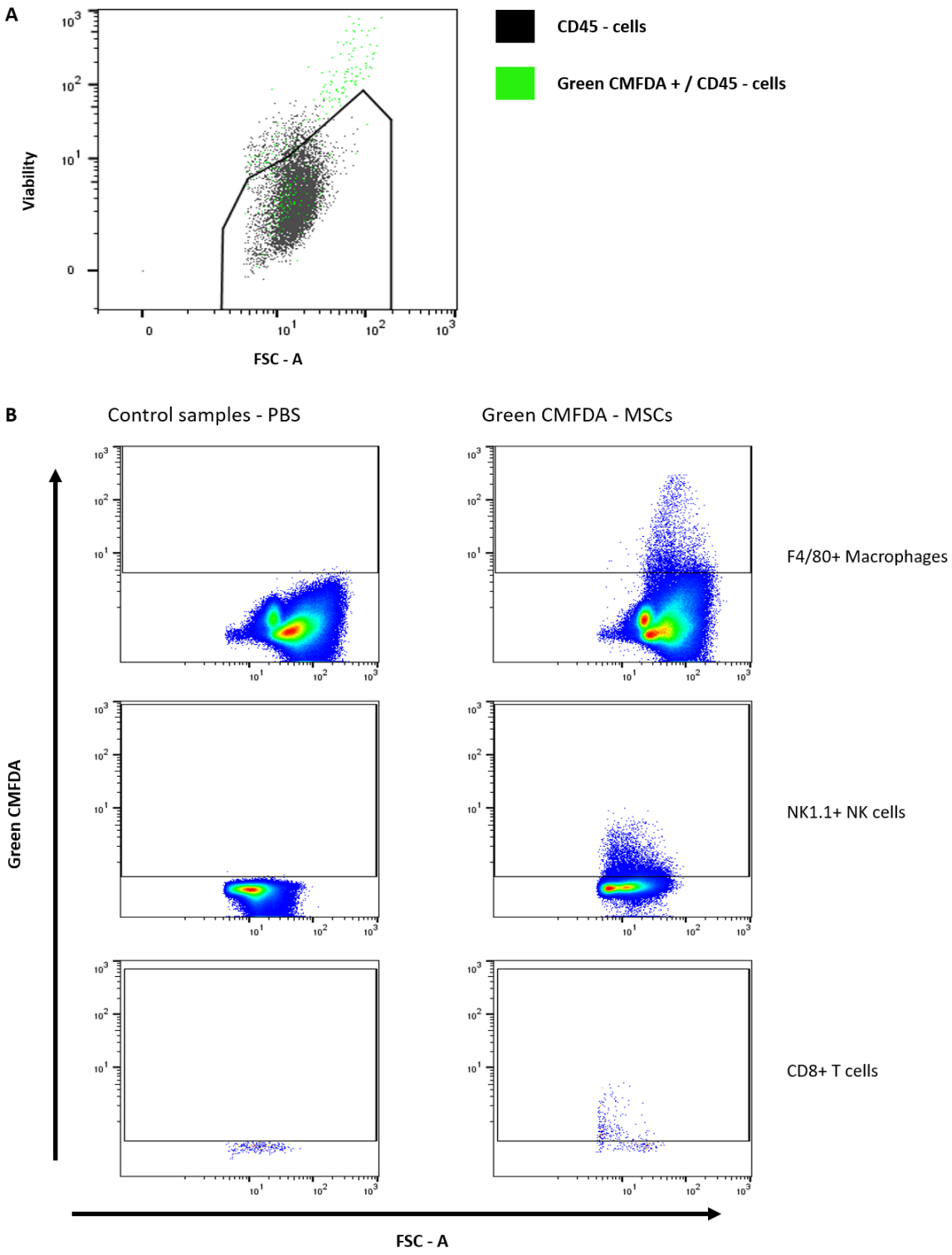


Figure 5-12. Identification of Green CMFDA positive staining from the air pouches and membranes of mice injected with Green CMFDA stained MSCs.
Air pouches were created on the dorsal of 8-week C57BL/6 female mice as explained in Section 2.4.2.1. The sixth day mice received an injection of pre-stained MSCs with the Cell-tracker Green CMFDA in 1 mL of sterile PBS or 1 mL sterile PBS. Mice were sacrificed and tissues were harvested 24 hours later. Flow cytometry was used to assess the immune cell infiltration into the air pouches of mice. CD45 negative population was studied for its Green CMFDA levels and its distribution between live and dead cells was assessed (**A**). The expression of Green CMFDA was assessed in F480^{+} macrophages, NK1.1^{+} NK cells and CD8^{+} T cells (**B**). PBS injected mice were used as a negative control to determine the Green CMFDA positive staining.

5.7 Discussion and conclusions

Using the air pouch model on C57BL/6 female mice, this chapter aimed to identify and examine the *in vivo* immune cell attraction profile of resting and pre-stimulated MSCs isolated from the bone marrow, Islets of Langerhans and adipose tissue via flow cytometry and to determine if tissue of origin influences the phenotype of the recruited cells.

As previously mentioned, MSCs have been shown to recruit immune cells but the mechanism by which this takes place is still not understood. It is important to point out that there are few studies that examine the immunogenicity of MSCs *in vivo* and to my knowledge, none that compares the immune attraction profile of MSCs isolated from different sources in a standardised manner. In addition, many of those studies have been conducted with human MSCs (Thirlwell, 2018) which usually lack a control for mismatched major histocompatibility complex molecule expression. Moreover, studies that did have a control for MHC expression determined that infusion of MHC-mismatched MSCs into an inflammatory environment led to both cell-mediated and humoral immune responses (Eliopoulos et al., 2005, Joswig et al., 2017). All these reasons made it essential to use autologous MSCs to study and compare the immune reactivity of MSCs from different sources. Allogenic stem cell-transplant and transplantation itself are heavily influenced by the sex of the donor and the recipient as sex-mismatched transplantation is linked to increased GVHD and even mortality (Nakasone et al., 2015, Kim et al., 2016). This is due to the mismatched minor histocompatibility antigens present on Y chromosome (H-Y) in males, increasing the rejection chances when the donor is male and the recipient is female, compared to female donor and male recipient (Kongtim et al., 2015). To avoid any kind of mismatch and study the immune attraction profile of MSCs in a highly controlled environment, MSCs were isolated from C57BL/6 female mice and infused into C57BL/6 female mice.

The current study found that resting BM, Is and Ad MSCs produced no immune cell infiltration into the air pouch, as the small number of immune cells present on it were the result of the creation of the air pouch itself, as demonstrated by the sterile PBS control mice (Figure 5-4 and Figure 5-7). However, licensing of MSCs with 40 ng/ mL of IFN-γ, TNF-α and IL-ß for 24 hours produced an

infiltration of CD45 positive cells, with a predominance for neutrophils, followed by macrophages and smaller numbers of eosinophils and monocytes regardless of the source of origin of the MSC. Moreover, on top of those immune cells, Is MSCs were also able to recruit $CD8^+$ T cells, while Ad MSCs were able to recruit $CD4^+$ and $CD8^+$ T cells and NK cells. Due to the lack of immune infiltration after the infusion of the resting MSCs into the air pouch, we can say with confidence that immune infiltration takes place as a result of the licensing of the cells and that the differences in the recruitment of immune cells by the different MSCs can only be explained as being related to the tissue of origin of MSCs. It is important to point out that the air pouch model had already been used to study the immune attraction profile of human stimulated Is MSCs (Thirlwell, 2018, Thirlwell et al., 2020). These cells produced a large infiltration of CD45 positive cells that consisted mostly of neutrophils, followed by macrophages and smaller numbers of monocytes and eosinophils. The much larger infiltration towards human MSCs in terms of cell number is likely explained by the mismatched major histocompatibility complex molecule expression.

It is important to mention that MSCs from different sources not only recruited different types of immune cells, but also recruited them in statistically significant different amounts. For this reason, the aim of this discussion will be focused on the mechanism by which the recruited cells could be detrimental or beneficial in a clinical setting and the relationship with the chemokine patterns described in Chapter 4.

5.7.1 MSCs and their *in vivo* attraction of neutrophils

Murine neutrophils express high levels of CXCR2 which is a receptor for several chemokines, mostly CXCL1 and CXCL2, but also CXCL3, CXCL5 and CXCL7, that lead to neutrophil recruitment and migration into target tissues (Futosi et al., 2013, Girbl et al., 2018). BM, Is and Ad MSCs were tested for the transcription of *CXCL1*, *CXCL2* and *CXCL5* and CXCL1 and CXCL2 protein levels. CXCL1 and CXCL2 were secreted at low levels under homeostatic conditions but their secretion was markedly upregulated upon MSC licensing in MSCs from every source, with Is MSCs secreting the most CXCL1 and Is and BM MSCs secreting the most CXCL2. As predicted by the increase of CXCL chemokine secretion, licensing of MSCs of every source led to a big neutrophil infiltration. Moreover, the role of human

CXCL8 (CXCL1 and CXCL2 in mouse) in neutrophil migration towards human MSCs has already been described; infusion of resting human Is MSCs produced a small neutrophil recruitment using the air pouch model, but this neutrophil recruitment was massively upregulated after stimulation of MSCs, which was correlated with increased secretion of CXCL8 (Joel et al., 2019, Thirlwell, 2018). However, despite the higher secretion of CXCL1 and CXCL2 by Is MSCs, no statistically significant differences were found when compared to the neutrophil recruitment by Is MSCs to BM and Ad MSCs (Figure 5-8 and Table 5-4). This finding suggests that even if CXCL1 and CXCL2 are strong neutrophil chemoattractants, there are other molecules involved in neutrophil migration or that only a low level of CXCL1 and CXCL2 is needed to induce neutrophil migration and once that is reached increased secretion will not further increase neutrophil recruitment.

Traditionally, the recruitment of neutrophils towards MSCs within a clinical setting could be considered to be detrimental due to the association of neutrophils with inflammatory diseases such as atherosclerosis, glomerulonephritis or rheumatoid arthritis, as well as being a marker for acute injury and transplant rejection (Prame Kumar et al., 2018). Neutrophils are fast responders to inflammatory signals and can exert their inflammatory role in three different manners: phagocytosis, degranulation and the formation of neutrophil extracellular traps (NETs). Neutrophils not only modulate the innate response but also the adaptive immune response as they produce and secrete several inflammatory factors that enable the recruitment of alloreactive $CD8^+$ T cells (Jones et al., 2010) and induce the expression of the pro-inflammatory cytokine IL-12 in DCs (Kreisel et al., 2011). However, in the last two decades the concept of neutrophil heterogeneity and plasticity has evolved, which suggests that once the neutrophils leave the bone marrow and infiltrate into inflammatory target sites, they can differentiate into discrete subsets with specific phenotypes and roles and can exert anti-inflammatory and pro-angiogenic functions (Rosales, 2018).

On the one hand, apoptosis of neutrophils is anti-inflammatory and pro-resolution not only because it avoids the secretion of proteolytic and oxidative mediators, but also because of the response it produces in phagocytic cells.

Phagocytosis of apoptotic neutrophils reduces IL-23 production and secretion by macrophages, which leads to a reduction in granulopoiesis and disabling of neutrophil degranulation within tissues (Stark et al., 2005). Moreover, phagocytosis of apoptotic neutrophils also results in the production and secretion of the anti-inflammatory mediators prostaglandin E2, TGF-ß, IL-10 and a novel class of lipid mediators called specialised pro-resolving mediators (Kasagi et al., 2014, Greenlee-Wacker, 2016, Serhan et al., 2015), which not only dampen the expression of inflammatory genes but also enforce tolerance through $CD4^+$ T cells (Kasagi et al., 2014). Furthermore, apoptotic neutrophils do not need to be phagocytosed to exert their anti-inflammatory effects, contact with monocytes is enough to suppress the production and secretion of pro-inflammatory cytokines and to increase the production of TGF-ß and IL-10 (Byrne and Reen, 2002).

Neutrophils could also inhibit graft inflammation and its subsequent rejection by enhancing wound and tissue repair coupled with neovascularization. Neutrophils generate barrier like dense clusters around necrotised tissues to prevent spreading and damaging of the healthy tissue surrounding it (Lammermann et al., 2013). Moreover, Pillay et al. described how the interaction between $CD16^{bright}$ $CD62^{low}$ neutrophil subset with T cells resulted in the suppression of proliferation (Pillay et al., 2012). In addition, Christoffersson et al. identified a $CD11b^+$/ $Gr\text{-}1^+$/ $CXCR4^{high}$ murine neutrophil subset that was recruited by VEGF-a into the site of islet engraftment that lead to the revascularization of transplanted islets (Christoffersson et al., 2012). As previously mentioned, this subset of neutrophils would be able to respond to the CXCL12 secretion by MSCs and they would be beneficial within a clinical setting. More importantly, this neutrophil subset produced matrix metallopeptidase 9 *(*MMP9*) and* the revascularization of transplanted islets was MMP9 dependent and $CD11b^+$/ $Gr\text{-}1^+$/ $CXCR4^{high}$ dependent, as MMP9 deficient mice were unable to revascularise the graft and the revascularization of the graft required the presence of neutrophils. MMP9 is most likely required for revascularization due to its role in increasing vascular density and blood flow (Christoffersson et al., 2010, Christoffersson et al., 2012).

Lastly, activated neutrophils are able to prevent T cell activation, and therefore, alloimmunity, by the release of the serine proteases cathepsin G and neutrophil elastase which cleave, and inactivate, the pro-inflammatory cytokines IL-2 and IL-6 (Bank et al., 1999).

Taking all this into account, we can conclude that the outcome of neutrophil infiltration into a site of inflammation could most likely be environment dependent and determining if they would be beneficial or detrimental within a clinical setting is not possible without the appropriate disease model. However, it is important to consider that granulocytic myeloid derived suppressor cells (GMDSCs) are anti-inflammatory immune cells that share surface marker expression with neutrophils. In fact, there is an ongoing debate on whether they are a neutrophil subset or a completely distinct cell or even if all suppressive neutrophils subsets should be considered GMDSCs. GMDSCs are able to suppress effector T cell responses and their function needs to be tightly regulated as they can lead to the development of chronic infection and tumour progression (Zilio and Serafini, 2016). Therefore, GMDSCs would most likely be beneficial within a transplant setting.

5.7.2 MSCs and their *in vivo* attraction of monocytes and macrophages

Murine monocytes and macrophages express high levels of CCR2 which is the receptor for several chemokines including CCL2, CCL7, CCL8, CCL12 and CCL13, that lead to extravasation and transmigration of monocytes and migration into target tissues (Chu et al., 2014). BM, Is and Ad MSCs were tested for the transcriptional and protein levels of CCL2 and CCL7. CCL2 and CCL7 were secreted at low levels under homeostatic conditions but their secretion was markedly upregulated upon MSC licensing in MSCs from every source, with Is MSCs secreting the most CCL2 and CCL7. As predicted by CCL chemokine secretion, licensed MSCs of every source induced a monocyte and macrophage infiltration into the air pouches. However, despite the higher secretion of CCL2 and CCL5 by Is MSCs, Ad MSCs produced a statistically significant higher monocyte and macrophage infiltration compared to BM and Is MSCs (Figure 5-8 and Table 5-4). This finding suggests that even if CCL2 and CCL5 are strong monocyte and macrophage chemoattractants, there are other molecules

involved in monocyte and macrophage migration or that only a low level of CCL2 and CCL5 is needed to induce monocyte and macrophage migration and once that amount is reached, an increased secretion will not produce an increment in monocyte and macrophage recruitment.

Like neutrophils, the recruitment of monocytes and macrophages towards MSCs within a clinical setting could be considered as detrimental due to the well-known association of monocytes and macrophages with inflammatory diseases such as systemic lupus erythematosus, systemic sclerosis and rheumatoid arthritis among others (Ma et al., 2019). However, within a transplant setting, the state of activation and macrophage phenotype may lead to a completely different outcome, from graft rejection due to tissue injury to tissue remodelling and anti-inflammatory effects. Macrophages are phagocytic immune cells essential in host defence and tissue homeostasis and like neutrophils, monocytes can differentiate into pro-inflammatory or anti-inflammatory macrophages depending on micro-environmental signals. Macrophages are classified as M1 and M2 macrophages. On the one hand, M1 macrophages are pro-inflammatory and are able to mediate transplant rejection by producing pro-inflammatory cytokines, such as IL-1, IL-12, IL-18, IL-6, IL-23, TNF-*α*, and IFN-*γ,* reactive oxygen species and reactive nitrogen species, which all together lead to acute rejection (Li et al., 2019a). On the other hand, M2 macrophages, are anti-inflammatory and are involved in tissue repair due to their roles in wound healing, angiogenesis, phagocytosis, fibrosis, and the resolution of inflammation (Li et al., 2019a). On top of this regulatory macrophages, a subset of macrophages that does not express most markers shared by M1 and M2 macrophages, are able to suppress allogeneic T cells by an inducible nitric oxide synthase *(*iNOS*)* dependent mechanism while promoting the expansion of $CD4^+$ $Foxp3^+$ regulatory T cells, thereby enabling graft tolerance (Conde et al., 2015, Riquelme et al., 2013).

However, it is important to mention that the infusion of bone marrow derived macrophages in a murine model of liver fibrosis led to the chemokine mediated recruitment of endogenous macrophages and neutrophils, which secreted MMP9 and IL-10, leading to the apoptosis of scar producing myofibroblasts and therefore, to reduced fibrosis (Thomas et al., 2011). Moreover, infusion of in

vitro differentiated $CD14^+$ monocytes isolated from cirrhotic patients into a murine liver fibrosis model led to the decrease of liver injury markers and to the increase of liver regeneration markers and, therefore, to reduced fibrosis (Moore et al., 2015).

Macrophages seem to be essential for liver repair however, to determine the outcome of macrophage infiltration within a clinical setting further characterisation of the recruited macrophages would be essential. The phenotype of macrophages will most likely be environment-dependent and determining if they would be beneficial or detrimental within a clinical setting is not possible without the appropriate disease model. Thorough analysis of gene signatures and flow cytometry validation enable a distinction to be made between M1 and M2 macrophages based on CD38/ Egr2 expression. M1 macrophages express CD38, Gpr18 and Fpr2 while Egr2 and c-Myc are exclusively expressed by M2 macrophages (Jablonski et al., 2015). DHRS9 has been established as human regulatory macrophage marker; however, DHRS9 is not upregulated in murine regulatory macrophages and therefore, can't be used to exclude other monocyte–derived cells (Riquelme et al., 2017).

5.7.3 MSCs and their *in vivo* attraction of eosinophils

Murine eosinophils express high levels of CCR3, which is the receptor for CCL2, CCL3, CCL4, CCL5, CCL11, CCL13, CCL14 and CCL16, that leads to migration into target tissues and degranulation (Chu et al., 2014, Nagase et al., 2001). BM, Is and Ad MSCs were tested for their transcription of *CCL3*, *CCL4*, *CCL5* and *CCL11* and protein levels of CCL2, CCL5 and CCL7. CCL2, CCL5 and CCL7 were secreted at low levels under homeostatic conditions. MSC licensing increased the production and secretion of the three CCLs but produced the largest upregulation in CCL2 in MSCs from the three sources. As predicted by the increase of CCL chemokine secretion, licensing of MSCs of every source led to a small upregulation of eosinophil infiltration. However, despite the higher secretion of CCL2, CCL5 and CCL7 by Is MSCs, Ad MSCs produced a statistically significant increased eosinophil infiltration compared to BM and Is MSCs (Figure 5-8 and Table 5-4), suggesting that even if CCL2, CCL5 and CCL7 are eosinophil chemoattractants, there are other factors involved in their migration.

The recruitment of eosinophils towards MSCs within a clinical setting could be considered as potentially detrimental due to their roles in allergic reactions. Eosinophils are involved in the secretion of pro-inflammatory molecules such as major basic protein (MBP), MBP2, eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and ß-glucuronidase. Moreover, they are also able to secrete cytokines (IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-16 and IL-18) and chemokines (CCL3, CCL5 and CL11), leading to the upregulation of the vascular adhesion system as well as to the increase of vascular permeability, enabling the recruitment of immune cells (Kita, 2011).

Eosinophilic infiltrates are a specific marker of rejection in liver allografts after discovering that successful treatment of acute rejection was correlated with a decrease on the number of infiltrated eosinophils (Nagral et al., 1998). In renal allografts eosinophilia is also a marker of graft damage and poor graft prognosis (Vanikar et al., 2017). However, Goldman et al. showed that the disappearance of the eosinophilic infiltrate did not stop the rejection of pig pancreatic islets in mice, suggesting that eosinophils could be bystanders in transplant rejection (Goldman et al., 2001). Although eosinophils can be found in the blood of patients transplanted with haematopoietic stem cells (Cromvik et al., 2014), eosinophilia after stem cell transplantation has been suggested to be a marker for a favourable outcome as the survival rate of patients with haematological disorders treated with stem cell transplantation was higher in those with eosinophilia compared to those without it (88.7 *vs* 43.0%) (Sato et al., 2005a). For these reasons, we hypothesised that eosinophilic infiltration produced by MSCs could be beneficial in a clinical setting, but an appropriate transplant model would be required to confirm this.

5.7.4 MSCs and their *in vivo* attraction of DCs

As previously described, CD11b and CD11c are co-expressed in myeloid-lineage DCs but these markers can be upregulated on activated cells irrespective of their naïve expression status. For this reason, despite the identification of a CD11b high CD11c$^+$ population the subset was not identified as DCs due to the lack of CD24 or other DC markers in the panel that would enable the exclusion of activated cells. However, we know that the CD11b high CD11c$^+$ myeloid contains,

among others, DCs and for this reason the role of DCs within a clinical setting is included in this discussion.

Immature murine DCs express high levels of CCR1, CCR2, CCR5, and CCR6, as well as CXCR3 and CXCR4; however, upon inflammatory stimulation they downregulate these receptors except for CXCR4 and CCR7, which are upregulated. CXCR4 interacts with CXCL12, while CCR7 interacts with CCL19 and CCL21 (Ricart et al., 2011). BM, Is and Ad MSCs were tested for transcription of *CCL19* and *CXCL12* and CXCL12 protein levels. CXCL12 was secreted at medium levels in BM and Is MSCs and at low levels in Ad MSCs under homeostatic conditions but its secretion was downregulated upon MSC licensing in MSCs from every source, with BM MSCs secreting the most CXCL12. Despite the decrease of CXCL12 secretion, licensing of MSCs from every source led to an increase in CD11b high $CD11c^+$ myeloid cells infiltration. However, despite the higher secretion of CXCL12 by BM MSCs, Ad MSCs recruited more CD11b high $CD11c^+$ myeloid cells upon stimulation (Figure 5-8 and Table 5-4); suggesting that even if CXCL12 is a strong CD11b high $CD11c^+$ myeloid cell chemoattractant, there are other molecules involved in their migration.

The recruitment of DCs towards MSCs in a clinical setting could be considered detrimental due to their role linking the innate and adaptive immune responses. Dendritic cells are mononuclear professional phagocytes that, unlike neutrophils and macrophages, do not digest the phagocytosed material but process it into peptide fragments and present self and nonself antigens to $CD4^+$ and $CD8^+$ T cells using MHC Class I and MHC Class II molecules respectively. In this way, DCs can activate the adaptive immune response; therefore, they connect the innate and the adaptive immune responses (Steinman, 2006). However, different DC subsets enable the balance between tolerance in the steady state and the induction of innate and adaptive immunity. DCs can initiate GVHD by activation of recipient T cells, for this reason removal of recipients DCs reduces the presence of effector T cells in the graft; thus, subsequently reducing rejection in solid organ transplantation (Zhuang et al., 2016). However, DCs are radioresistant, therefore they survive to the irradiation that precedes stem cell transplantation and can initiate GVHD by activation of recipient T cells (Duffner et al., 2004).

The absence of functional DCs lead to survival of iPSCs transplanted under the kidney capsule in a syngeneic C57BL/6 mouse stem cell transplantation model, whilst when iPSCs were co-transplanted with mature DCs immune rejection took place (Todorova et al., 2016). Moreover, prevention of DC migration towards the lymph nodes, inhibition of the maturation of DCs and downregulation of co-stimulatory molecules required for T cell activation, has been shown to alleviate GVHD. However, semi-mature DCs are able to decrease the $CD4^+$ T cell infiltration, prolonging survival of transplanted cells; and tolerogenic DCs induce regulatory T cells, promoting immune tolerance (Zhang et al., 2017). Taking all this into account, we cannot conclude that DC migration towards MSCs would be beneficial or detrimental within a clinical setting.

5.7.5 MSCs and their *in vivo* attraction of T cells

T cells express most of the CC and CXC chemokine receptors at different levels according to their state of differentiation, as an example, T cells can express high levels of CCR4, CCR5, CCR7, CCR9 and CCR10, as well as CXCR3 and CXCR4 and respond to CCL5, CCL17, CCL19, CCL21, CCL25, CCL27, CCL28 and CXCL9, CXCL10 and CXCL12, which shows the complex regulation of T cell migration (Nolz et al., 2011). BM, Is and Ad MSCs were tested for transcription of *CCL5*, *CCL19*, *CXCL10* and *CXCL12* and CCL5, CXCL10 and CXCL12 protein levels. CCL5 and CXCL12 were secreted at medium levels under homeostatic conditions, while CXCL10 was secreted at lower levels. However, their secretion was upregulated upon MSC licensing in MSCs from every source, with BM MSCs secreting the most CCL5 and CXCL12 and Is MSCs secreting the most CXCL10. This increase in the secretion of CCL5, CXCL10 and CXCL12 was associated with increased T cell migration towards MSCs from all sources. However, despite the higher secretion of CCL5 and CXCL12 by BM MSCs and CXCL10 by Is MSCs, Ad MSCs recruited the most $CD8^+$ and $CD4^+$ T cells upon stimulation, followed by Is MSCs, while BM MSCs did not produce a $CD8^+$ and $CD4^+$ T cell infiltration (Figure 5-8 and Table 5-4).

The recruitment of $CD4^+$ and $CD8^+$ T cells towards MSCs within a clinical setting could be considered detrimental due to the role of $CD4^+$ T cells in activation and proliferation of $CD8^+$ T cells, which have cytotoxic and inflammatory functions.

As previously mentioned, T cell mediated graft rejection starts with the recognition and presentation of antigens by DCs, leading to the stimulation of T cells and to the subsequent secretion of cytotoxic molecules, such as perforin and granzyme-B by $CD8^+$ T cells, as well as pro-inflammatory cytokines, such as IFN-γ and IL–2 by $CD4^+$ and $CD8^+$ T cells to induce an increased immune response. Th1 cells produce IL-2, promoting the proliferation of cytotoxic $CD8^+$ T cells and $CD8^+$ T cells secrete IFN-γ, acting as positive feedback as it promotes Th1 responses. Bishop et al. found high number of $CD8^+$ T cells within the rejected grafts in a murine cardiac allograft model. However, depletion of $CD8^+$ T cells did not resolve rejection as it led to Th1 and Th2 mediated rejection, proving that both $CD4^+$ and $CD8^+$ T cells are involved in graft rejection (Chan et al., 1995). In addition, memory $CD8^+$ T cells need to be depleted to be able to induce tolerance towards the graft. On the contrary, $CD8^+$ regulatory T cell populations are able to suppress the function of effector $CD8^+$ T cells promoting tolerance and avoiding graft rejection in a rat liver transplantation model (Liu et al., 2007). $CD4^+$ regulatory T cells secrete IL-9, which recruits mast cells mediating immune tolerance in a skin graft model (Lu et al., 2006). Moreover, the presence of $CD8^+$ T cells in a BM graft promoted engraftment of the haematopoietic and progenitor cells via a poorly understood mechanism, as $CD8^+$ T cells also increased the risk for acute and chronic GVHD (Martin et al., 1999). Anyhow, recipient cytotoxic cells are essential to initiate MSC-mediated immunosuppression in a murine model of GVHD as MSCs need to be targeted by cytotoxic T cells to undergo apoptosis to be engulfed by macrophages and produce indoleamine 2,3-dioxygenase (IDO) (Galleu et al., 2017). For this reason, we conclude that T cell migration towards MSCs may be beneficial within a clinical setting.

5.7.6 MSCs and their *in vivo* attraction of NK cells

NK cells express CXCR1, CXCR3, CXCR4 and different NK cell subsets also express CCR1, CCR4, CCR5, CCR6, CCR9, CXCR5 and CXCR6; for this reason NK cells are able to interact with a wide range of chemokines including CCL2, CCL5, CCL7, CCL19, CCL21 and CXCL10, CXCL11 and CXCL12 among others (Berahovich et al., 2006). BM, Is and Ad MSCs were tested for transcription of *CCL5*, *CCL19*, *CXCL10* and *CXCL12* and CCL5, CXCL10 and CXCL12 protein levels. CCL5 and CXCL12 were secreted at medium levels under homeostatic conditions, while CXCL10 was

secreted at lower levels. However, their secretion was upregulated upon MSC licensing in MSCs from every source, with BM MSCs secreting the most CCL5 and CXCL12 and Is MSCs secreting the most CXCL10. This increase in the secretion of CCL5, CXCL10 and CXCL12 led to an increase of NK cell infiltration by MSCs from all sources. However, despite the higher secretion of CCL5 and CXCL12 by BM MSCs and CXCL10 by Is MSCs, Ad MSCs recruited the most NK cells upon stimulation, followed by Is MSCs, while BM MSCs did not produce a NK cell infiltration (Figure 5-8 and Table 5-4).

NK cells interact with MHC Class I molecules and promote self-tolerance when a self-antigen is recognised; however, recognition of nonself-antigen leads to activation of NK cells and subsequent cytotoxicity due to the secretion of cytoplasmic granules containing perforins and granzymes (Warren and Smyth, 1999). For this reason, the recruitment of NK cells towards MSCs within a clinical setting could be considered detrimental. NK cell infiltration is considered detrimental in kidney and lung transplants due to the production of granzyme A and B along with IFN-γ, leading to cytotoxic T cell recruitment and GVHD (Tötterman et al., 1989). However, not only the NK cells from the recipient itself can lead to graft rejection, but also the NK cells within the graft as they could recognise the recipient as nonself, leading to graft rejection. To support this, Espinoza et al. showed that NK cell response was ameliorated in recipients in which the graft was HLA matching (Peraldi et al., 2015). On the other side, NK cells are associated with better prognosis in patients with haematological malignancies that have received stem cell transplantation as they promote graft versus leukaemia instead of GVHD (Cooley et al., 2018).

Despite its cytotoxic effector functions, NK cells are also able to secrete cytokines that can modulate the immune response. As an example, a subset of IL-10 secreting NK cells has been described to suppress T cell proliferation and secretion of IFN-γ and other pro-inflammatory cytokines *in vitro* (Deniz et al., 2008). Moreover, there is growing evidence suggesting that NK cells can interact with myeloid cells and lymphocytes supressing alloimmunity and promoting tolerance. NK cells can interact and promote perforin mediated death of donor allogeneic DCs in the lymph nodes; therefore, avoiding the activation of T cells and promoting skin graft survival (Laffont et al., 2008). In addition, Beilke et al.

showed that perforins secreted by NK cells are essential for islet allograft tolerance as perforin competent NK cells were able to restore graft tolerance in perforin-recipient recipients (Beilke et al., 2005). Taking all this into account, we cannot conclude whether NK cell migration towards MSCs would be beneficial or detrimental within a clinical setting.

5.7.7 Conclusions

To summarise and conclude, this chapter aimed to determine the *in vivo* immune cell attraction profile of resting and licensed BM, Is and Ad MSCs to determine the effect of the differential chemokine secretion on the immunoreactivity of MSCs. Under resting conditions MSCs did not produce many chemokines which predicted, if any, a small infiltrate of leukocytes, while MSC licensing led to the production of large quantities of CCL2, CCL5, CXCL1 and CXCL10, which predicted the migration of large amounts of monocytes/ macrophages and neutrophils. Despite Is MSCs secreting the most chemokines, Ad MSCs produced the biggest infiltrate, suggesting that there are more elements involved in the immune attraction profile of MSCs. As an example, MSCs could induce a secondary response in the surrounding tissues resulting in chemokine production. MSCs were not only able to recruit large numbers of neutrophils and macrophages, but also eosinophils, monocytes and DCs and in some cases, $CD4^+$ and $CD8^+$ T cells and NK cells. These cells have been shown to have a dual role promoting inflammation but also graft tolerance, which makes difficult to determine whether immune cell migration towards MSCs would be detrimental or beneficial in a clinical setting.

It is important to bear in mind that MSCs are well known for their immunomodulatory properties, which suggests that MSCs could recruit these leukocytes and use their immunomodulatory properties to exert an anti-inflammatory phenotype. Secretion of IDO, iNOS in mouse, PGE2 and tumour necrosis factor-inducible gene 6 (TSG-6), by IFN-γ mediated licensed MSCs, promoted the switch of monocytes and macrophages towards an anti-inflammatory phenotype (Choi et al., 2011, Chiossone et al., 2016, Németh et al., 2009) and IDO has also been shown to restrain the proliferation, IFN-γ secretion and cytotoxic activity of NK cells (Spaggiari et al., 2008). Licensing of MSCs with TNF-α has been described to induce cyclooxygenase 2 (COX2), which is

essential for the synthesis of PGE2 and promotes M2 macrophage polarization as COX2 inhibition promotes the M1 phenotype (Francois et al., 2012). MSCs are also able to supress the immune response by regulating DCs. Co-culture of MSCs and DCs promotes the secretion of the anti-inflammatory cytokine IL-10 by DCs while decreasing the production of TNF-α by these cells (Aggarwal and Pittenger, 2005). Moreover, MSCs suppress maturation and the antigen presentation potential of DCs, reducing $CD4^+$ T cell proliferation (Aggarwal and Pittenger, 2005). Regarding their role in neutrophils, MSCs enhance their migration and phagocytosis potential while suppressing the production of nitric oxide and hydrogen peroxide by active neutrophils, thereby diminishing their inflammatory potential and neutrophil extracellular trap formation (Joel et al., 2019, Jiang et al., 2016).

Another important aspect relates to the absence of detection of MSCs after administration into the air pouch. Inability to detect MSCs after administration has already been described and understanding the fate of these cells is essential to assess their immunoregulatory potential (Thirlwell, 2018, Galleu et al., 2017). Uptake of the Green CMFDA cell-tracker by macrophages, NK cells and $CD8^+$ T cells suggests an uptake of MSCs. MSCs have been described to not only alter the activation of immune cells via active mechanisms such as the secretion of molecules, but also by passive mechanisms. A murine model of GVHD demonstrated that recipient's cytotoxic cells must target MSCs promoting their perforin-dependent apoptosis in order to initiate immunosuppression. This hypothesis was confirmed when it was shown that after MSC infusion, only patients that had high toxicity responded to the treatment (Galleu et al., 2017). Phagocytosis of MSCs by macrophages increases the production of IL-10 and IDO while decreasing the production of IL-6 (Braza et al., 2016). Phinney et al. also showed that MSCs undergo mitophagy, which is the degradation of mitochondria by autophagy, enabling the macrophages to engulf the mitochondria and the secretion of exosomes containing miRNA that activate NF-κB signalling pathway increasing the production of IL-1ß, PGE2, TNF-α and IL-10 by macrophages (Phinney et al., 2015).

Taking all this into account, we have shown the ability of licensed MSCs to induce leukocyte migration and we have observed interactions between MSCs,

cytotoxic cells and macrophages, which may explain the rapid clearance of infused MSCs within the air pouch. However, we have not been able to explain the mechanism by which the MSCs could benefit from the leukocyte recruitment. For this reason, determining the secretion of immunomodulatory mediators potentially involved in this process by MSCs would provide a better understanding of their role in a clinical setting.

Chapter 6

Profiling of MSC toll-like receptors, complement system and other immunoregulatory and anti-inflammatory molecules expression at transcript and protein level

6 Profiling of MSC toll-like receptors, complement system and other immunoregulatory and anti-inflammatory molecules expression at transcript and protein level

6.1 Introduction and aims

In Chapter 5, we demonstrated that licensing of BM, Is and Ad MSC with 40 ng/mL of TNF-α, IL-1β and IFN-ϒ lead to the recruitment of not only neutrophils and macrophages, but also eosinophils, monocytes and DCs and in some cases $CD4^+$ and $CD8^+$ T cells and NK cells *in vivo*. These immune cells have been shown to have a dual role promoting inflammation but also graft tolerance, which makes it difficult to determine whether MSC-mediated immune cell infiltration would be detrimental or beneficial in a clinical setting. But MSCs are well described for their tissue repairing and immunomodulatory properties. As an example, in the presence of IFN-γ and inflammatory cytokines such as TNF-α, IL-1α or IL-1β, MSCs have been shown to increase the expression of adhesion molecules and the secretion of chemokines and nitric oxide (NO), producing an immune cell infiltrate and enhancing their immunosuppressive activity (Ren et al., 2010, Ren et al., 2008). However, low NO production by MSCs leads to enhanced immune responses by the recruited immune cells promoting inflammation. For this reason, it has been proposed that the immunomodulatory properties of MSCs might depend on the level of inflammation surrounding them as well as their tissue of origin (Li et al., 2012).

Toll-like receptors (TLRs) are essential in the regulation of the cross talk between the innate and adaptive immune response; TLRs can recognise damage-associated molecular pattern molecules (DAMPs) leading to the expression of pro-inflammatory genes involved in the innate immune response and at the same time, TLRs promote the secretion of cytokines involved in the recruitment and maturation of the adaptive immune response. TLRs are expressed on progenitor cells and they have been described to be involved in proliferation, differentiation, self-renewal and immunomodulation (Sallustio et al., 2019). TLR signalling by MSCs has been described to promote the production of both pro- and anti-inflammatory cytokines by MSCs and TLR expression and the subsequent immune response appear to have a strong association with tissue of origin of the

MSCs. The expression of TLR3 and TLR4 has been associated with the immunosuppressive properties of human BM MSCs by suppressing T cell proliferation (Opitz et al., 2009), while other studies show that expression of these TLRs decrease the ability to suppress T cell proliferation (Liotta et al., 2008). TLR4 expression has been linked with the secretion of the pro-inflammatory mediators IL-6 and CXCL8, while TLR3 expression has been associated with the secretion of the anti-inflammatory molecules IL-4, IDO and PGE2 by human MSCs (Shirjang et al., 2017).

The complement system is a component of the innate immune system and it is involved in the amplification of the immune system through synergy in collaboration with TLRs. Interaction of MSCs with the complement system has been described but it is not fully understood. Infusion of MSCs leads to complement activation after their contact with serum (Li and Lin, 2012), but at the same time, licensing of MSCs with TNF-α and IFN–γ leads to the secretion of complement factor H, which inhibits the activation of the complement system and its subsequent inflammation (Tu et al., 2010a). Moreover, BM MSCs can secrete C3 protein, inhibiting immunoglobulin production by B cells (Lee et al., 2014).

The overall aim of this thesis was to gain understanding of the *in vivo* behaviour of MSCs by studying which immune cells MSCs attract and to gain understanding of the anti-inflammatory and immunomodulatory effects of MSCs on recruited immune cells. Thus, the expression of TLRs, the complement system and several immunomodulatory and anti-inflammatory genes were assessed at a transcriptional level, including IL-6, IL-10, TSG-6, iNOS, COX2, hepatocyte growth factor (HGF), MMP9 and granulocyte-macrophage colony-stimulating factor (GMCSF) among others. All in all, the aims of this chapter were to i) characterise the transcriptional levels of TLRs, complement system and other relevant immunomodulatory molecules in murine MSCs, ii) identify the effect of MSC tissue source of origin on the expression of these molecules and iii) study how MSC licensing alters the expression of these molecules.

Results

Throughout this results section, the transcription and expression profile of toll-like receptors, complement system and other immunoregulatory and anti-inflammatory molecules are described in detail in each section under resting and inflammatory conditions. Transcriptional profiling enabled the identification of the molecules that had undergone the most significant variations at transcriptional level upon stimulation. The secretion profile of those molecules by MSCs from the three sources was then analysed under resting and stimulatory conditions.

6.2 Analysis of the effect of a single inflammatory stimulus on the transcription of toll-like receptors, complement system and other immunoregulatory and anti-inflammatory molecules

To understand the mechanisms involved in MSC-mediated immunomodulation and tissue repair, it was important to study the expression of the TLRs, the complement system and other immunomodulatory genes by MSCs. To do this, TLRs, the complement system and a selection of the most studied immunomodulatory molecules were chosen, and their transcript analysis was carried out. BM, Is and Ad MSCs were transcriptionally assessed under resting and inflammatory conditions (40 ng/mL of TNF-α, IL-1ß and IFN-ϒ), to determine the effect of tissue source and/or inflammatory conditions in the transcriptional profile of these molecules my MSCs.

As explained in Chapter 4, transcriptional data were normalised to the house keeping gene *beta-2 microglobulin* (*B2M*) to control for variations in RNA quality and quantity. As this study did not have a reference sample, data were represented as $2^{(-\Delta CT)}$, which enables the visualization of expression levels of specific genes normalised to *B2M* for each sample. Due to the nature of normalisation, genes that generated a CT of 35 or above resulted in $2^{(-\Delta CT)}$ less than, or equivalent to ~0.0001. Genes with $2^{(-\Delta CT)}$ values similar to ~0.0001 are marked with a red box on the following graphs and are likely not transcribed at meaningful levels by MSCs (Thirlwell, 2018).

6.2.1 Transcription of toll-like receptors under homeostatic and inflammatory conditions

Under resting conditions, BM, Is and Ad MSCs transcribed very few, if any, TLRs (Figure 6-1). No clear pattern of transcriptional regulation was observed upon MSC licensing and the outcome of licensing was not affected by tissue of origin of MSCs. The expression of *TLR1* (A), *TLR4* (D), *TLR6* (F) and *TRL7* (G) was downregulated after 24-hour cytokine-mediated stimulation in MSCs isolated from all sources, *TLR2* (B) expression was upregulated in BM, Is and Ad MSCs after 24–hour cytokine-mediated stimulation, while cytokine-mediated stimulation produced no effect on the expression of *TLR3* (C), *TLR5* (E), *TLR8* (H), *TLR9* (I), *TLR11* (J), *TLR12* (K) and *TLR13*. Fold transcript changes upon cytokine-mediated licensing of MSCs are detailed in Table 6-1.

BM MSCs expressed the highest levels of *TLR1* (A) under resting conditions and inflammatory stimulation resulted in *TLR1* downregulation in MSCs from every source, with BM and Ad MSCs showing the biggest fold change. BM MSCs expressed the highest levels of *TLR2* (B) under resting and stimulatory conditions; *TLR2* was the only TLR that was downregulated after cytokine-mediated licensing in MSCs from the three sources. BM MSCs expressed the highest levels of *TLR3* (C), *TLR5* (E) and *TLR12* (K) under resting conditions and inflammatory stimulation did not significantly alter the expression of these receptors. BM MSCs expressed the highest levels of *TLR4* (D) and *TLR6* (F) under resting conditions and inflammatory stimulation resulted in reduction of these transcript levels in MSCs from every source, with BM MSCs showing the biggest fold change. Is MSCs expressed the highest levels of *TLR7* (G) under resting conditions and inflammatory stimulation resulted in downregulation in MSCs from every source, but this downregulation was statistically significant only in Is MSCs. *TLR8* (H), *TLR9* (I), *TLR11* (J) and *TLR13* (L) were expressed at very low levels by MSCs from all sources and 24-hour cytokine-mediated stimulation produced no alteration in the expression of these TLRs.

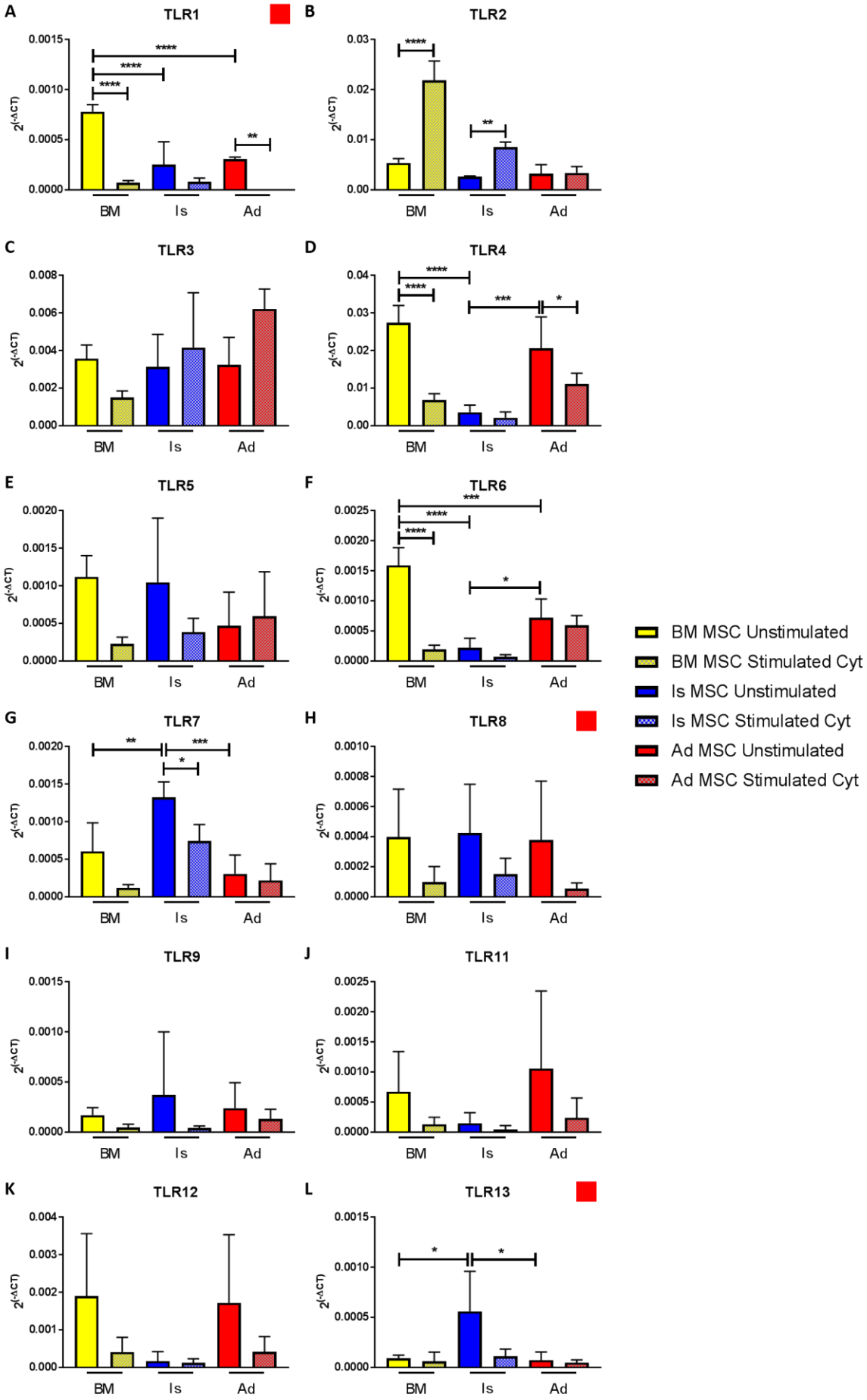


Figure 6-1. Inflammation and MSC tissue origin impacts TLR transcript levels in MSCs.

MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with a cocktail of cytokines, 40 ng/ mL of IFN-γ, TNF-α and IL-1β, for 24 hours. Unstimulated cells were left growing in MSC culture medium as a control. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate TLR transcripts in BM, Is and Ad MSCs under homeostatic and inflammatory conditions. Each bar represents an n of 3 independent experiments and is graphed as mean ± SEM. Data are normalised to the housekeeping gene *B2M* and expressed as $2^{(-\Delta CT)}$. Appropriate statistical analysis was performed and includes Students paired T test between one MSC tissue source (Resting vs Inflammatory Conditions) and One Way ANOVA with Tukey's multiple comparisons post-test to compare all MSC sources. Statistically significant differences are marked with the appropriate number of asterisks. p = 0.05 was considered the limit for statistical significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Table 6-1. Fold change in TLR transcript levels of cytokine-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.
Following the experimental set up explained in Figure 6-1, fold change in transcript levels of TLR is represented as mean of fold change ± standard deviation. Statistically significant differences are marked with a colour code, where $p < 0.05$ is represented by green, $p < 0.01$ is represented by orange, $p < 0.001$ is represented by blue and $p < 0.0001$ is represented by red.

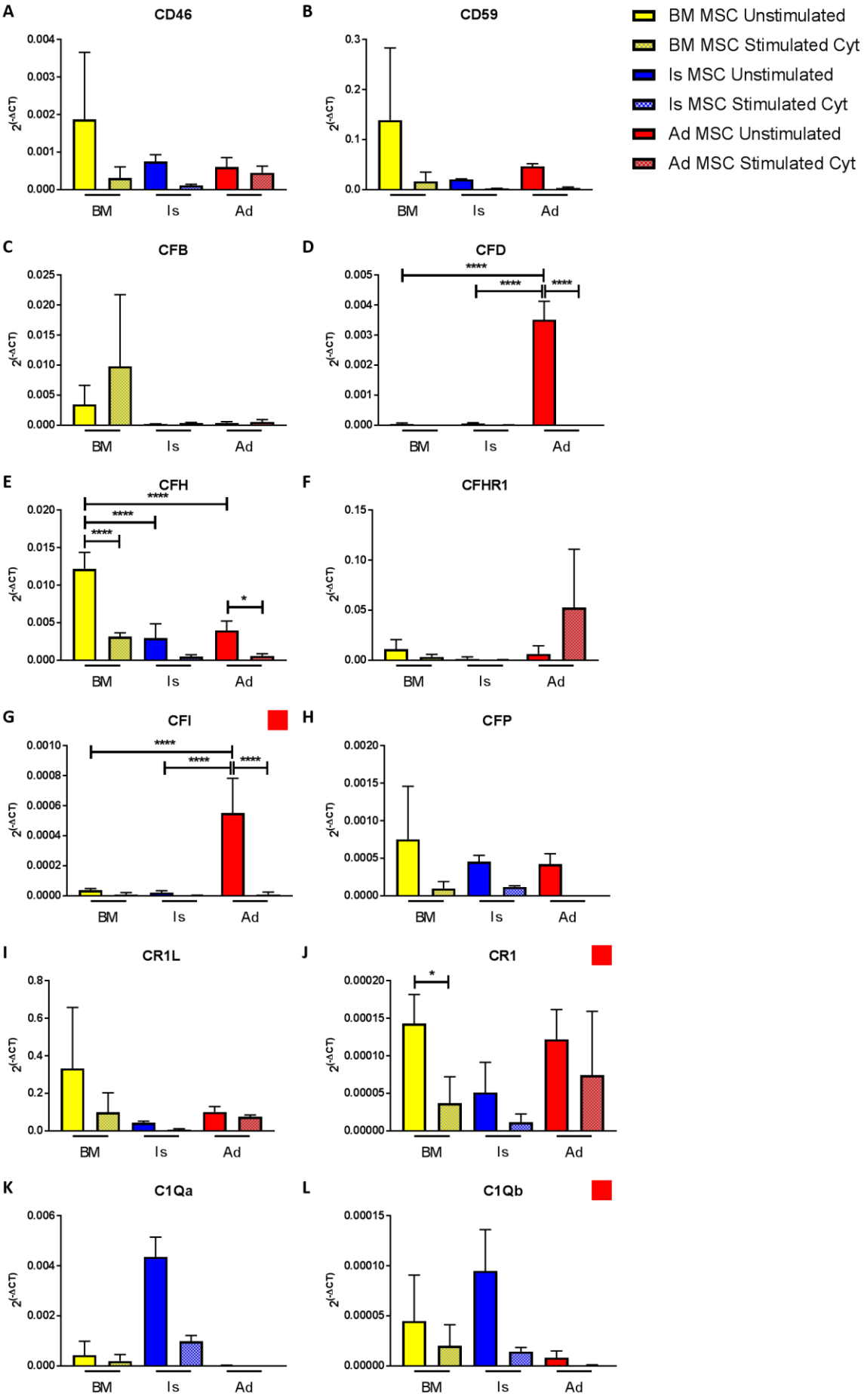
Gene	BM MSCs	Is MSCs	Ad MSCs
TLR1	**0.09 ± 0.02**	0.44 ± 0.16	**0.01 ± 0.00**
TLR2	**4.10 ± 0.66**	**3.28 ± 0.44**	1.14 ± 0.27
TLR3	0.43 ± 0.10	1.28 ± 0.34	2.18 ± 0.81
TLR4	**0.25 ± 0.06**	0.53 ± 0.18	**0.58 ± 0.16**
TLR5	0.21 ± 0.06	0.62 ± 0.38	3.20 ± 4.10
TLR6	**0.12 ± 0.03**	0.35 ± 0.06	0.99 ± 0.44
TLR7	0.21 ± 0.04	0.57 ± 0.17	0.52 ± 0.27
TLR8	0.21 ± 0.06	0.37 ± 0.04	0.37 ± 0.50
TLR9	0.23 ± 0.08	0.49 ± 0.36	1.04 ± 1.11
TLR11	0.22 ± 0.08	0.44 ± 0.28	1443.42 ± 1440.99
TLR12	0.21 ± 0.05	2.20 ± 1.39	0.23 ± 0.11
TLR13	0.51 ± 0.55	0.22 ± 0.00	0.78 ± 0.27

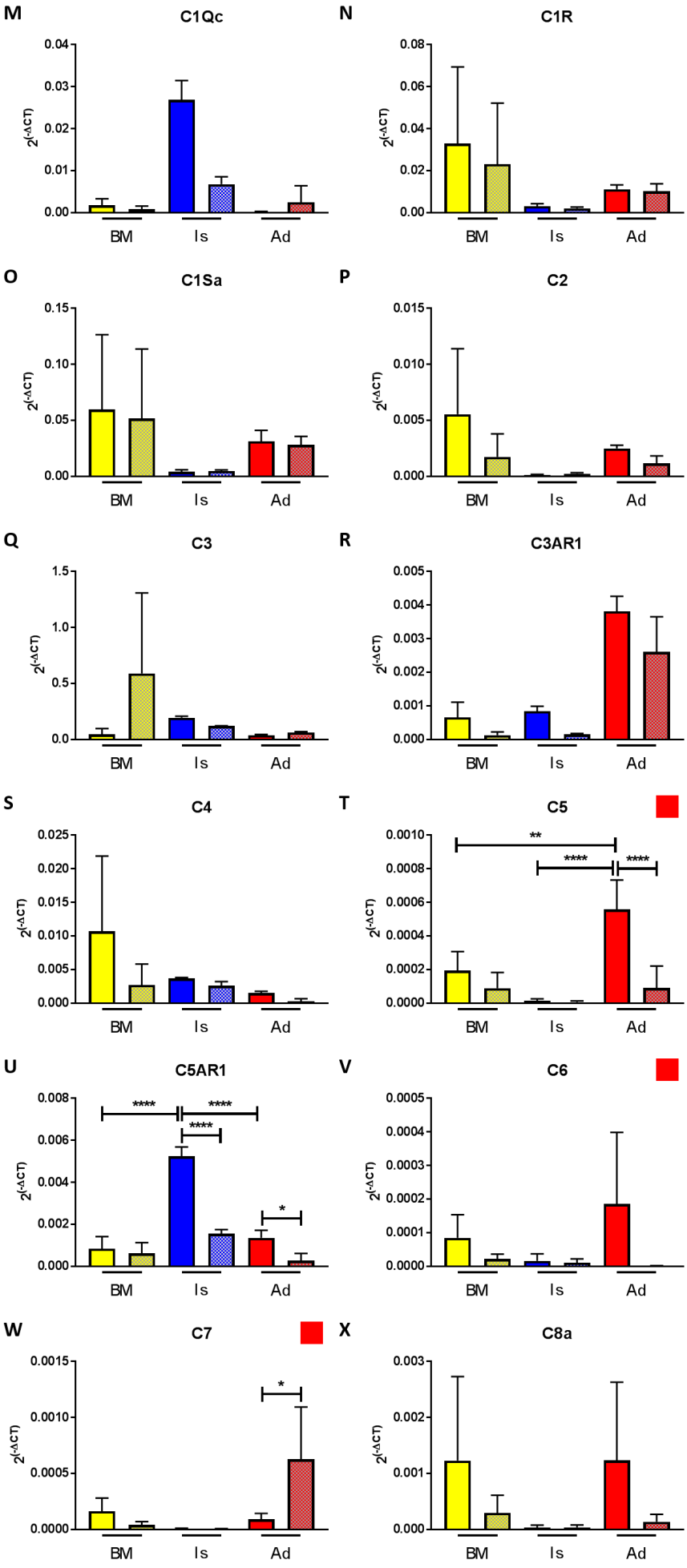
6.2.2 Transcription of the complement system under homeostatic and inflammatory conditions

Under resting conditions, BM, Is and Ad MSCs transcribed very few, if any, elements of the complement system with the exception of *CFH* (Figure 6-2, E), which was transcribed at higher levels by BM MSCs, and *C1Qc*, which was expressed at higher levels by Is MSCs (Figure 6-2, M). A pattern of transcriptional downregulation was observed in *CFD* (D), *CFH* (E), *CFI* (G), *CR1* (J), *C5* (T) and *C5AR1* (U) after 24-hour cytokine-mediated stimulation while cytokine-mediated stimulation did not produce a statistically significant effect in the expression of *CD46* (A), *CD59* (B), *CFB* (C), *CFHR1* (F), *CFP* (H), *CR1L* (I), *C1Qa* (K), *C1Qb* (L), *C1Qc* (M), *C1R* (N), *C1Sa* (O), *C2* (P), *C3* (Q), *C3AR1* (R), *C4* (S), *C6* (V), *C7* (W),

*C8*a (X), *C8b* (Y) and *C8g* (Z). Fold transcript upon cytokine-mediated licensing of MSCs are detailed in Table 6-2.

CD46 (Figure 6-2, A), CFB (C), *CFHR1* (F), CFP (H), CR1 (J), *C1Qa* (K), *C1Qb* (L), *C1R* (N), *C1Sa* (O), *C2* (P), *C4* (S), *C6* (V), *C8*a (X), *C8b* (Y) and *C8g* (Z) were expressed at very low levels by MSCs from all sources and 24-hour cytokine-mediated stimulation produced no variation in the levels of these elements of the complement system. BM MSCs expressed the highest levels of *CD59* (B) under resting and inflammatory conditions and inflammatory stimulation resulted in downregulation in MSCs from every source, with Is and Ad MSCs showing the biggest fold change. *CFD* and *CFI* were expressed at very low levels by MSCs from all sources but Ad MSCs expressed statistically significant higher levels of *CFD* (D) and *CFI* transcripts (G). BM MSCs expressed the highest levels of *CFH* (E) under resting and stimulatory conditions and inflammatory stimulation resulted in downregulation in MSCs from every source. BM MSCs expressed the highest levels of *CR1L* (I) under resting and inflammatory conditions and inflammatory stimulation resulted in downregulation in MSCs from every source. Is MSCs expressed the highest levels of *C1Qc* (M) and *C5AR1* (U) under resting and inflammatory conditions and inflammatory stimulation resulted in downregulation of these transcript levels in MSCs from every source except Ad MSCs, where cytokine-mediated stimulation increased the expression of *C1Qc*. Is MSCs expressed the highest transcript levels of *C3* (Q) under resting conditions but the effect of cytokine-mediated stimulation depended on the tissue of origin of MSCs; BM and Ad MSCs increased their *C3* transcript levels after stimulation while Is MSCs decreased their transcript levels. Ad MSCs expressed the highest levels of *C3AR1* (R) and *C5* (T) under resting and inflammatory conditions and inflammatory stimulation resulted in downregulation in MSCs from every source. Under resting conditions *C7* (W) was expressed at very low levels but BM MSCs expressed the highest levels; inflammatory stimulation led to a reduction of *C7* transcript levels in BM MSCs while producing an upregulation of transcript levels in Is and Ad MSCs.


A
CD46
B
CD59
C
CFB
D
CFD
E
CFH
F
CFHR1
G
CFI
H
CFP
I
CR1L
J
CR1
K
C1Qa
L
C1Qb
2^(-ΔCT)
BM
Is
Ad
****
*
BM MSC Unstimulated
BM MSC Stimulated Cyt
Is MSC Unstimulated
Is MSC Stimulated Cyt
Ad MSC Unstimulated
Ad MSC Stimulated Cyt


M
C1Qc
N
C1R
O
C1Sa
P
C2
Q
C3
R
C3AR1
S
C4
T
C5
U
C5AR1
V
C6
W
C7
X
C8a
2^(-ΔCT)
BM
Is
Ad
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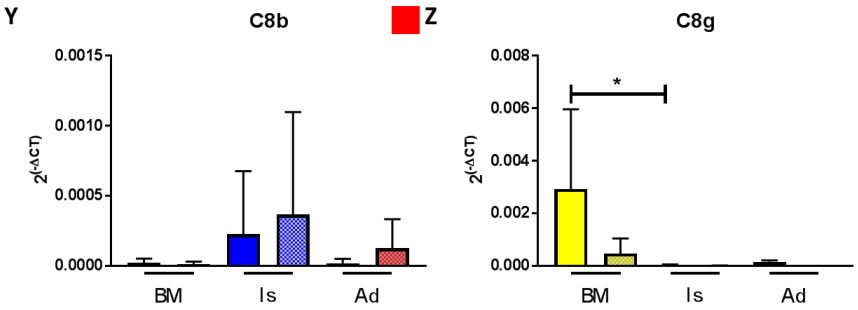


Figure 6-2. Inflammation and MSC tissue origin impacts complement system molecules transcript levels in MSCs.
MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with a cocktail of cytokines, 40 ng/ mL of IFN-Ɣ, TNF-α and IL-1β, for 24 hours. Unstimulated cells were left growing in MSC culture medium as a control. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate complement system transcripts in BM, Is and Ad MSCs under homeostatic and inflammatory conditions. Each bar represents an n of 3 independent experiments and is graphed as mean ± SEM. Data are normalised to the housekeeping gene *B2M* and expressed as $2^{(-\Delta CT)}$. Appropriate statistical analysis was performed and includes Students paired T test between one MSC tissue source (Resting vs Inflammatory Conditions) and One Way ANOVA with Tukey's multiple comparisons post-test to compare all MSC sources. Statistically significant differences are marked with the appropriate number of asterisks. p = 0.05 was considered the limit for statistical significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Table 6-2. Fold change in the complement system molecules transcript levels of cytokine-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.
Following the experimental set up explained in Figure 6-2, fold change in transcript levels of the complement system molecules is represented as mean of fold change ± standard deviation. Statistically significant differences are marked with a colour code, where $p < 0.05$ is represented by green, $p < 0.01$ is represented by orange, $p < 0.001$ is represented by blue and $p < 0.0001$ is represented by red.

Gene	BM MSCs	Is MSCs	Ad MSCs
CD46	0.24 ± 0.20	0.16 ± 0.07	0.82 ± 0.30
CD59	0.13 ± 0.06	0.10 ± 0.02	0.07 ± 0.03
CFB	2.62 ± 1.29	1.99 ± 0.66	1.31 ± 0.35
CFD	0.05 ± 0.04	0.14 ± 0.04	**0.00 ± 0.00**
CFH	**0.27 ± 0.09**	0.16 ± 0.01	**0.14 ± 0.03**
CFHR1	0.46 ± 0.27	0.27 ± 0.21	10.31 ± 4.12
CFI	0.30 ± 0.38	0.19 ± 0.12	**0.04 ± 0.07**
CFP	0.13 ± 0.04	0.27 ± 0.07	0.00 ± 0.00
CR1L	0.32 ± 0.15	0.14 ± 0.08	0.78 ± 0.17
CR1	**0.23 ± 0.15**	0.21 ± 0.20	0.78 ± 0.92
C1Qa	0.43 ± 0.07	0.23 ± 0.03	0.04 ± 0.02
C1Qb	0.63 ± 0.43	0.17 ± 0.00	0.13 ± 0.13
C1Qc	0.48 ± 0.17	0.25 ± 0.05	12.36 ± 10.94
C1R	0.72 ± 0.34	0.70 ± 0.12	0.89 ± 0.14
C1Sa	0.91 ± 0.44	1.33 ± 0.35	0.93 ± 0.20
C2	0.33 ± 0.17	1.99 ± 0.60	0.46 ± 0.23
C3	12.97 ± 5.57	0.62 ± 0.05	1.68 ± 0.38
C3AR1	0.19 ± 0.07	0.18 ± 0.05	0.67 ± 0.20

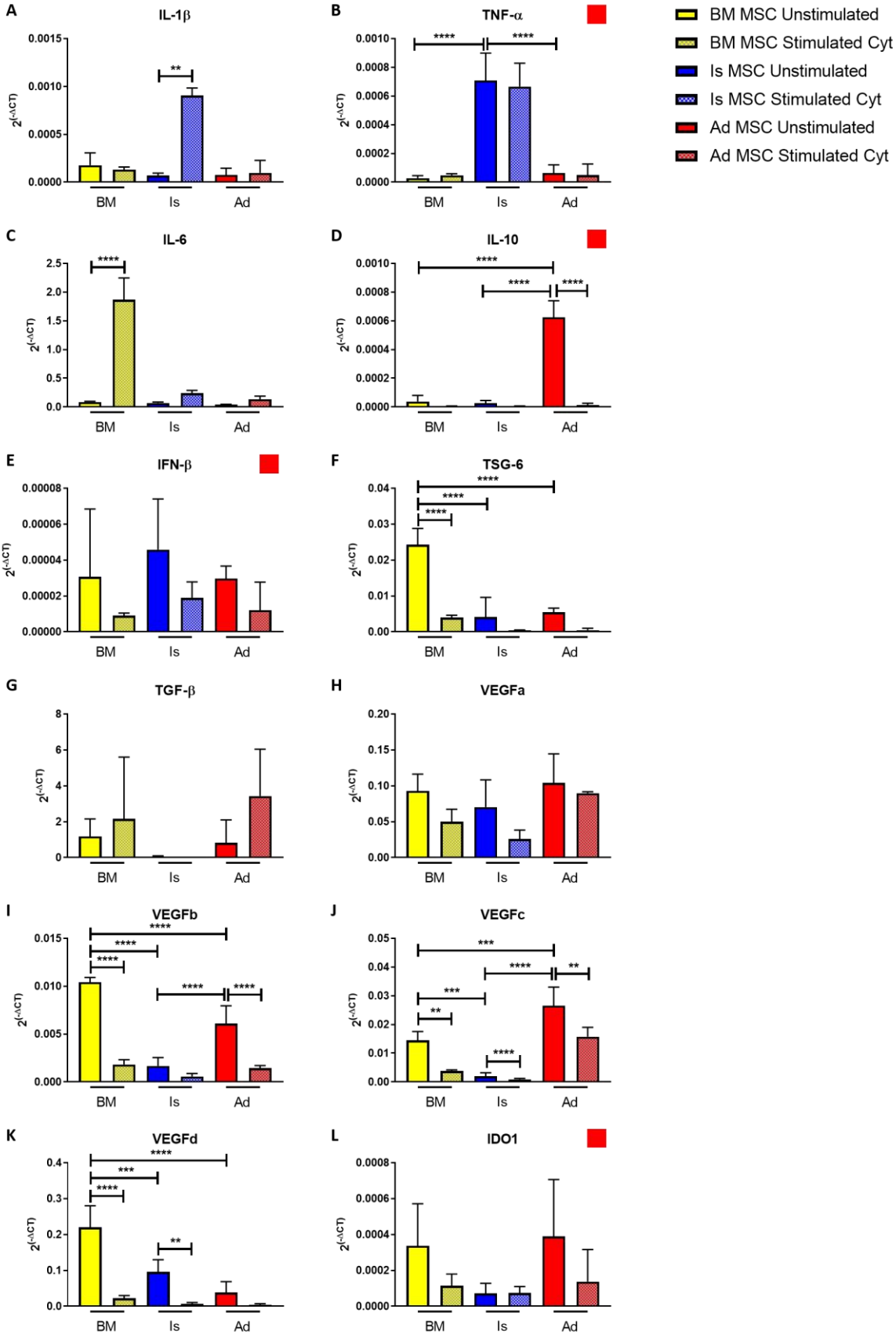
Gene	BM MSCs	Is MSCs	Ad MSCs
C4	0.26 ± 0.14	0.70 ± 0.15	0.18 ± 0.28
C5	0.36 ± 0.20	1.20 ± 1.92	**0.15 ± 0.20**
C5AR1	0.80 ± 0.40	**0.30 ± 0.04**	**0.17 ± 0.16**
C6	0.32 ± 0.25	0.89 ± 0.34	0.04 ± 0.05
C7	0.26 ± 0.15	8.73 ± 10.12	**6.81 ± 3.71**
C8a	0.24 ± 0.19	1.09 ± 0.00	0.08 ± 0.04
C8b	0.93 ± 0.94	0.44 ± 0.69	9.68 ± 6.53
C8g	0.16 ± 0.07	0.20 ± 0.07	0.01 ± 0.02

6.2.3 Transcription of other immunoregulatory and anti-inflammatory molecules under homeostatic and inflammatory conditions

Under resting conditions BM, Is and Ad MSCs transcribed very little *IL-1β* (Figure 6-3, A), *TNF-α* (B), *IL-10* (D), *IFN-β* (E), *IDO 1* (L), *IDO 2*, *MMP9* (S), *ITGAX* (U) and *ITGB2* (V) while *TGF-β* (G), *COX2* (O), *HGF* (Q) and *CD142* (R) were transcribed at high levels. The effect of inflammatory licensing was gene, and tissue of origin, dependent. Fold changes in transcript levels upon cytokine-mediated licensing of MSCs are detailed in Table 6-3.

IFN-β (E), *IDO 1* (L), *IDO 2*, *CD274* (P), *MMP9* (S) and *ITGAX* (U) were expressed at very low levels by MSCs from all sources and 24-hour cytokine-mediated stimulation produced no variation in the transcript levels of these genes. BM MSCs expressed the highest levels of *IL-1β* (A) under resting conditions and inflammatory stimulation led to increased levels of *IL-1β* transcription only in Is MSCs. *TNF-α* (B) was expressed at very low levels in MSCs from every source and 24-hour cytokine-mediated stimulation led to no effect but Is MSCs had statistically significant higher transcript levels than BM and Ad MSCs. BM MSCs expressed the highest levels of *IL-6* (C) under resting and stimulatory conditions and inflammatory stimulation led to an upregulation of *IL-6* in MSCs from every source, especially in BM MSCs where it produced 21–times more *IL-6* transcript. *IL-10* (D) was expressed at very low levels by MSCs from all sources but Ad MSCs expression was higher in a statistically significant manner and cytokine-mediated stimulation led to a downregulation of *IL-10* transcript levels in MSCs from all sources. BM MSCs expressed the highest levels of *TSG-6* (F), *VEGFb* (I), *VEGFd* (K), *HGF* (Q) and *CD142* (R) under resting and inflammatory conditions and 24-

hour stimulatory licensing led to the downregulation of these transcripts in MSCs from all sources. Ad MSCs expressed the highest levels of *VEGFc* (J) under resting and inflammatory conditions and stimulatory licensing led to the downregulation of *VEGFc* in MSCs from all sources. BM MSCs expressed the highest levels of *iNOS* (N) under resting conditions; stimulatory licensing led to no effect in BM MSCs while it produced a marked upregulation in Is and Ad MSCs, with Is MSCs expressing the highest transcript levels. Ad MSCs expressed the highest levels of *COX2* (O) under resting and inflammatory conditions and stimulatory licensing led to no effect on *COX2* transcript levels. Ad MSCs expressed the highest levels of *GMCSF* (T) under resting conditions; stimulatory licensing led to an upregulation of *GMCSF* transcript levels in MSCs from all sources, with Is MSCs showing the biggest fold change. Is MSCs expressed the highest levels of *ITGB2* (V) under resting and inflammatory conditions and stimulatory licensing led to no effect in BM MSCs while it produced a statistically significant downregulation in Is and Ad MSCs.


A
IL-1β
B
TNF-α
C
IL-6
D
IL-10
E
IFN-β
F
TSG-6
G
TGF-β
H
VEGFa
I
VEGFb
J
VEGFc
K
VEGFd
L
IDO1
BM
Is
Ad
2^(-ΔCT)
BM MSC Unstimulated
BM MSC Stimulated Cyt
Is MSC Unstimulated
Is MSC Stimulated Cyt
Ad MSC Unstimulated
Ad MSC Stimulated Cyt

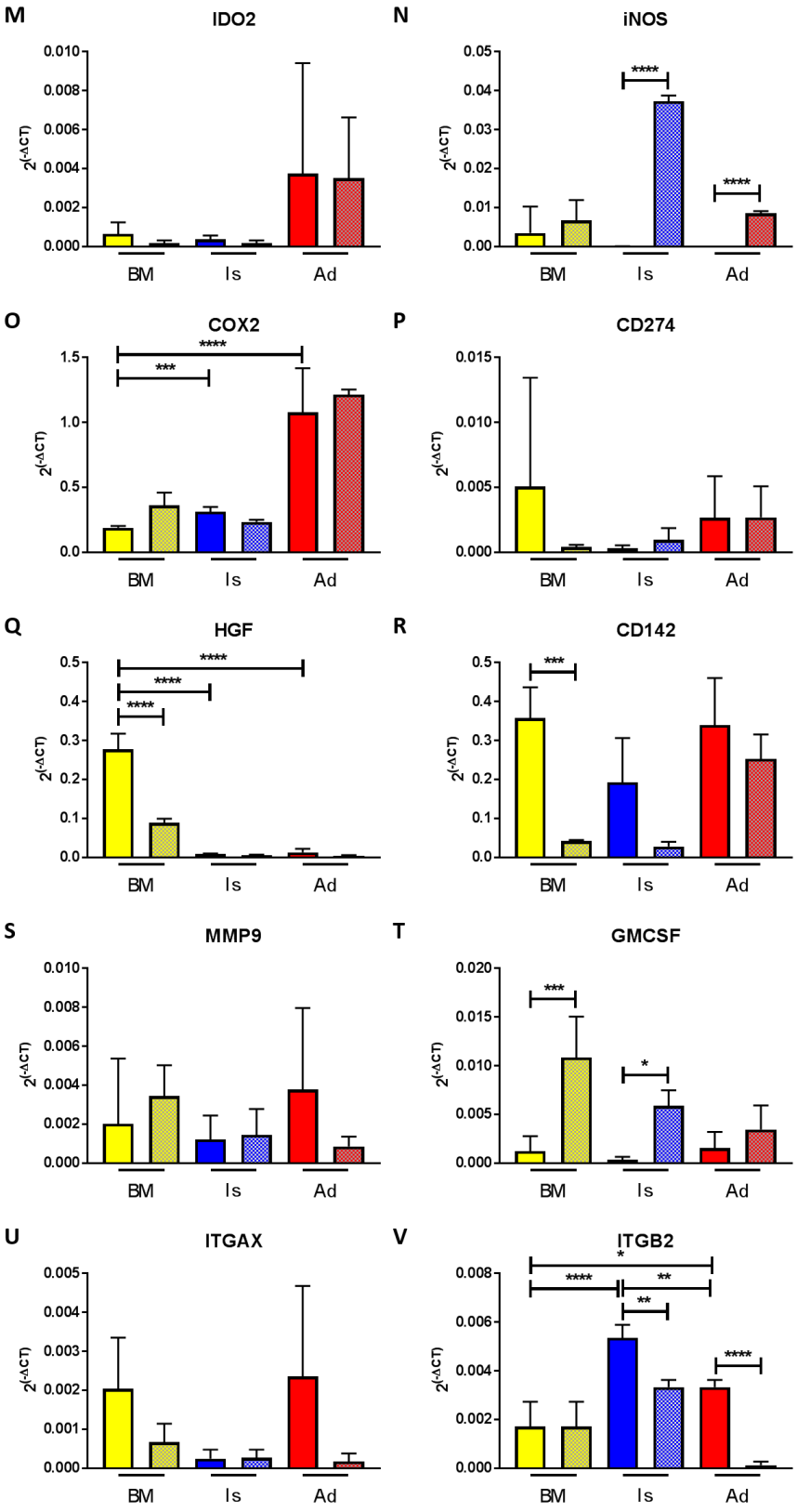


Figure 6-3. Inflammation and MSC tissue origin impacts immunomodulatory molecules transcript levels in MSCs.
MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with a cocktail of cytokines, 40 ng/ mL of IFN-Ɣ, TNF-α and IL-1β, for 24 hours. Unstimulated cells were left growing in MSC culture medium as a control. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate immunomodulatory molecule transcripts in BM, Is and Ad MSCs under homeostatic and inflammatory conditions. Each bar represents an n of 3 independent experiments and is graphed as mean ± SEM. Data are normalised to the housekeeping gene *B2M* and expressed as $2^{(-\Delta CT)}$.

Appropriate statistical analysis was performed and includes Students paired T test between one MSC tissue source (Resting vs Inflammatory Conditions) and One Way ANOVA with Tukey's multiple comparisons post-test to compare all MSC sources. Statistically significant differences are marked with the appropriate number of asterisks. p = 0.05 was considered the limit for statistical significance; * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.

Table 6-3. Fold change in immunomodulatory molecules transcript levels of cytokine-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.
Following the experimental set up explained in Figure 6-3, fold change in transcript levels of immunomodulatory molecules represented as mean of fold change ± standard deviation. Statistically significant differences are marked with a colour code, where p < 0.05 is represented by green, p < 0.01 is represented by orange, p < 0.001 is represented by blue and p < 0.0001 is represented by red.

Gene	BM MSCs	Is MSCs	Ad MSCs
IL-1β	1.09 ± 0.62	14.03 ± 3.49	0.95 ± 0.88
TNF-α	2.79 ± 2.06	0.95 ± 0.11	0.42 ± 0.53
IL-6	21.85 ± 4.62	3.56 ± 0.45	3.42 ± 1.25
IL-10	0.27 ± 0.28	0.07 ± 0.07	0.02 ± 0.02
IFN-β	2.73 ± 4.12	0.51 ± 0.28	0.45 ± 0.49
TSG-6	0.17 ± 0.03	0.26 ± 0.15	0.11 ± 0.13
TGF-β	1.66 ± 1.62	0.12 ± 0.14	65.03 ± 93.49
VEGFa	0.53 ± 0.04	0.38 ± 0.04	0.96 ± 0.31
VEGFb	0.17 ± 0.04	0.35 ± 0.06	0.24 ± 0.04
VEGFc	0.27 ± 0.03	0.51 ± 0.14	0.60 ± 0.03
VEGFd	0.10 ± 0.03	0.06 ± 0.02	0.12 ± 0.01
IDO1	0.44 ± 0.28	1.49 ± 0.00	0.29 ± 0.20
IDO2	0.40 ± 0.21	0.52 ± 0.28	2.97 ± 2.02
iNOS	174.63 ± 159.27	727.41 ± 163.85	4528.28 ± 6706.77
COX2	1.92 ± 0.53	0.76 ± 0.10	1.22 ± 0.36
CD274	0.55 ± 0.42	6.08 ± 5.42	1.65 ± 0.64
HGF	0.33 ± 0.06	0.70 ± 0.22	0.21 ± 0.09
CD142	0.12 ± 0.03	0.16 ± 0.03	0.77 ± 0.10
MMP9	21.85 ± 23.09	3.21 ± 2.39	1.73 ± 1.94
GMCSF	16.71 ± 8.28	71.23 ± 63.81	3.98 ± 2.62
ITGAX	0.33 ± 0.24	1.23 ± 0.68	0.09 ± 0.04
ITGB2	1.06 ± 0.95	0.62 ± 0.04	0.04 ± 0.04

6.3 Analysis of the effect of a double inflammatory stimulus over time on the transcription of the complement system and other immunoregulatory and anti-inflammatory molecules

Several reports in the literature indicate that the immunomodulatory activities of MSCs are not spontaneous, that priming by inflammatory cytokines is essential

for MSC-mediated immunosuppression, irrespective of the species of origin (Ren et al., 2009). Within a clinical setting, MSCs are usually infused within an inflammatory environment, which provides the required licensing for increasing the immunomodulatory properties of MSCs. When the anti-inflammatory properties of MSCs are studied in vitro, this licensing is mimicked in an *in vitro* culture with the addition of proinflammatory cytokines; in this specific case with 40 ng/ mL of IFN-ϒ, TNF-α and IL-1ß.

As described in the previous section, pro-inflammatory cytokine-mediated MSC licensing produced a transcriptional regulation of genes involved in the regulation of the immune response and immunomodulatory mediators, which could explain why MSCs are more immunosuppressive upon activation. Within clinical settings, it has been reported that pre-treatment of MSCs with inflammatory cytokines prior to administration within animal models of inflammatory diseases boosts the therapeutic effect of MSCs (Duijvestein et al., 2011, Noronha et al., 2019). For this reason, we wondered if pre-licensing the MSCs prior to exposing them to an inflammatory environment *in vitro* would lead to even bigger variations in chemokine transcript levels and therefore, in enhanced therapeutic potential. Following the experimental set up explained in Chapter 4, cells were pre-licensed for 48 hours, after which cells were washed twice with PBS and stimulated again for another 24 hours. The first stimulation primes the MSCs while the second stimulation would mimic the inflammatory environment MSCs would face when infused into a patient with an inflammatory disorder. Figure 6-4 illustrates the time points at which supplemented medium was added. Culture medium was removed at the time cells were harvested and was kept for experimental procedures.

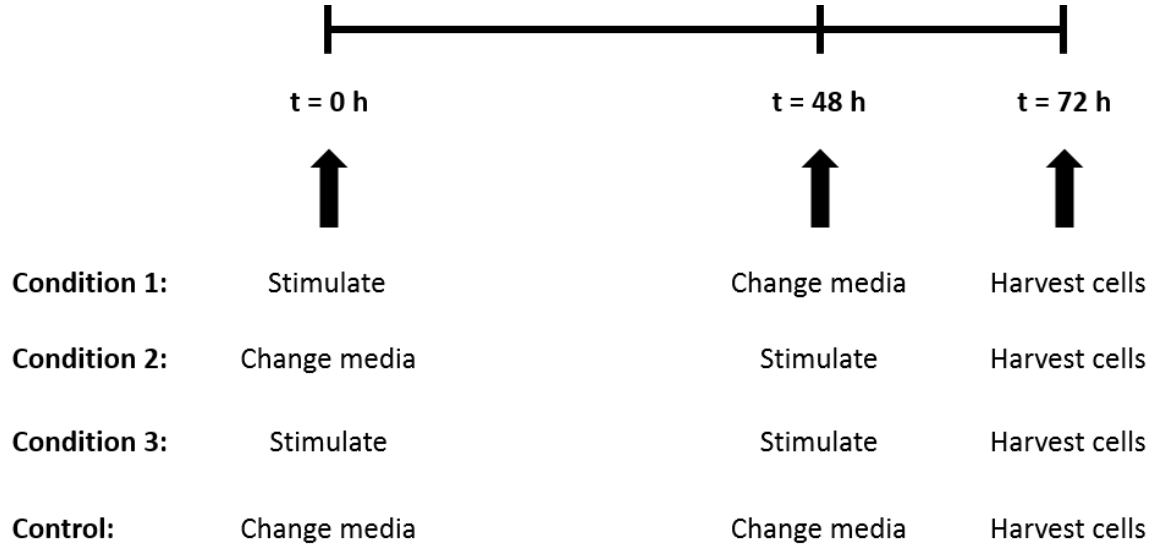


Figure 6-4. Diagrammatic illustration of the time course of the MSC licensing.

Two different control conditions were used. In the first one, cells were stimulated for 48 hours, after which cells were washed twice with PBS and fresh culture medium was added; cells were harvested 24 hours later. In the second condition, cells were washed twice with PBS, the culture medium was replaced with fresh medium and the cells were left to grow for 48 hours. Cells were then washed twice with PBS, the culture medium was replaced with supplemented medium and the cells were harvested 24 hours later. There is extensive literature about how cytokine-mediated licensing enhances the potential therapeutic efficacy of MSCs however, little is known about the role of TLR ligand-mediated activation on the secretion of chemotactic cytokines by MSCs. For this reason, 100 ng/mL lipopolysaccharide *(*LPS), 100 ng/mL lipoteichoic acid *(*LTA*)* and 4 mg/ ml polyinosinic-polycytidylic acid (poly I:C), as well as the previously described cytokine cocktail, was used for MSC licensing.

According to the results described in Section 6.2, the genes that regulated their transcript levels in a statistically significant manner upon stimulation were selected and their transcriptional levels were assessed after licensing with different inflammatory molecules and different inflammatory conditions.

It is important to bear in mind that in this section, the influence of four different inflammatory agents (cytokine cocktail with TNF-α, IL-1ß and IFN-ϒ, LPS, LTA and Poly I:C) at four different time points (control, 72 hours, 24 hours and double stimulation) in 14 different genes was studied. This study was

performed in MSCs isolated from three different sources (BM, Is and Ad MSCs), which gave us a total of 546 data points per experiment ([4 inflammatory agents × 3 time points + 1 control] × 14 genes × 3 MSC sources).

Among all the licensing molecules tested, the combination of 40 ng/ mL of TNF-α, IL-1ß and IFN-ϒ led to the biggest change in immunomodulatory mediators and among all the conditions tested, harvesting the cells 24 hours after the inflammatory stimulation led to the most significant results. These conditions have already been discussed in Section 6.2, for this reason, due to the large amount of data presented in this chapter, we decided to move the less relevant results to the Appendix. Thus, this result section will only display the fold transcript changes upon licensing of MSCs in tables, while the explanatory text and figures are displayed in Section 8.2.

6.3.1 Transcription of the complement system under homeostatic and inflammatory conditions

As outlined in Section 6.2.2, under resting conditions, BM, Is and Ad MSCs transcribed very little, if any, of the elements of the complement system and only the transcription of *CFH, C1Qc* and *C5AR1* were affected by MSC licensing in a statistically significant manner (Section 8.2). A pattern of transcriptional upregulation was observed in *CFH, C1Qc* and *C5AR1* after cytokine, LPS and Poly I:C-mediated licensing while LTA licensing led to a pattern of transcriptional upregulation; however, these genes were upregulated differentially in MSCs according to their tissue of origin. Fold changes of transcriptional regulation upon licensing of MSCs are detailed in Table 6-4.

Table 6-4. Fold change in complement system molecules transcript levels of cytokine, LPS, LTA or Poly I:C-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.

MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with either a cocktail of cytokines (40 ng/ mL of IFN-ϒ, TNF-α and IL-1β), 100 ng/ mL LPS, 100 ng/ mL LTA or 4 µg/ mL Poly I:C. Unstimulated cells were left growing in MSC culture medium as a control. Three different licensing conditions were tested. In the first one, cells were stimulated for 48 hours, after which cells were washed twice with PBS and fresh culture medium was added; cells were harvested 24 hours later. In the second condition, cells were washed twice with PBS, the culture medium was replaced with fresh one and the cells were left growing for 48 hours. Cells were then washed twice with PBS, the culture medium was replaced with supplemented one and the cells were harvested 24 hours later. In the last condition, cells were stimulated for 48 hours, after which cells were washed twice with PBS and were stimulated again for another 24 hours. Figure 6-4 illustrates the time points at which supplemented medium was added. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate complement system molecules transcripts in BM, Is and Ad MSCs under

resting and inflammatory conditions. Data are normalised to the housekeeping gene *B2M* and expressed as $2^{(-\Delta CT)}$. Fold change in transcript levels is represented as mean of fold change ± standard deviation. One Way ANOVA with Tukey's multiple comparisons post-test was performed to compare all MSC sources and the different conditions. Statistically significant differences are marked with a colour code, where $p < 0.05$ is represented by green, $p < 0.01$ is represented by orange, $p < 0.001$ is represented by blue and $p < 0.0001$ is represented by red.

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
CFH	1	Cyt	0.21 ± 0.07	0.10 ± 0.12	0.69 ± 0.50
		LPS	0.37 ± 0.10	0.32 ± 0.26	0.30 ± 0.04
		LTA	3.40 ± 2.12	46.04 ± 25.45	0.72 ± 0.24
		Poly I:C	0.03 ± 0.01	0.24 ± 0.23	0.46 ± 0.10
	2	Cyt	0.38 ± 0.26	0.03 ± 0.00	0.24 ± 0.04
		LPS	0.22 ± 0.07	0.15 ± 0.11	0.17 ± 0.12
		LTA	0.91 ± 0.34	15.74 ± 7.32	0.18 ± 0.06
		Poly I:C	0.09 ± 0.01	0.08 ± 0.04	0.19 ± 0.03
	3	Cyt	0.26 ± 0.17	0.03 ± 0.02	0.27 ± 0.07
		LPS	0.63 ± 0.37	0.20 ± 0.14	0.43 ± 0.09
		LTA	1.26 ± 0.71	149.39 ± 91.04	1.24 ± 0.35
		Poly I:C	0.03 ± 0.02	0.28 ± 0.24	0.64 ± 0.13
C1Qc	1	Cyt	0.59 ± 0.25	0.15 ± 0.07	0.94 ± 0.23
		LPS	0.92 ± 0.42	0.12 ± 0.07	0.47 ± 0.05
		LTA	2.19 ± 0.86	0.50 ± 0.18	0.63 ± 0.10
		Poly I:C	0.22 ± 0.08	0.21 ± 0.18	0.35 ± 0.05
	2	Cyt	0.52 ± 0.19	0.04 ± 0.02	0.18 ± 0.03
		LPS	0.32 ± 0.04	0.08 ± 0.05	0.27 ± 0.07
		LTA	2.04 ± 1.26	0.40 ± 0.17	0.25 ± 0.05
		Poly I:C	0.40 ± 0.17	0.08 ± 0.03	0.14 ± 0.04
	3	Cyt	0.52 ± 0.26	0.13 ± 0.05	1.04 ± 0.18
		LPS	0.67 ± 0.33	0.21 ± 0.13	0.14 ± 0.02
		LTA	1.59 ± 0.51	2.52 ± 0.75	0.36 ± 0.08
		Poly I:C	0.39 ± 0.35	0.52 ± 0.65	0.07 ± 0.01
C5AR1	1	Cyt	1.59 ± 0.26	0.09 ± 0.06	1.06 ± 0.44
		LPS	3.14 ± 0.44	0.24 ± 0.09	0.74 ± 0.14
		LTA	6.45 ± 0.57	0.40 ± 0.10	0.88 ± 0.16
		Poly I:C	0.89 ± 0.07	0.22 ± 0.12	0.46 ± 0.13
	2	Cyt	2.71 ± 0.51	0.06 ± 0.03	0.81 ± 0.13
		LPS	2.49 ± 0.92	0.22 ± 0.04	0.48 ± 0.06
		LTA	8.17 ± 0.42	0.41 ± 0.06	0.25 ± 0.07
		Poly I:C	1.72 ± 0.70	0.13 ± 0.07	0.34 ± 0.09
	3	Cyt	1.63 ± 0.12	0.08 ± 0.03	0.01 ± 0.01
		LPS	2.87 ± 0.24	0.40 ± 0.17	1.00 ± 0.11
		LTA	8.49 ± 1.17	1.52 ± 0.82	1.44 ± 0.38
		Poly I:C	0.61 ± 0.02	0.22 ± 0.19	0.29 ± 0.08

6.3.2 Transcription of other immunoregulatory and anti-inflammatory molecules under homeostatic and inflammatory conditions

As previously mentioned, under resting conditions BM, Is and Ad MSCs transcribed very little, if any, of the immunoregulatory and anti-inflammatory genes analysed in Section 6.2.3 with the exception of *IL-6*, *VEGFa*, *VEGFd* and *CD142* (Section 8.2). The effect of inflammatory licensing in these genes was tissue of origin and stimulatory agent dependent and fold changes of transcriptional regulation upon licensing of MSCs are detailed in Table 6-5.

Table 6-5. Fold change in immunomodulatory molecules transcript levels of cytokine, LPS, LTA or Poly I:C-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.
MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with either a cocktail of cytokines (40 ng/ mL of IFN-Ɣ, TNF-α and IL-1β), 100 ng/ mL LPS, 100 ng/ mL LTA or 4 μg/ mL Poly I:C. Unstimulated cells were left growing in MSC culture medium as a control. Three different licensing conditions were tested. In the first one, cells were stimulated for 48 hours, after which cells were washed twice with PBS and fresh culture medium was added; cells were harvested 24 hours later. In the second condition, cells were washed twice with PBS, the culture medium was replaced with fresh one and the cells were left growing for 48 hours. Cells were then washed twice with PBS, the culture medium was replaced with supplemented one and the cells were harvested 24 hours later. In the last condition, cells were stimulated for 48 hours, after which cells were washed twice with PBS and were stimulated again for another 24 hours. Figure 6-4 illustrates the time points at which supplemented medium was added. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate immunomodulatory molecule transcripts in BM, Is and Ad MSCs under resting and inflammatory conditions. Data are normalised to the housekeeping gene *B2M* and expressed as $2^{(-\Delta CT)}$. Fold change in transcript levels is represented as mean of fold change ± standard deviation. One Way ANOVA with Tukey's multiple comparisons post-test was performed to compare all MSC sources and the different conditions. Statistically significant differences are marked with a colour code, where $p < 0.05$ is represented by green, $p < 0.01$ is represented by orange, $p < 0.001$ is represented by blue and $p < 0.0001$ is represented by red.

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
IL-6	1	Cyt	1.15 ± 0.90	1.56 ± 0.97	0.11 ± 0.05
		LPS	0.26 ± 0.22	2.35 ± 1.05	0.42 ± 0.06
		LTA	0.17 ± 0.12	1.24 ± 0.62	**0.22 ± 0.02**
		Poly I:C	0.15 ± 0.11	1.92 ± 1.31	**0.23 ± 0.03**
	2	Cyt	**53.97 ± 44.65**	**8.04 ± 4.83**	0.65 ± 0.06
		LPS	2.02 ± 0.64	3.09 ± 1.63	0.89 ± 0.29
		LTA	0.49 ± 0.06	1.38 ± 0.51	1.07 ± 0.13
		Poly I:C	0.78 ± 0.72	1.46 ± 0.36	0.66 ± 0.11
	3	Cyt	**76.21 ± 61.41**	3.41 ± 1.35	**10.74 ± 1.08**
		LPS	1.72 ± 0.72	2.26 ± 1.29	0.51 ± 0.02
		LTA	0.30 ± 0.20	0.88 ± 0.21	0.70 ± 0.18
		Poly I:C	0.86 ± 0.43	1.04 ± 0.79	0.80 ± 0.13
TSG-6	1	Cyt	**0.08 ± 0.03**	0.74 ± 0.14	3.98 ± 2.09
		LPS	**0.13 ± 0.01**	2.86 ± 0.90	0.66 ± 0.21
		LTA	**0.17 ± 0.02**	2.24 ± 0.34	0.54 ± 0.18
		Poly I:C	**0.07 ± 0.03**	1.53 ± 0.40	0.42 ± 0.21

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
	2	Cyt	0.06 ± 0.03	0.62 ± 0.17	0.51 ± 0.28
		LPS	0.23 ± 0.09	2.34 ± 1.30	0.56 ± 0.17
		LTA	0.76 ± 0.36	1.40 ± 0.39	0.31 ± 0.30
		Poly I:C	0.07 ± 0.03	1.09 ± 0.25	0.37 ± 0.21
	3	Cyt	0.13 ± 0.09	0.29 ± 0.19	1.12 ± 0.40
		LPS	0.66 ± 0.35	1.44 ± 0.36	0.51 ± 0.20
		LTA	0.54 ± 0.31	4.18 ± 1.52	1.06 ± 0.53
		Poly I:C	0.08 ± 0.05	0.82 ± 0.58	1.27 ± 0.66
VEGFa	1	Cyt	0.40 ± 0.05	0.22 ± 0.10	2.43 ± 0.51
		LPS	0.54 ± 0.17	0.93 ± 0.19	0.63 ± 0.06
		LTA	1.04 ± 0.14	1.77 ± 0.57	1.16 ± 0.10
		Poly I:C	0.24 ± 0.04	0.46 ± 0.14	0.47 ± 0.05
	2	Cyt	0.76 ± 0.12	0.37 ± 0.27	1.04 ± 0.17
		LPS	0.65 ± 0.19	0.68 ± 0.05	1.01 ± 0.35
		LTA	1.07 ± 0.26	1.14 ± 0.29	0.67 ± 0.05
		Poly I:C	0.41 ± 0.29	1.02 ± 0.31	0.46 ± 0.07
	3	Cyt	0.49 ± 0.04	0.38 ± 0.13	3.17 ± 0.47
		LPS	0.98 ± 0.07	0.73 ± 0.17	0.80 ± 0.08
		LTA	0.92 ± 0.21	1.25 ± 0.17	1.10 ± 0.26
		Poly I:C	0.37 ± 0.02	0.83 ± 0.08	1.19 ± 0.29
VEGFb	1	Cyt	0.35 ± 0.10	0.16 ± 0.06	0.61 ± 0.14
		LPS	3.30 ± 4.00	0.69 ± 0.17	0.34 ± 0.13
		LTA	2.10 ± 0.97	0.71 ± 0.22	0.48 ± 0.18
		Poly I:C	0.19 ± 0.10	0.36 ± 0.21	0.35 ± 0.04
	2	Cyt	0.25 ± 0.11	0.21 ± 0.13	0.66 ± 0.19
		LPS	0.21 ± 0.07	0.38 ± 0.03	0.38 ± 0.11
		LTA	1.21 ± 0.66	0.82 ± 0.07	0.27 ± 0.04
		Poly I:C	0.14 ± 0.04	0.31 ± 0.29	0.34 ± 0.07
	3	Cyt	0.19 ± 0.11	0.09 ± 0.02	4.45 ± 0.52
		LPS	0.71 ± 0.31	0.30 ± 0.12	0.75 ± 0.11
		LTA	1.45 ± 0.76	0.94 ± 0.34	1.10 ± 0.27
		Poly I:C	0.11 ± 0.06	0.28 ± 0.07	0.60 ± 0.10
VEGFc	1	Cyt	0.27 ± 0.15	1.00 ± 0.28	0.29 ± 0.18
		LPS	0.56 ± 0.23	3.07 ± 0.34	0.67 ± 0.13
		LTA	0.67 ± 0.17	2.50 ± 1.14	0.56 ± 0.13
		Poly I:C	0.22 ± 0.06	1.83 ± 0.68	0.47 ± 0.10
	2	Cyt	0.45 ± 0.05	0.96 ± 0.36	1.12 ± 0.07
		LPS	0.63 ± 0.08	1.05 ± 0.26	0.86 ± 0.31
		LTA	0.62 ± 0.22	1.64 ± 0.92	0.38 ± 0.12
		Poly I:C	0.53 ± 0.28	1.60 ± 0.71	0.54 ± 0.11
	3	Cyt	0.20 ± 0.08	0.61 ± 0.21	7.80 ± 1.71
		LPS	0.74 ± 0.46	1.09 ± 0.51	1.05 ± 0.28
		LTA	0.65 ± 0.25	0.91 ± 0.39	1.14 ± 0.36
		Poly I:C	0.38 ± 0.10	0.65 ± 0.19	1.14 ± 0.23
VEGFd	1	Cyt	0.15 ± 0.04	0.05 ± 0.03	1.44 ± 0.35
		LPS	0.27 ± 0.03	0.25 ± 0.10	1.10 ± 0.41

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
		LTA	**0.37 ± 0.07**	**0.42 ± 0.07**	1.30 ± 0.13
		Poly I:C	**0.10 ± 0.03**	**0.11 ± 0.10**	0.66 ± 0.11
	2	Cyt	**0.16 ± 0.07**	**0.01 ± 0.00**	**0.29 ± 0.07**
		LPS	**0.54 ± 0.10**	**0.09 ± 0.03**	**0.20 ± 0.07**
		LTA	1.15 ± 0.37	**0.30 ± 0.07**	**0.31 ± 0.08**
		Poly I:C	**0.12 ± 0.03**	**0.13 ± 0.02**	**0.40 ± 0.07**
	3	Cyt	**0.15 ± 0.08**	**0.00 ± 0.00**	**0.23 ± 0.03**
		LPS	**0.61 ± 0.08**	**0.16 ± 0.11**	**0.38 ± 0.05**
		LTA	**0.72 ± 0.19**	**0.46 ± 0.08**	**2.06 ± 0.41**
		Poly I:C	**0.10 ± 0.04**	**0.09 ± 0.02**	**0.41 ± 0.08**
iNOS	**1**	Cyt	18.43 ± 14.56	4.52 ± 3.84	4.06 ± 3.80
		LPS	5.04 ± 7.56	0.54 ± 0.40	1.99 ± 1.86
		LTA	0.09 ± 0.09	0.23 ± 0.24	1.01 ± 0.93
		Poly I:C	0.57 ± 0.40	0.16 ± 0.15	1.88 ± 1.73
	2	Cyt	191.3 ± 171.7	**170.15 ± 178.95**	49.31 ± 47.41
		LPS	2.25 ± 1.86	0.20 ± 0.21	271.90 ± 239.85
		LTA	0.25 ± 0.22	0.25 ± 0.04	1.04 ± 1.13
		Poly I:C	135.1 ± 222.2	0.14 ± 0.05	22.15 ± 31.04
	3	Cyt	77.75 ± 55.07	83.97 ± 86.59	**40476 ± 37122**
		LPS	4.61 ± 3.70	1.99 ± 1.83	32.53 ± 51.05
		LTA	0.22 ± 0.09	45.06 ± 53.10	28.72 ± 37.91
		Poly I:C	4.38 ± 3.46	0.15 ± 0.16	147.15 ± 153.43
COX2	**1**	Cyt	0.85 ± 0.16	5.46 ± 1.93	**2.45 ± 0.86**
		LPS	1.00 ± 0.46	**29.93 ± 4.88**	0.46 ± 0.05
		LTA	1.39 ± 0.19	11.11 ± 7.88	0.69 ± 0.17
		Poly I:C	0.50 ± 0.07	5.41 ± 1.65	0.37 ± 0.05
	2	Cyt	1.65 ± 0.67	5.16 ± 1.41	**3.34 ± 0.19**
		LPS	1.48 ± 0.67	3.96 ± 2.32	1.52 ± 0.53
		LTA	1.19 ± 0.59	6.46 ± 0.44	0.79 ± 0.06
		Poly I:C	1.31 ± 0.47	6.15 ± 4.01	0.75 ± 0.08
	3	Cyt	0.93 ± 0.09	8.71 ± 3.77	**23.83 ± 2.96**
		LPS	1.57 ± 0.59	7.92 ± 5.36	1.52 ± 0.15
		LTA	0.97 ± 0.35	6.73 ± 2.58	0.89 ± 0.54
		Poly I:C	0.57 ± 0.06	4.33 ± 1.81	1.88 ± 0.27
HGF	**1**	Cyt	**0.09 ± 0.01**	0.17 ± 0.06	**2.48 ± 0.99**
		LPS	**0.17 ± 0.05**	0.87 ± 0.36	1.13 ± 0.25
		LTA	**0.37 ± 0.07**	1.40 ± 1.24	**1.68 ± 0.56**
		Poly I:C	**0.04 ± 0.01**	1.75 ± 0.53	0.87 ± 0.19
	2	Cyt	**0.32 ± 0.24**	0.48 ± 0.28	0.67 ± 0.16
		LPS	**0.39 ± 0.28**	1.68 ± 1.01	1.27 ± 0.74
		LTA	1.06 ± 0.69	**5.03 ± 0.70**	0.61 ± 0.11
		Poly I:C	**0.11 ± 0.05**	0.43 ± 0.08	0.33 ± 0.08
	3	Cyt	**0.22 ± 0.13**	1.24 ± 1.02	**2.20 ± 0.38**
		LPS	0.74 ± 0.56	1.01 ± 0.63	0.52 ± 0.10
		LTA	0.97 ± 0.45	2.12 ± 0.69	0.65 ± 0.24
		Poly I:C	**0.12 ± 0.07**	0.35 ± 0.22	**0.53 ± 0.14**

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
CD142	1	Cyt	0.32 ± 0.03	0.11 ± 0.00	0.93 ± 0.11
		LPS	0.74 ± 0.12	0.47 ± 0.14	**0.42 ± 0.05**
		LTA	0.70 ± 0.12	0.79 ± 0.32	**0.55 ± 0.06**
		Poly I:C	0.27 ± 0.06	0.40 ± 0.18	**0.35 ± 0.03**
	2	Cyt	0.45 ± 0.26	0.18 ± 0.11	**0.53 ± 0.06**
		LPS	0.59 ± 0.17	0.90 ± 0.37	**0.39 ± 0.14**
		LTA	1.19 ± 0.25	0.78 ± 0.27	**0.42 ± 0.04**
		Poly I:C	0.32 ± 0.05	0.18 ± 0.07	**0.49 ± 0.08**
	3	Cyt	0.33 ± 0.20	0.13 ± 0.06	1.48 ± 0.15
		LPS	0.79 ± 0.21	0.27 ± 0.03	**0.50 ± 0.06**
		LTA	0.83 ± 0.25	0.37 ± 0.11	1.44 ± 0.40
		Poly I:C	0.55 ± 0.37	0.53 ± 0.36	0.87 ± 0.13
GMCSF	1	Cyt	1.56 ± 1.30	6.07 ± 8.72	14.53 ± 12.02
		LPS	0.66 ± 0.30	13.17 ± 22.47	1.65 ± 1.05
		LTA	14.41 ± 24.03	10.08 ± 9.00	2.62 ± 1.26
		Poly I:C	145.6 ± 250.5	7.51 ± 11.31	1.07 ± 0.86
	2	Cyt	8.85 ± 8.64	2.75 ± 2.17	1.22 ± 0.60
		LPS	0.74 ± 0.40	1.59 ± 1.77	1.98 ± 2.17
		LTA	1.31 ± 0.84	10.35 ± 16.41	1.31 ± 1.05
		Poly I:C	0.87 ± 0.75	0.78 ± 0.27	0.55 ± 0.28
	3	Cyt	1.26 ± 0.95	8.57 ± 13.07	365.9 ± 300.6
		LPS	1.25 ± 0.67	1.59 ± 1.99	1.53 ± 1.14
		LTA	0.72 ± 0.40	8.85 ± 11.71	**2.35 ± 1.37**
		Poly I:C	0.74 ± 0.40	3.32 ± 5.56	4.37 ± 3.01

6.4 Analysis of the immunoregulatory and anti-inflammatory molecules secretion profile by MSCs under homeostatic and inflammatory conditions

Under resting conditions, BM, Is and Ad MSCs secreted less than 0.6 pg of IL-6 and less than 0.2 pg of VEGF per mg of total protein (Figure 6-5). A pattern of upregulation was observed in IL-6 (A) under every condition in MSCs from the three sources after cytokine-mediated licensing, while the effect mediated by cytokine licensing on VEGF (B) depends on condition and tissue of origin. Fold changes of IL-6 and VEGF secretion upon cytokine-mediated licensing of MSCs are specified in Table 6-6.

MSCs isolated from the three tissues produced very little IL-6 under resting conditions but Ad MSCs secreted the highest levels of IL-6 (BM = 0.09 pg/ mg; Is = 0.23 pg/ mg; Ad = 0.60 pg/ mg). Cytokine-mediated licensing produced an

upregulation of the secretion in all the conditions tested in MSCs of the three tissue sources. After 72 hours of stimulation (Condition 1), IL-6 secretion was upregulated in MSCs from the three tissues but was only statistically significant in Is and Ad MSCs. However, 24 hours of stimulation (Condition 2) produced a statistically significant upregulation of IL-6 secretion in MSCs from the three sources, with the three sources secreting close to 2 pg of IL-6 per mg of total protein. A second cytokine-mediated stimulation 48 hours after the first one (Condition 3) led to the same level of secretion of IL-6 as Condition 1 in MSCs from the three sources. The lower limit of quantification for IL-6 in the Mouse Magnetic Luminex Assay was 29.55 pg/mL and all the analysed samples were above this value.

MSCs isolated from the three tissues produced very little VEGF under resting conditions but Ad MSCs secreted the highest levels of VEGF (BM = 0.07 pg/ mg; Is = 0.19 pg/ mg; Ad = 0.11 pg/ mg). 72 hours of stimulation with the cytokine cocktail (Condition 1) led to no effect in VEGF secretion by MSCs from the three sources. However, 24 hours of stimulation (Condition 2) produced a statistically non-significant upregulation of VEGF secretion by BM MSCs and no effect in Is and Ad MSCs. A second cytokine-mediated stimulation 48 hours after the first one (Condition 3) led to a statistically non-significant upregulation of VEGF secretion by BM MSCs, to a statistically significant downregulation in Is MSCs and to no effect in the secretion of VEGF by Ad MSCs. The lower limit of quantification for VEGF in the Mouse Magnetic Luminex Assay was 4.2 pg/mL and all the analysed samples were above this value.

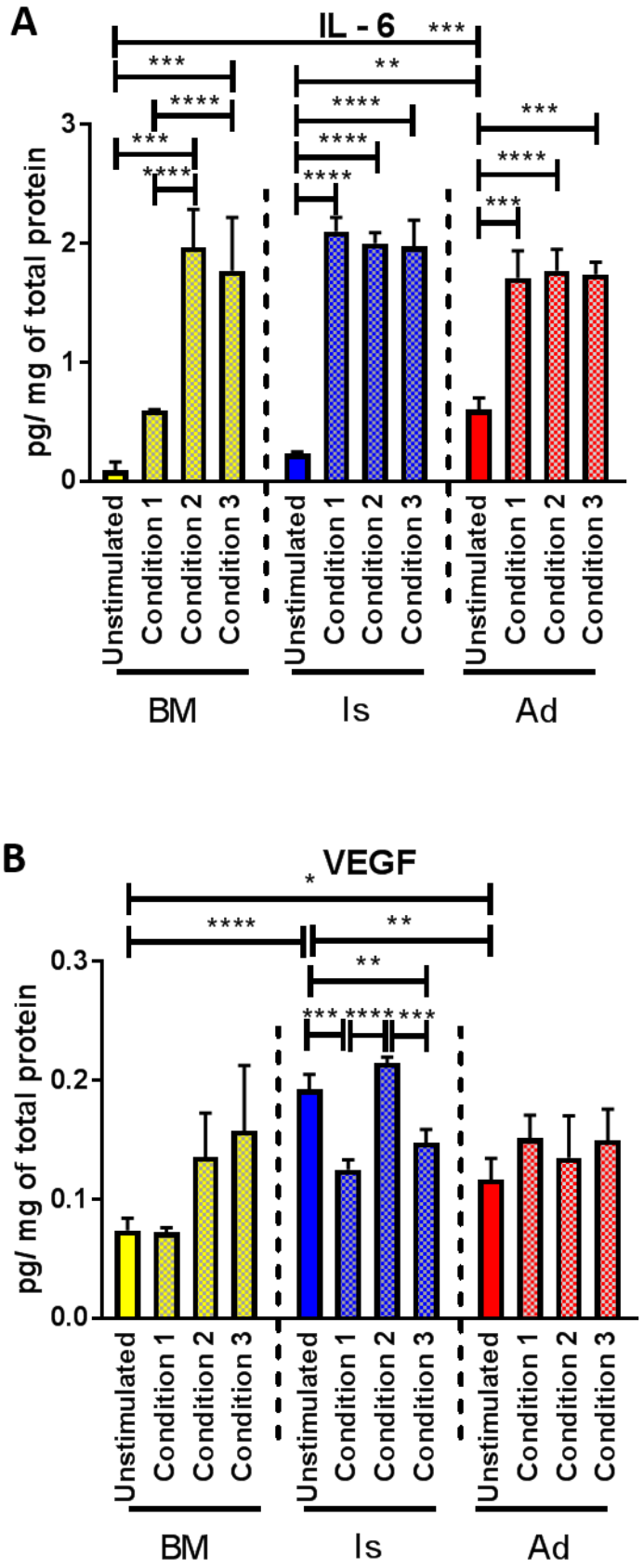


Figure 6-5. Cytokine-mediated stimulation, repetitive stimulus and MSC tissue origin impacts IL-6 and VEGF secretion in MSCs.
MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with a cocktail of cytokines (40 ng/ mL of IFN-ϒ, TNF-α and IL-1β). Unstimulated cells were left growing in MSC culture medium as a control. Three different licensing conditions were tested. In the first one, cells were stimulated for 48 hours, after

which cells were washed twice with PBS and fresh culture medium was added; cells were harvested 24 hours later. In the second condition, cells were washed twice with PBS, the culture medium was replaced with fresh one and the cells were left growing for 48 hours. Cells were then washed twice with PBS, the culture medium was replaced with supplemented one and the cells were harvested 24 hours later. In the last condition, cells were stimulated for 48 hours, after which cells were washed twice with PBS and were stimulated again for another 24 hours. Figure 6-4 illustrates the time points at which supplemented medium was added. Luminex was performed to evaluate protein secretion in BM, Is and Ad MSCs under homeostatic and inflammatory conditions. Each bar represents an n of 3 independent experiments and is graphed as mean ± SEM. Data are normalised total amount of protein in medium and expressed as picograms of protein of interest per mg of total protein. Appropriate statistical analysis was performed and includes Students paired T test between one MSC tissue source (Resting vs Inflammatory Conditions) and One Way ANOVA with Tukey's multiple comparisons post-test to compare all MSC sources. Statistically significant differences are marked with the appropriate number of asterisks. p = 0.05 was considered the limit for statistical significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Table 6-6. Fold change in IL-6 and VEGF secretion of cytokine-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.
Following the experimental set up explained in Figure 6-5, fold change in IL-6 and VEGF protein levels is represented as mean of fold change ± standard deviation. Statistically significant differences are marked with a colour code, where $p < 0.05$ is represented by green, $p < 0.01$ is represented by orange, $p < 0.001$ is represented by blue and $p < 0.0001$ is represented by red.

Protein	Condition	BM MSCs	Is MSCs	Ad MSCs
IL-6	1	15.35 ± 15.43	9.04 ± 0.77	2.93 ± 0.68
	2	54.67 ± 57.57	8.59 ± 0.26	2.99 ± 0.49
	3	51.14 ± 55.55	8.54 ± 1.18	2.91 ± 0.25
VEGF	1	1.00 ± 0.15	0.65 ± 0.06	1.33 ± 0.28
	2	1.90 ± 0.60	1.12 ± 0.07	1.20 ± 0.35
	3	0.31 ± 0.16	0.77 ± 0.08	1.28 ± 0.04

6.5 Discussion and conclusions

It is largely assumed that MSCs elicit their immunomodulatory effects via the inhibition of lymphocyte activation and proliferation and the secretion of pro-inflammatory cytokines, while at the same time, promoting a regulatory phenotype. Several molecules, including IL-6, IL-10, TGF-β, TSG-6, iNOS, COX2, HGF, MMP9 and GMCSF have been described to be important for their efficacy in the clinic but there is controversy in the literature and the immunomodulatory mechanism of MSCs remains unknown. For this reason, the aim of this chapter was to gain an understanding of the anti-inflammatory and immunomodulatory mechanisms of MSCs by studying the expression of TLRs, the complement system and several genes described in the literature to have essential roles. Transcript levels 24 hours after cytokine-mediated stimulation are summarised in Figure 6-6.

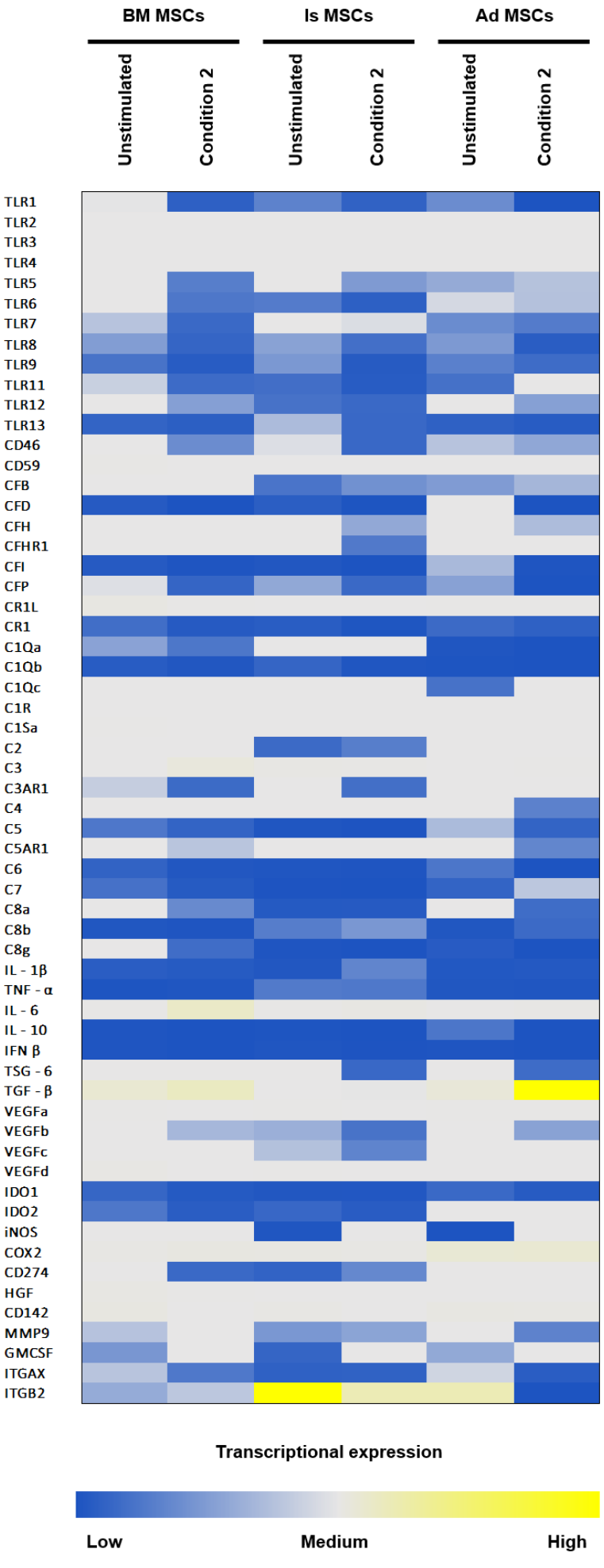

BM MSCs
Is MSCs
Ad MSCs
Unstimulated
Condition 2
Unstimulated
Condition 2
Unstimulated
Condition 2
TLR1
TLR2
TLR3
TLR4
TLR5
TLR6
TLR7
TLR8
TLR9
TLR11
TLR12
TLR13
CD46
CD59
CFB
CFD
CFH
CFHR1
CFI
CFP
CR1L
CR1
C1Qa
C1Qb
C1Qc
C1R
C1Sa
C2
C3
C3AR1
C4
C5
C5AR1
C6
C7
C8a
C8b
C8g
IL - 1β
TNF - α
IL - 6
IL - 10
IFN β
TSG - 6
TGF - β
VEGFa
VEGFb
VEGFc
VEGFd
IDO1
IDO2
iNOS
COX2
CD274
HGF
CD142
MMP9
GMCSF
ITGAX
ITGB2
Transcriptional expression
Low
Medium
High

Figure 6-6. Heat map representing the transcriptional expression of TLRs, the complement system and several immunomodulatory and anti-inflammatory genes by MSCs from different sources under resting and inflammatory conditions.
Data from Figure 6-1, Figure 6-2 and Figure 6-3 are combined and presented as a heat map to illustrate the transcriptional profile of TLRs, the complement system and several immunomodulatory and anti-inflammatory genes by MSCs maintained under resting and inflammatory conditions. The heat map summarises each tissue source of MSC highest and lowest transcribed genes under resting conditions and inflammatory stimulation. Genes with low $2^{(-\Delta CT)}$ values are highlighted in blue, genes with intermediate $2^{(-\Delta CT)}$ values are highlighted in grey and genes with high $2^{(-\Delta CT)}$ values are highlighted in yellow.

Priming by inflammatory cytokines has been described to be essential for MSC-mediated immunosuppression (Ren et al., 2009), but to my knowledge, there are no studies comparing different inflammatory agents and their potential to increase the clinical efficacy of MSCs. The establishment of standardised MSC isolation and culture protocols (Chapter 3) enables an objective comparison of the transcriptional and protein profile of BM, Is and Ad MSCs. For this reason, in this study we decided to examine the potential of different immunostimulants, cytokines and TLR 2, TLR3 and TLR4 agonists, to prime MSCs and increase their immunomodulatory functions. However, the use of these inflammatory agents, as well as the different conditions in which MSCs were stimulated with the same inflammatory agent, did not show any trend of up or down regulation of genes that could suggest a better inflammatory agent for MSC licensing. For these reasons, instead of discussing the results per condition, we have decided to discuss the results by gene of interest, focusing on the results obtained harvesting the cells 24 hours after licensing with 40 ng/ml of TNF-α, IL-1β and IFN-ϒ (Condition 2). In this manner, it is possible to compare the expression of all the genes analysed in this study in Section 6.2, which is summarised in Figure 6-6. More importantly, careful analysis of the transcript data enabled the identification of genes of interest to be targeted for protein assays; the secretion of IL-6 and VEGF by BM, Is and Ad MSCs under resting and inflammatory conditions is summarised in Figure 6-7.

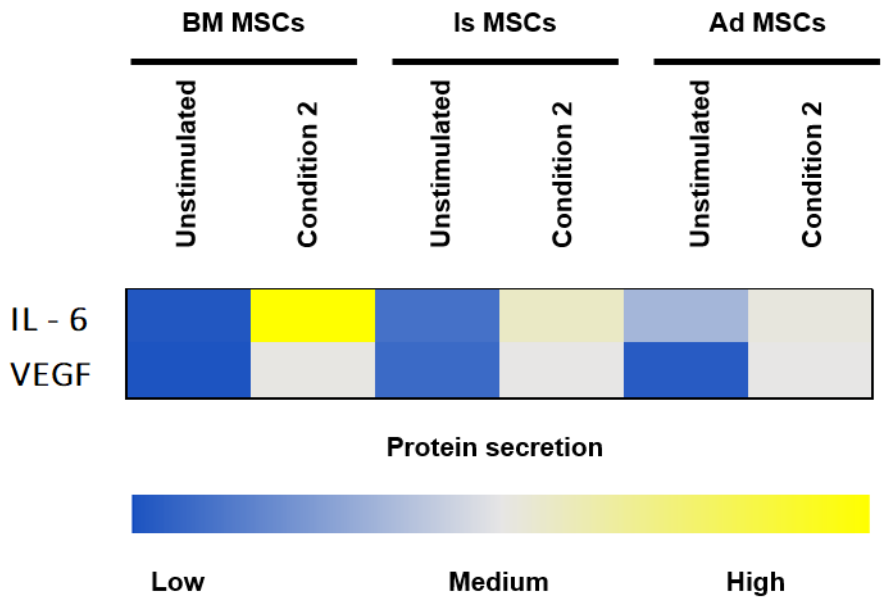


Figure 6-7. Heat map representing the secretion of IL-6 and VEGF by MSCs from different sources under homeostatic and inflammatory conditions.

Data from Figure 6-5 are combined and presented as a heat map to illustrate the IL-6 and VEGF secretion profile of MSCs maintained under homeostatic and inflammatory conditions. The heat maps summarise each tissue source of MSC highest and lowest transcribed genes under homeostatic conditions and inflammatory stimulation. Proteins with low secretion are highlighted in blue, proteins with intermediate secretion are highlighted in grey and proteins with high secretion are highlighted in yellow.

After analysis and comparison of the transcript and protein data described in this thesis with other studies in the literature, this discussion will focus on 5 genes, *IL-6*, *IL-10*, *TGF-B*, *VEGF*, and *iNOS*, and the clinical implications of the expression and regulation upon inflammatory stimulus of these molecules will be examined.

Interleukin 6 (IL-6) is a cytokine with pleiotropic effects on inflammation and immune response as well as regenerative processes and haematopoiesis. Different names, including B-cell stimulatory factor 2 and hepatocyte-stimulating factor, were provided to this soluble factor based on all these distinct biological roles and it was not until the molecular cloning of these molecules that it was determined they were all the same and subsequently renamed IL-6 (Kishimoto, 1989, Tanaka et al., 2014). IL-6 is secreted after inflammation by most nucleated cells, including monocytes and macrophages, endothelial cells, T cells, B cells, granulocytes and osteoclasts among others and is involved in host defence through induction of acute phase responses,

haematopoiesis and immune reactions (Tanaka et al., 2014). IL-6 exerts its functions by targeting hepatocytes, leukocytes, T cells, B cells and haematopoietic cells (Akdis et al., 2011). Circulating IL-6 levels are upregulated in several chronic inflammatory disorders including rheumatoid arthritis, psoriasis, systemic lupus erythematosus and Crohn's disease (Gabay, 2006). BM, Is and Ad MSCs transcribed (Figure 6-3, C) and secreted (Figure 6-5, A) moderate levels of IL-6 under resting conditions. These transcript and protein levels were significantly upregulated after inflammatory stimulation by MSCs isolated from all tissue sources. BM MSCs secreted the most IL-6 after cytokine-mediated inflammatory stimulation. Secretion of IL-6 by human BM and Ad MSCs has previously been reported (Ivanova-Todorova et al., 2012, Mi and Gong, 2017), but to my knowledge differences in IL-6 transcription and secretion by mouse BM, Is and Ad MSCs has not been documented.

It is difficult to determine whether the secretion of IL-6 by MSCs would be beneficial or detrimental in a clinical environment. IL-6 promotes the differentiation of monocytes into macrophages instead of DCs by inducing the expression of colony stimulating factor receptors (Chomarat et al., 2000) and at the same time, IL-6 modulates the DCs towards an immunosuppressive phenotype (Pasare and Medzhitov, 2003). IL-6 upregulates the secretion of CCL2, CCL4, CCL5, CCL11, CCL17 promoting the recruitment of monocytes, macrophages, $CD4^+$ T cells, $CD8^+$ T cells, NK cells and DCs, while downregulating the secretion of CXCL1 and CXCL8, thereby supressing neutrophil infiltration (McLoughlin et al., 2004, Akdis et al., 2011). IL-6 promotes anti-inflammatory Th2 cell differentiation (Diehl et al., 2002) and increases IL-27 secretion by monocytes and macrophages promoting the maturation of regulatory T cells (Pyle et al., 2017).

However, IL-6 has also been described to inhibit TGF-ß-induced Treg differentiation and to promote the differentiation of the pro-inflammatory Th17 cells from naïve T cells, disrupting immunological tolerance and leading to autoimmune and chronic inflammatory diseases (Bettelli et al., 2006, Tanaka et al., 2014). IL-6 promotes the differentiation of $CD8^+$ T cells into cytotoxic T cells (Okada et al., 1988) and induces the differentiation of activated B cells into antibody producing plasma cells (Okada et al., 1988, Kishimoto, 1989).

Within a transplant setting, IL-6 has been shown to mediate allograft rejection as blockage of the IL-6 axis prolonged graft survival in a mouse model of cardiac allograft rejection (Zhao et al., 2012). Despite its inflammatory effects, IL-6 is required for MSC proliferation and 'stemness' maintenance as it suppresses differentiation of MSCs, which results in loss of immune privilege and rejection (Pricola et al., 2009, Li et al., 2013). More importantly, IL-6 also induces the secretion of VEGF, enhancing angiogenesis, which is essential for the repair and regenerative processes required for effective engraftment after transplantation (Mathe et al., 2006). Taking all this into account, IL-6 secretion by MSCs would most likely be beneficial within a clinical setting and as BM MSCs secreted the most IL-6 under inflammatory conditions, BM MSCs could be a better choice than Is and Ad MSC to be used as cellular therapeutics.

Interleukin 10 (IL-10), also known as cytokine synthesis inhibitory factor, is an anti-inflammatory cytokine involved in Th1 cell, NK cell and macrophage inhibition to avoid tissue damage after acute inflammation. IL-10 was first described to be produced by Th2 cells, but it is also secreted by macrophages, DCs, B cells and subsets of regulatory T cells (Li et al., 1999, Kamanaka et al., 2006, Fiorentino et al., 1989). IL-10 inhibits the expression of MHC Class II and co-stimulatory molecules as well as secretion of pro-inflammatory cytokines and chemokines by macrophages and in this manner, it supresses T cells and NK cells (Couper et al., 2008). Due to its roles in resolving inflammation, IL-10 can also ameliorate autoimmune pathologies and its expression has been found altered in several autoimmune diseases including systemic lupus erythematosus and rheumatoid arthritis, where high levels of IL-10 correlate with low disease activity (Moore et al., 2001). BM, Is and Ad MSCs transcribed (Figure 6-3, D) very low levels of *IL-10* under resting conditions and cytokine-mediated stimulation downregulated *IL-10* transcript levels in MSCs isolated from all tissue sources. Ad MSCs transcribed the most *IL-10* under resting and inflammatory conditions.

Secretion of IL-10 by murine MSCs has already been described and it attenuated acute liver failure in a D–galactosamine–mediated acute liver failure mouse model. However, IL-10 secretion levels by MSCs need to be very high as infusion of MSCs alone into mouse and rat models of inflammatory rheumatoid arthritis produced no effect, while injection of IL-10 transduced MSCs significantly

decreased the severity of arthritis in a mouse model of antigen-induced arthritis (Hughes et al., 2014, Peruzzaro et al., 2019). IL-10 secretion may be highly beneficial in any clinical setting to dampen inflammation; thus, we could hypothesise that Ad MSCs could have an increased therapeutic potential compared to BM and Is MSCs. However, due to the very low transcript levels, IL-10 secretion should be study as the increased transcript levels by Ad MSCs may not be biologically significant.

The TGF-ß family of growth factors is involved in development and homeostasis by tightly regulating cell proliferation, differentiation and apoptosis in most cell types including fibroblasts, immune cells and haematopoietic cells (Kubiczkova et al., 2012). The most relevant effect of TGF-ß on target cells is based on its ability to suppress the expression and function of c-myc and cyclin-dependent kinases to suppress proliferation. For this reason, the TGF-ß pathway, and sensitivity to this family of growth factors, is altered in a number of diseases including solid and haematopoietic tumours and psoriasis. Under these conditions, TGF-ß switches from promoting an anti-proliferative response to induce proliferation, promoting tumour growth and keratinocyte hyperproliferation in psoriasis, thus, increasing the severity of the disease (Han et al., 2010, Kubiczkova et al., 2012).

BM and Ad MSCs transcribed (Figure 6-3, G) high levels of *TGF-ß*, while Is MSCs transcribed low levels of *TGF-ß* under resting conditions. Cytokine-mediated stimulation upregulated *TGF-ß* transcript levels in BM and Ad MSCs, while it led to a downregulation in Is MSCs. BM MSCs transcribed the highest *TGF-ß* transcript levels under resting conditions, however, cytokine-mediated stimulation resulted in Ad MSCs transcribing the highest levels of *TGF-ß*. Human MSCs isolated from the bone marrow, adipose tissue, Wharton's jelly and placenta have already been described to produce and secrete TGF-ß (Ryan et al., 2007, Carrillo-Galvez et al., 2015, Tomic et al., 2011, Zhou et al., 2011, Heo et al., 2016).

TGF-ß treatment of MSCs has been widely described to alter their *in vitro* and *in vivo* behaviour (Ghosh et al., 2017); however, TGF-ß secreted by MSCs also plays an important role in the context of tissue regeneration and modulation of the immune responses. TGF-ß is well known to inhibit T cell responses and its secretion by MSCs has been demonstrated to be partially involved in MSC-

mediated T cell inhibition *in vitro* (Nasef et al., 2007, Gao et al., 2016). Moreover, MSC-secreted TGF-ß induces the proliferation of regulatory T cells as TGF-ß deficient MSCs could not generate regulatory T cells *in vitro* (Wang et al., 2017). MSC-derived TGF-ß promotes Th2 phenotypes while inhibiting Th17 responses in vitro (Kong et al., 2009). To further confirm this role, infusion of engineered TGF-ß overexpressing BM MSCs increased Th2 response in a model of type 1 diabetes, which resulted in increased therapeutic potential compared to control BM MSCs (Daneshmandi et al., 2017).

MSC-derived TGF-ß not only modulates T cells, but also macrophages as it promotes anti-inflammatory phenotypes. As an example, Song et al. demonstrated that infusion of MSCs into an experimental asthma model alleviated the symptoms by promoting the polarization of M2 macrophages in the lungs and it was TGF-ß-mediated, as inhibition of TGF-ß signalling abolished the therapeutic effect of infused MSCs (Song et al., 2015). Altogether, TGF-ß secretion would be highly beneficial in any clinical setting to dampen inflammation; thus, according to the increased transcript levels by Ad MScs under inflammatory conditions we could again hypothesise that Ad MSCs could have an increased therapeutic potential compared to BM and Is MSCs. However, protein secretion would have to be assessed to further confirm this hypothesis.

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), is secreted by most cell types including macrophages, platelets and keratinocytes, but usually not by endothelial cells themselves (Maharaj and D'Amore, 2007). VEGF was originally described to promote endothelial cell permeability, proliferation and angiogenesis however, its roles are not only associated with the vascular system but also with the formation of bone (Gerber et al., 1999), haematopoiesis (Ferrara et al., 1996), wound healing (Chintalgattu et al., 2003) and development (Reichardt and Tomaselli, 1991). However, upregulation of VEGF is also involved in the development of inflammatory diseases as rheumatoid arthritis (Lee et al., 2001), psoriasis (Detmar et al., 1994), atherosclerosis (Zhao and Zhang, 2018) and cancer (Carmeliet, 2005).

BM, Is and Ad MSCs transcribed high levels of *VEGFa* (Figure 6-3, H) and *VEGFd* (K) while they had low *VEGFb* (I) and *VEGFc* (J) transcript levels under resting conditions. MSCs from the three sources were secreting close to 0.1 pg of VEGF

per mg of total protein (Figure 6-5, B) and despite the downregulation of *VEGF* transcript levels after stimulatory licensing, 24 hour cytokine licensing led to the upregulation of VEGF secretion by MSCs from all sources. Is MSCs secreted the most VEGF under resting and stimulatory conditions. Secretion of VEGF by murine BM and human BM and Ad MSCs has previously been reported (Ge et al., 2018, Lu et al., 2018, Kagiwada et al., 2008), but to my knowledge differences in VEGF transcription and secretion by murine BM, Is and Ad MSCs has not been documented.

Secretion of VEGF by MSCs is most likely beneficial within a clinical setting due to its role in angiogenesis. VEGF is a key mediator of angiogenesis in cancer enabling development and growth of the tumour; for this reason, anti-VEGF is used to reduce the production of tumour vasculature and reduce the growth and progression potential. However, this leads to hypertension, proteinuria, impaired wound healing, haemorrhage and thrombosis, endocrine dysfunction and cardiac impairment, which shows how essential VEGF is for maintaining homeostasis (Kamba and McDonald, 2007).

Liver transplantation is commonly followed by hepatic ischemia/ reperfusion injury, which is treated with endothelial precursor cell transplantation. Administration of exogenous VEGF prior to endothelial precursor cell transplantation significantly reduced liver transaminase levels, hepatocellular injury levels and hepatic apoptosis levels significantly decreasing hepatic ischemia/ reperfusion injury and increasing graft survival (Cao et al., 2017).

Transfection of *VEGF* gene into bone MSCs led to a better therapeutic outcome on endometrial regeneration and endometrial receptivity compared to MSC-only controls in a thin endometrium rat model (Jing et al., 2018). Moreover, transplantation of VEGF-overexpressing BM MSCs promoted the neovascularization and decreased senile plaques in hippocampal specific layers in a murine model of Alzheimer's disease (Garcia et al., 2014). VEGF has been described to promote the therapeutic efficacy of MSC derived extracellular vesicles against neonatal hyperoxic lung injury (Ahn et al., 2018). VEGF secreted by MSCs has been described to improve myocardial survival and to improve the engraftment of infused MSCs within infarcted hearts (Tang et al., 2011); moreover, VEGF is also involved in decreasing cardiac apoptosis in a rat model of

myocardial infarction (Song et al., 2017). For these reasons, we consider that expression of VEGF by MSCs could be therapeutically beneficial within a clinical setting and as Is MSCs secreted the most VEGF, infusion of MSCs isolated from the islets could have a better clinical outcome in transplantation than infusion of BM and Ad MSCs.

iNOS is an inducible nitric oxide synthase expressed by macrophages, T cells and DCs that upon inflammatory activation produces large quantities of nitric oxide, an important pro-inflammatory cytotoxic agent involved in immunity. However, iNOS is also involved in the regulation of differentiation and function of immune cells and therefore, iNOS has an important immunomodulatory role. iNOS inhibits the production of IL-12 in DCs and macrophages and in this manner, it inhibits differentiation towards Th1 phenotype (Xiong et al., 2004). Moreover, iNOS expression by T cells also inhibits Th17 differentiation and pro-inflammatory phenotypes in macrophages and DCs as iNOS knock out mice exhibit increased Th17 subsets and pro-inflammatory macrophages and DCs (Xue et al., 2018). Due to its immunomodulatory role, iNOS has been described to be deregulated in several disease models including hepatocellular carcinoma and Parkinson's disease (Calvisi et al., 2008, Koppula et al., 2012).

BM, Is and Ad MSCs transcribed (Figure 6-3, N) low levels of *iNOS* under resting conditions and cytokine-mediated stimulation upregulated *iNOS* transcript levels in MSCs isolated from all tissue sources. Under resting conditions, BM MSCs transcribed higher *iNOS* transcript levels than Is and Ad MSCs, while Is MSCs transcribed the highest *iNOS* transcript levels after cytokine-mediated stimulation.

MSC-mediated iNOS expression and secretion could be beneficial within a clinical setting due to its role suppressing allogeneic effector T cells as well as promoting the expansion of $CD4^+$ $Foxp3^+$ regulatory T cells (Conde et al., 2015, Riquelme et al., 2013). In fact, in a model of systemic sclerosis induced by hypochlorite in mice, MSCs isolated from iNOS knocked out mice were unable to exert the anti-fibrotic properties exerted by WT MSCs, and thus, did not promote a reduction of skin thickness and collagen deposition as compared to MSCs isolated from WT mice (Maria et al., 2018). Furthermore, MSCs isolated from iNOS KO mice were unable to suppress T cell proliferation *in vitro* and their co-

administration with ovalbumin in ovalbumin-immunised mice enhanced the immune response compared to WT MSCs (Ren et al., 2008). It is important to bear in mind that there are more mechanisms involved in MSC-mediated immunosuppression as blocking of IDO, the human equivalent to iNOS in mouse, was only able to partially inhibit the immunosuppressive functions of amnion MSCs (Meesuk et al., 2016). However, expression of iNOS could be highly beneficial within a clinical setting. Is MSCs transcribed the highest levels of *iNOS* transcript levels under inflammatory conditions, however, iNOS secretion and its T cell proliferation potential would have to be assessed to allow us to conclude that Is MSCs could be a better source for MSC isolation to use as cellular immunomodulatory therapeutics.

To summarise and conclude, this chapter aimed to determine if murine MSCs isolated from different tissues had a specific pattern of TLRs, complement system and other immunoregulatory molecule expression at a transcriptional, and most importantly, protein level.

BM, Is and Ad MSCs all had very low transcript levels of TLRs under resting and stimulatory conditions, which suggests that they are not key regulators of MSC function. Regarding the complement system, these molecules were transcribed at low levels and no differences where observed except for *CFH*, *C1Qc* and *C5AR1*. BM MSCs expressed the highest *CFH* transcript levels while *C1Qc* and *C5AR1* transcript levels were higher in Is MSCs under resting conditions. 24-hour cytokine-mediated licensing led to the downregulation of these transcripts in MSCs from every source.

Among the immunomodulatory molecules studied, only IL-6 and VEGF were transcribed at high levels and were affected by MSC licensing. Under resting conditions Ad MSCs secreted the most IL-6 but 24-hour cytokine-mediated licensing led to high levels of IL-6 secretion by BM, Is and Ad MSCs. Under resting conditions Is MSCs secreted the most VEGF but 24-hour cytokine-mediated licensing led to a higher secretion by Is MSCs. This chapter showed differential immunoregulatory and angiogenic potential of MSCs depending on their tissue origin and suggests that MSCs isolated from various tissues may act differently *in vivo*. For this reason, to have a better understanding of the behaviour of MSCs from different sources in vivo, co-transplantation of MSCs to evaluate the fate of

the graft would be essential. Moreover, knocking out IL-6 and VEGF would help to understand the role of these molecules in a clinical setting.

Chapter 7

General Discussion

7 General Discussion

7.1 Introduction

MSCs have the ability to self-renew and differentiate, have low levels of MHC Class I expression and lack the expression of MHC Class II and co-stimulatory molecules, which has raised the biologic and clinic interest in MSCs. BM MSCs were the first MSCs to be isolated and are therefore the most researched and clinically used source of MSCs. However, their isolation involves an invasive and painful procedure. As MSCs can be isolated from a large variety of tissues, alternative sources which are more easily accessible could be more suitable for clinical use. Adipose tissue, umbilical cord and islets of Langerhans are easily accessible, they are clinical waste so there is no burden on the donor and MSCs have an increased frequency in these sources. As an example, adipose tissue yields a 500-fold higher frequency of colony forming units (CFU-F) than bone marrow and can be obtained easily (Fraser et al., 2006); MSCs from the umbilical cord could also be a good choice as they are very abundant and they are ontogenically primitive (Choudhery et al., 2013). Different isolation, enrichment and culture protocols, as well as the intrinsic variability among MSCs derived from different donors/ tissues has led to contradictory results in the literature regarding their immunomodulatory and regenerative potential. In addition, MSCs are often infused within an inflammatory environment, which makes it essential to study their properties both under resting and inflammatory conditions. As a result of inflammatory stimulation, MSCs increase MHC Class I and MHC Class II expression, leading to both cell-mediated and humoral immune responses when human MSCs are infused in murine models, which confounds interpretation of results (Eliopoulos et al., 2005, Joswig et al., 2017, Thirlwell, 2018).

Thus, considering the limitations of studies carried out with human MSCs, the aim of this study was to isolate, study and compare, through a stringent set of standardised techniques, the potential *in vivo* function of murine BM, Is and Ad MSCs under resting and inflammatory conditions. This would provide insight into the role of tissue source in the therapeutic potential of MSCs and would potentially allow the identification of a preferred tissue source for MSC isolation for use within the clinic, with a particular focus on the co-transplantation of MSCs with islets of Langerhans to treat individuals with type 1 diabetes mellitus

(T1DM), which is a major area of study in the SNBTS group. To address this, four key questions were considered:

- Are cells isolated from murine bone marrow, islets of Langerhans and adipose tissue true MSCs? Do they have the same phenotype?

- What is the migratory potential of murine MSCs when infused into a mouse? Does the tissue origin of MSCs impact this?

- Are MSCs able to recruit immune cells? Does MSC microenvironment (stimulation) impact the recruitment capability of MSCs? Does tissue source of origin affect MSCs recruitment potential?

- How do MSCs interact and immunomodulate surrounding tissues? Does MSC microenvironment (stimulation) and tissue origin influence their behaviour?

Chapter 3 of this thesis focused on question number one and studied whether cells isolated from the bone marrow, islets of Langerhans and adipose tissue satisfied the minimum criteria outlined by the International Society of Cellular Therapy (ISCT) for human MSCs (Dominici et al., 2006). Although adherence and trilineage differentiation are characteristics of murine MSCs, the surface marker phenotype is not so well characterised, and variations might apply. The ability to adhere to plastic and the phenotypic properties of these cells was assessed throughout passage. As required by the ISCT, the surface molecule expression, size and granularity of the cells was studied under resting and stimulatory conditions. Moreover, trilineage differentiation potential into adipocytes, chondrocytes and osteocytes was assessed in cells isolated from the three tissues.

Chapter 4 addressed question number two. Transcript levels of chemokine receptors expressed by MSCs were assessed under resting and inflammatory conditions to understand if MSCs possessed migratory capacity and if MSC tissue origin could affect their migratory potential.

Chapters 4 and 5 addressed question number three. An extensive assessment of the chemokine ligands expressed by MSCs at a transcript level under resting and inflammatory conditions highlighted genes of interest to be assessed at a protein level (Chapter 4). Chemokine secretion by MSCs was examined to predict the leukocyte recruitment potential of MSCs under resting and inflammatory conditions. After showing that MSCs transcribed and secreted a distinct set of chemokines under homeostatic and inflammatory conditions, chemokine function was studied *in vivo* (Chapter 5) by assessing the immune cell attraction profile of MSCs.

Chapter 6 answered question number four. An extensive assessment of toll like receptors, complement system and anti-inflammatory and immunomodulatory genes expressed by MSCs at transcript level, under resting and inflammatory conditions, highlighted genes of interest to be assessed at the protein level. This knowledge provided a better understanding into how MSCs might interact with and immunomodulate the immune cells they recruit. The following section will discuss these findings and their relevance within specific areas of clinical relevance.

7.2 The tissue origin of MSCs could impact their performance within the clinic

Cells isolated from bone marrow, islets of Langerhans and adipose tissue were plastic adherent, expressed high levels of the widely used CD90, CD105 and CD73 MSC markers and had trilineage differentiation potential. In contrast to human MSCs, cells isolated from the bone marrow and islets of Langerhans were $CD45^+$ $CD11b^+$ and, for this reason, did not meet ISCT criteria. ISCT criteria were established for the identification of human MSCs and although adherence and trilineage differentiation potential are characteristics of murine MSCs, the surface marker phenotype of murine MSCs is not so well characterised. In fact, induction of CD45 expression on murine BM MSCs has already been described (Yeh et al., 2006). The lack of CD64 expression rules out a myeloid origin of these cells, while CD146 positive expression enables to discriminate MSCs from fibroblasts. Under resting conditions, MSCs from the three tissues were

expressing very low levels of MHC Class I and II molecules, which is essential to avoid recognition by the recipient's immune system and subsequent rejection. Inflammatory conditions upregulated the expression of MHC Class I, which avoids recognition and cytotoxic activity by NK cells. Thus, based on plastic adherence, trilineage differentiation potential and CD90, CD105 and CD73 expression, we determined that cells isolated from the bone marrow, islets of Langerhans and adipose tissue were MSCs regardless of CD45 and CD11b expression. Moreover, specific criteria to enable the identification of murine MSCs should be established.

MSCs are often infused within an inflammatory environment and their clinical performance is often monitored by the resolution of symptoms, or in the case of transplantation, the promotion of graft survival (Ankrum et al., 2014, English and Wood, 2013). The efficacy of MSCs within the clinic depends on several factors including the ability of MSCs to migrate into target tissues and the potential of MSCs to interact and immunomodulate the surrounding tissues and immune cells (Kean et al., 2013). One of the most critical aspects of MSC use as cellular therapeutics is related to MSC homing and engraftment within the target tissue following their administration. MSCs can be infused systemically, where MSCs are administered intra-venously or intra-arterially and the cells must exit the circulation and migrate to the site of injury, or can be administered locally at the target tissue and are then guided to the site of injury (Nitzsche et al., 2017). After systemic administration, the majority of the cells are found in the lungs and only a small percentage of the cells home to, and remain in, the target tissue.

The size of the cells could be an essential factor involved in lung trapping. For this reason, the size of MSCs was compared among tissue sources (Figure 3-17). Murine BM MSCs are smaller than Is and Ad MSCs, which suggests that they could be a better source of MSC if being delivered intravenously as their smaller size may prevent lung entrapment. However, in humans, size of MSCs does not vary significantly between tissue sources and thus, it is not valid interpretation for human therapy (Thirlwell, 2018). MSCs are well described for their anti-inflammatory and immunomodulatory properties. Many of these molecules are stored in granules; BM MSC are more granular than Is and Ad MSCs, suggesting

more extensive storage of clinically beneficial molecules (Figure 3-17). These data correlate with human MSCs, as BM MSCs have previously been described to be more granular than Is and Ad MSCs under both resting and inflammatory conditions (Thirlwell, 2018).

Specific chemokines have been shown to target immune cells to specific tissues. For example, CCR7 targets MSCs to secondary lymphoid organs (Li et al., 2014, Ma et al., 2016), CXCR3-deficient MSCs fail to infiltrate into the nephritic kidney (Lee et al., 2018) and CXCR4 receptor overexpression in MSCs improves treatment of acute lung injury (Yang et al., 2015). Moreover, the expression of CCR1 in macrophages and neutrophils leads to kidney infiltration in renal ischemia-reperfusion injury (Furuichi et al., 2008); CCR5 directs $CD8^+$ T cells towards the brain (Martin-Blondel et al., 2016); CCR3, CCR4 and CCR10 are highly expressed by T cells in skin (Fujimoto et al., 2008, Ma et al., 2002); CXCR3 mediates T cell recruitment into the kidney (Panzer et al., 2007); and CXCR6 is highly expressed by liver-infiltrating $CD8^+$ T cells (Sato et al., 2005b). Therefore, we hypothesise that the expression of these chemokines could potentially target MSCs into those tissues. BM MSCs transcribed significantly higher levels of *CCR7*, *CXCR6* and *ACKR4*; Is MSCs transcribed significantly higher levels of *CCR1*, *CCR10* and *CXCR3*, while Ad MSCs transcribed significantly higher levels of *CCR3* (Figure 4-10). Therefore, we hypothesise that CCR7 and CXCR6 expression would direct BM MSCs towards lymphoid organs such as the spleen, the lymph nodes and the liver and thus, BM MSCs would be ideal candidates for dampening inflammation in the liver after transplantation. Is MSCs have an enhanced potential to migrate towards the kidneys due to their CCR1 and CXCR3 expression; thus, islets of Langerhans would be the best tissue source for MSC isolation when used as cell therapeutics to dampen inflammation within the kidneys. Lastly, Ad MSCs transcribed statistically significantly more CCR3 than Is and BM MSCs and therefore, they have a greater potential to migrate to the skin and to be more beneficial dampening and regulating excessive inflammation in psoriasis and other skin conditions. In contrast, due to the increased transcription of CXCR4 by Ad MSCs, if infused systemically Ad MSCs are more likely to remain in the lungs or to migrate towards the bone marrow. However, Ad MSCs transcribed the lowest levels of chemokines receptors apart from CXCR4, which suggests that if infused at the target tissue they are more likely to

remain in place. These hypotheses have been formulated from transcript analysis data in murine MSCs. Thus, experiments on chemokine receptor expression and functionality and a critical comparison with the expression of chemokine receptors on human MSCs would be essential to translate these results into human therapy. Based on chemokine receptor expression at transcript level, the tissue of choice to isolate MSCs to enhance migration and retention towards specific anatomical location is represented in Figure 7-1.

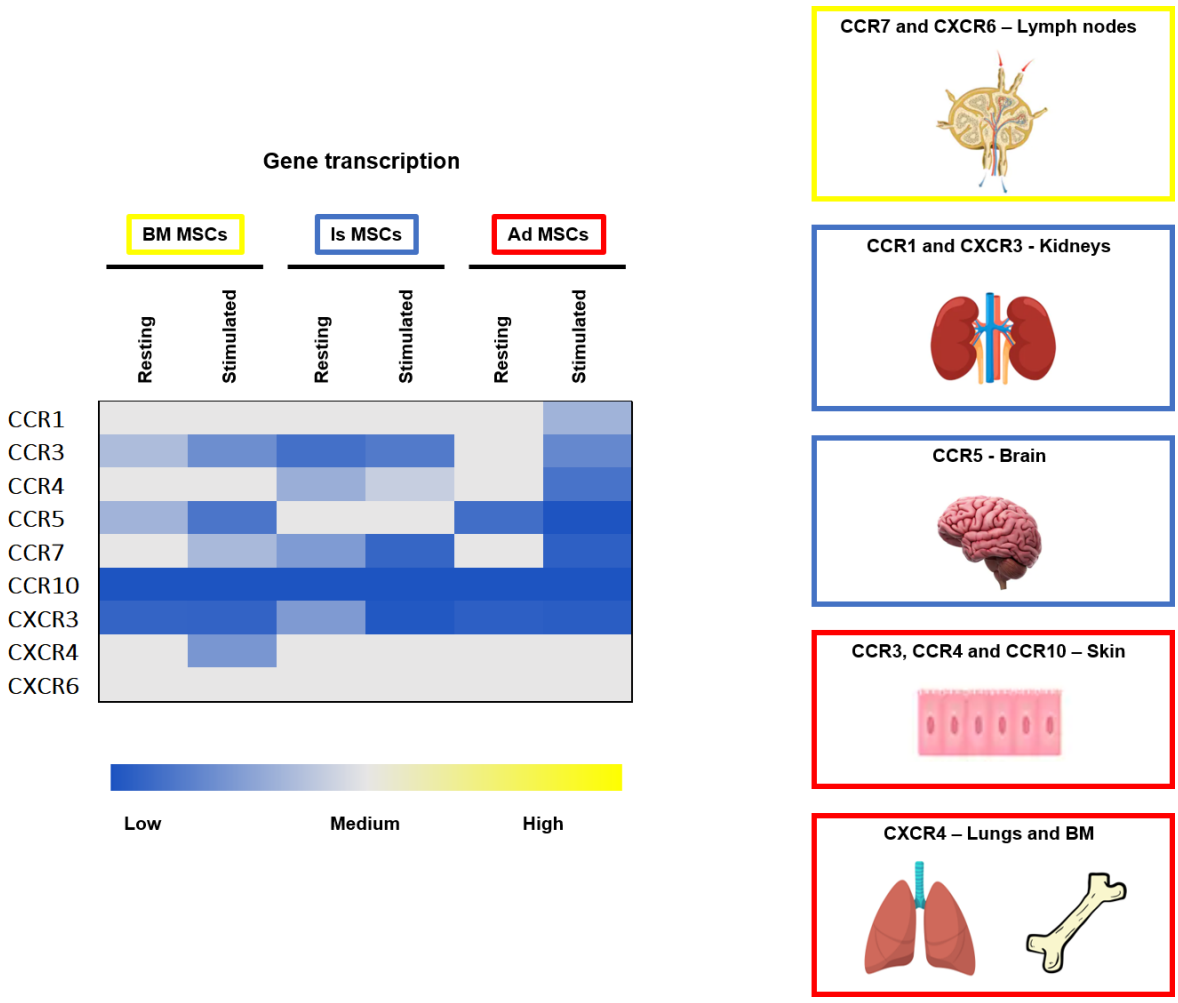


Figure 7-1. Overview of chemokine receptor expression by MSCs at transcript level under resting and inflammatory conditions.
Chemokine receptor expression by BM, Is and Ad MSCs were compared to each other in resting and inflammatory conditions to understand which tissue source of MSC expressed the highest levels of chemokine receptors. Colour coding highlights tissue sources that transcribed receptors at the highest (yellow), medium (grey) and lowest (blue) levels. BM MSCs transcribed the highest levels of *CCR7* and *CXCR6*, suggesting that bone marrow might be the preferred tissue to isolate MSCs to enhance migration towards the lymph nodes. Conversely, Is MSCs transcribed the highest levels of *CCR1*, *CXCR3* and *CCR5*, suggesting that islets of Langerhans might be the desired source of MSCs to promote trafficking towards the kidneys and brain, respectively. Ad MSCs, on the contrary, transcribed the highest levels of *CCR3* and *CXCR4*, suggesting that adipose tissue could be the preferred tissue for MSC isolation to enhance migration towards the skin and the lungs and BM, respectively. Transcriptional expression does not always correlate to protein expression and therefore, a rigorous testing of receptors at protein level is required. Transcript data shown here is summarised from Chapter 4, Figure 4-3 and Figure 4-4.

Through the assessment of chemokine ligand expression at transcript and protein level this study highlighted that MSCs isolated from different tissue sources were able to secrete pro-inflammatory, angiogenic and angiostatic chemokines that have the potential to recruit and interact with the surrounding cells. Under resting conditions, all MSCs regardless of tissue source of origin had a similar chemokine secretion profile, where CCL2 was the top chemokine secreted by all MSC populations (Figure 4-11). Furthermore, CCL2 is also the top chemokine secreted by human MSCs regardless of the tissue of origin, under resting conditions (Thirlwell, 2018). Despite the low secretion of chemokines, infusion of MSCs from the three sources into the air pouch did not lead to a recruitment of leukocytes above control levels (Figure 5-5).

Inflammatory stimulation of MSCs led to the upregulation of transcription and secretion of chemokines in MSCs from the three sources (Figure 4-11). CCL2, CCL5, CXCL1 and CXCL10 were the most upregulated chemokines and secretion levels depended on tissue source of origin. Inflammatory stimulation with a cytokine cocktail also resulted in increased secretion of CCL2, CXCL1 and CXCL8, the equivalent of CXCL1 in mice, by human BM, Is and Ad MSCs (Thirlwell, 2018). Is MSCs secreted the highest levels of the chemokines studied except for CXCL12, which was secreted at higher levels by BM MSCs. CCL2, CCL5 and CXCL1 are strong chemoattractants, however, despite higher secretion by Is MSCs under inflammatory conditions, Ad MSCs were able to recruit significantly more leukocytes into the air pouches of mice than BM and Is MSCs. An overview of the chemokines that MSCs expressed at protein level and the subsequent *in vivo* immune cell recruitment by MSCs under resting and inflammatory conditions is provided in Figure 7-2. More importantly, Ad MSCs were the only MSCs able to support the recruitment of small numbers of T cells. Recipient cytotoxic cells are essential to initiate MSC-mediated immunosuppression in a murine model of GVHD as MSCs need to be targeted by cytotoxic T cells to undergo apoptosis, be engulfed by macrophages and produce IDO (Galleu et al., 2017). As Ad MSCs are the only MSCs able to support the recruitment of T cells we could hypothesise that they have a greater immunosuppressive potential than BM and Is MSCs (Figure 5-5).

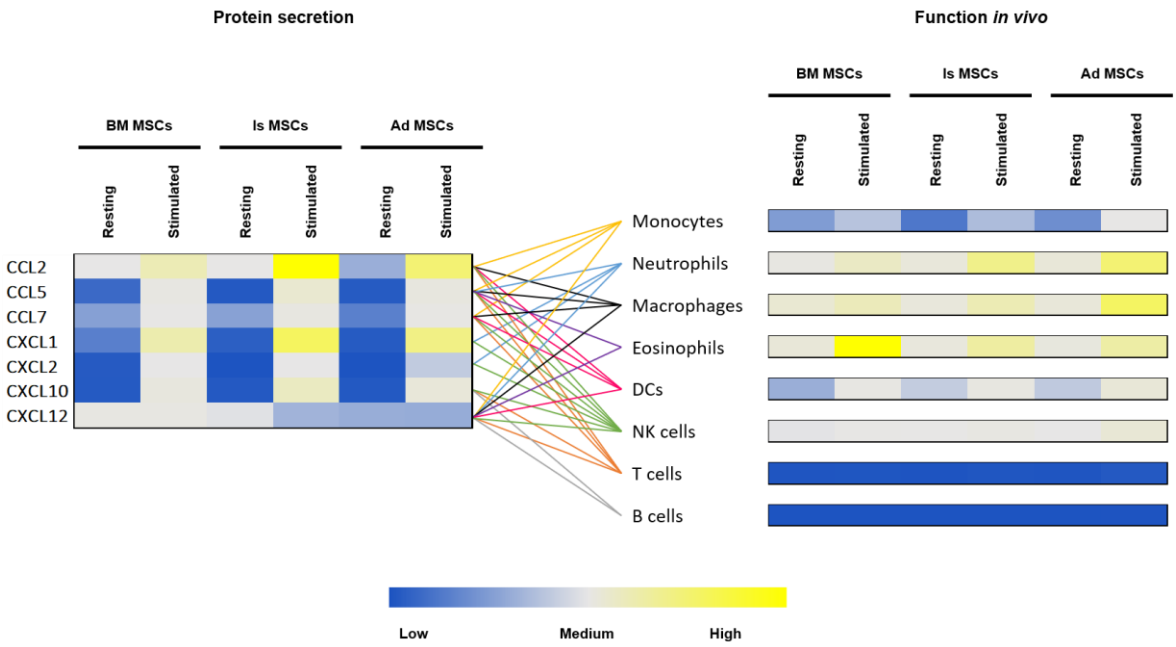


Figure 7-2. Summary of the CC and CXC chemokines MSCs transcribed, secreted and the subsequent immune cell attraction under homeostatic and inflammatory conditions.
Colour coding highlights tissue sources that secreted chemokines at the highest (yellow), medium (grey) and lowest (blue) levels. The immune cells that each chemokine could potentially attract are highlighted by colour coded lines that match specific immune cells. The same colour coding system was implemented (blue-grey-yellow) based on the number of immune cells MSCs attracted *in vivo* under resting and stimulatory conditions.

Chemokines do not only have the potential to recruit leukocytes but also have angiogenic and angiostatic roles. CXCL1, CXCL2 and CXCL12 have angiogenic properties, while CXCL10 has angiostatic potential. Is MSCs have the highest secretion of these four chemokines and it is therefore difficult to predict the overall effect of the secretion of the four chemokines altogether. On the one hand, Is MSCs have the potential to promote angiogenesis the most, while they have the potential to inhibit it the most compared to BM and Ad MSCs. High secretion of angiogenic chemokines would be of importance in transplantation to promote graft re-vascularization. However, if MSCs were infused within a tumour environment, high secretion of angiogenic chemokines would be detrimental, while the secretion of CXCL10 would be beneficial to suppress tumour growth.

Chemokines are not the only molecules secreted by MSCs with the potential to regulate angiogenesis. VEGF, hepatocyte growth factor (HGF) and matrix metalloproteinases (MMP) are well-known pro-angiogenic mediators involved in revascularisation (Cheng et al., 2007, Golocheikine et al., 2010, Olsson et al., 2006). Moreover, IL-6 can increase VEGF expression and can therefore, promote

angiogenesis (Huang et al., 2004). In Chapter 6, the transcription of *IL-6*, *VEGF*, *HGF* and MMP9 (Figure 6-3), as well as the secretion of IL-6 and VEGF (Figure 6-7) were studied.

IL-6 transcription levels were similar in MSCs from the three sources under resting conditions; inflammatory conditions upregulated the transcription in MSCs from every source but BM MSCs upregulated *IL-6* transcription the most. Despite similar levels of transcription under resting conditions, Ad MSCs secreted the most IL-6, while inflammatory stimulation led to similar levels of secretion by MSCs from the three sources. Therefore, MSCs from the three sources have the same potential for VEGF production and secretion.

VEGFa, *VEGFb*, *VEGFc* and *VEGFd* were downregulated after inflammatory stimulation in MSCs from the three sources. Ad MSCs transcribed the most *VEGFa* and *VEGFc*, while BM MSCs had the highest transcript levels of *VEGFb* and *VEGFd*. However, transcript levels do not correlate with protein level in this case, as Is MSCs secreted the most VEGF under resting and stimulatory conditions and 24-hour cytokine-mediated stimulation increased the secretion of VEGF in MSCs from the three sources. The increased secretion of VEGF suggests that Is MSCs would be the preferential tissue source of MSC to promote angiogenesis.

Under resting conditions, BM MSCs transcribed the highest levels of *HGF* and the transcript levels decreased after inflammatory stimulation in the three sources of MSCs. *MMP9* was transcribed at similar levels by MSCs from the three sources and inflammatory stimulation did not influence its transcription. Due to the increased secretion of CXCL1, CXCL2 and CXCL12 as well as VEGF, we hypothesise that Is MSCs could have greater re-vascularisation potential.

More importantly, MSCs not only regulate angiogenesis but also immunomodulate their surroundings by inhibition of effector T cell proliferation as well as promoting a regulatory phenotype of leukocytes via the secretion of anti-inflammatory and immunomodulatory molecules, including IL-10, TSG-6, TGF-ß, IDO and CD274 (Choi et al., 2008, Li et al., 2019b, Niu et al., 2017, Davies et al., 2017, Chinnadurai et al., 2018). IL-10, and TSG-6 secretion not only suppresses T cell proliferation, but also secretion of IFN-γ and other pro-inflammatory cytokines (Deniz et al., 2008, Choi et al., 2008, Li et al., 2019b, Chinnadurai et

al., 2018). Moreover, MSCs secrete TGF-ß, which upon interaction with glycoprotein A repetitions predominant (GARP) in regulatory T cells, activates Tregs, which is indispensable for effector T cells suppression (Niu et al., 2017). Similarly, iNOS, the murine equivalent of IDO is able to suppress allogeneic T cells as well as to promote the expansion of $CD4^+$ $Foxp3^+$ regulatory T cells, enabling in this way graft tolerance (Conde et al., 2015, Riquelme et al., 2013). CD274, also known as programmed death 1 ligand, is an important regulator of T cell activation and in addition to MSC-surface expression, MSCs are also able to secrete it to mediate immunosuppression in a contact-independent manner (Davies et al., 2017).

In Chapter 6, the transcription of *IL-10*, *TSG-6*, *TGF-ß*, *iNOS* and *CD274* was assessed. *IL-10*, *TSG-6* and *TGF-ß* transcript levels were downregulated after inflammatory stimulation, while *iNOS* and *CD274* transcript levels were upregulated. BM MSCs transcribed the most *TSG-6*, Is MSCs transcribed the most *iNOS* and Ad MSCs transcribed the most *IL-10*, *TGF-ß* and *CD274* (Figure 6-3). Among all these molecules, *iNOS* was transcribed at the highest levels and IDO is at least in part, involved in the suppressive effect of *MSCs* on cytotoxic cell proliferation, and thus, immunomodulation. For this reason, Is MSCs could have an increased immunosuppressive potential compared to BM and Ad MSCs.

Complement factor H is a complement regulatory protein that regulates complement mediated cell destruction by disrupting C3b and suppressing, in this way, the formation of C3 convertases. MSCs have been shown to secrete CFH under resting conditions and this secretion has been documented to increase under inflammatory conditions, where human BM MSCs were secreting significantly higher amounts of CFH than Is and Ad MSCs (Thirlwell, 2018). In this study, we showed that BM MSCs transcribed statistically significantly more *CFH* than Is and Ad MSCs under resting and stimulatory conditions; however, inflammatory stimulation resulted in the downregulation of *CFH* transcript levels in MSCs from every source (Figure 6-2). Despite RNA levels not always correlating with protein levels, if we assume that transcription levels of *CFH* are proportionately maintained at the protein level, BM MSCs would be secreting the most CFH. Therefore, we could hypothesise that BM MSCs would have better therapeutic potential than Is and Ad MSCs due to their increased potential to

suppress complement system activation. A summary of the expression at transcript and protein level of angiogenic, angiostatic and anti-inflammatory molecules by BM, Is and Ad MSCs under resting and inflammatory conditions is provided in Figure 7-3.

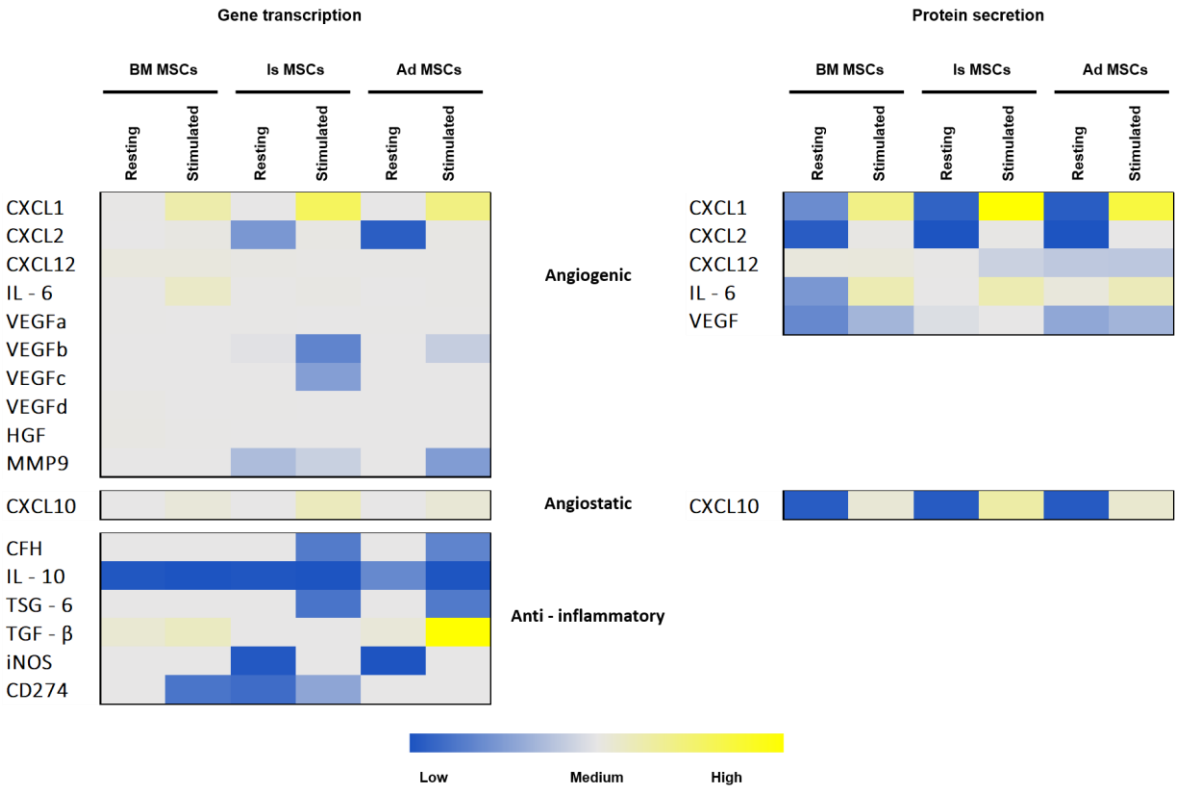


Figure 7-3. Summary of the angiogenic, angiostatic and anti-inflammatory molecules transcribed and secreted by MSCs under resting and inflammatory conditions.
Colour coding highlights tissue sources that transcribed (left hand side) and secreted (right hand side) angiogenic, angiostatic and anti-inflammatory molecules at the highest (yellow), medium (grey) and lowest (blue) levels.

To summarise, the answers to the questions outlined in the introduction of this chapter:

1) **Are cells isolated from murine bone marrow, islets of Langerhans and adipose tissue MSCs? Do they have the same phenotype?** Cells isolated from the three sources are spindle-shaped plastic-adherent cells with trilineage potential. However, tissue origin affects size and granularity as well as CD45 and CD11b expression. ISCT criteria were established for the identification of human MSCs and the surface marker phenotype of murine MSCs is not so well characterised. For this reason, based on plastic adherence, trilineage

differentiation potential and CD90, CD105 and CD73 expression, we determined that cells isolated from the bone marrow, islets of Langerhans and adipose tissue were MSCs and that specific criteria to enable the identification of murine MSCs should be established. More importantly, unlike BM and Ad MSCs, cells isolated from the islets of Langerhans undergo an epithelial to mesenchymal transition to give rise to MSCs and we hypothesise that these cells lose the CD45 and CD11b positive expression as a result of this transition.

2) **What is the migratory potential of murine MSCs when infused into a mouse? Does the tissue origin of MSCs impact this?** MSCs from the three sources transcribed very low levels of chemokine receptors. However, based on these transcripts MSCs could migrate to the lymph nodes, kidneys, brain, skin and lungs. More importantly, the source of MSC isolation affects the transcription levels of chemokine receptors suggesting that particular tissue sources might increase the potential to get entrapped in the lungs or to migrate towards specific anatomical sites.

3) **Are MSCs able to recruit immune cells? Does MSC microenvironment (stimulation) impact the recruitment capability of MSCs? Does tissue source of origin affect MSCs recruitment potential?** Under resting conditions, all MSCs, regardless of tissue source of origin, had a similar chemokine secretion profile, where CCL2 was the top chemokine secreted by all MSC populations. CCL2 has the potential to recruit monocytes and macrophages with high affinity however, CCL2 secretion did not support leukocyte recruitment above background levels *in vivo*. Inflammatory stimulation of MSCs resulted in upregulated secretion of neutrophil and monocyte/ macrophage chemoattractants, which was mirrored by the recruitment of these cells towards MSCs from the three sources. However, the tissue of origin dictated the chemokine secretion levels and more importantly, secretion levels did not correlate with recruitment levels *in vivo*, suggesting that more factors are involved in leukocyte recruitment.

4) **How do MSCs interact and immunomodulate surrounding tissues? Does MSC microenvironment (stimulation) and tissue origin influence their behaviour?** Under resting conditions, MSCs from the three sources

transcribed and secreted angiogenic and immunomodulatory mediators. However, transcription and expression levels were tissue source of origin dependent. Moreover, inflammatory stimulation influenced each gene and protein in a different manner and no trends were observed. These findings show the complexity of this field and suggest that MSCs isolated from specific tissue sources might possess greater immunomodulatory capacity on the immune cells they attract as well as on the tissue resident cells.

Altogether, this thesis has shown that BM, Is and Ad MSCs had differential transcription levels of chemokine receptors that could influence their migratory potential, as well as differential chemokine, anti-inflammatory and angiogenic molecule secretion profiles. Thus, the data presented in this thesis suggest that MSCs isolated from specific tissues might be more beneficial than other tissue sources of MSCs in distinct clinical settings. For the purpose of this discussion, I will focus on three clinical settings, transplantation and the autoimmune diseases psoriasis and arthritis, and I will discuss the potential benefits of MSCs as a cellular therapy in these settings and whether tissue source of origin could influence outcome.

7.2.1 The optimal tissue for MSC isolation to co-transplant with islets of Langerhans

As T1DM results from the destruction of insulin-producing cells within the islets of Langerhans in the pancreas, pancreas solid organ transplantation (SOT) seems like a reasonable approach. However, it involves major surgery that carries considerable risk of complications, including organ rejection, and is therefore only performed with simultaneous kidney transplantation in diabetic patients in end-stage kidney disease who would already be administered immunosuppressive drugs due to the kidney graft (Chiang et al., 2014). Thus, islet transplantation is an attractive alternative to SOT because the transplantation of a smaller tissue mass makes it minimally invasive and reduces the need for immunosuppression after transplantation (Figliuzzi et al., 2014). The success of islet transplantation expressed as insulin independence 5 years after transplantation is 25% to 50% in international cohort studies (Barton et al., 2012, Bellin et al., 2012). Graft

rejection and failure of islets re-vascularisation are the causes of the low success rate (Ritz-Laser et al., 2002, Blondet et al., 2007, Wood and Goto, 2012). Both innate and adaptive immune responses are involved in graft rejection and involve multiple processes including activation of complement system, antibody mediated rejection, alloantigen specific induction of T cell proliferation and the activation of T cell effector functions (Tjernberg et al., 2008). The anti-inflammatory, immunomodulatory and pro-regenerative properties of MSCs make them a promising candidate for co-transplantation with islets of Langerhans to improve long-term islet transplant function by reducing graft rejection, whilst increasing survival. In fact, diabetic immunodeficient and immunocompetent mouse models support the success of this approach (Forbes et al., 2020).

The liver is the favoured islet transplantation site due to its high vascularization and the bolus of islets is typically infused into the hepatic portal vein. However, islet infusion through this vein can increase the blood pressure, activating the endothelium and promoting leukocyte recruitment into the liver leading to portal vein thrombosis (Kariya et al., 2016, Lalor et al., 2002). For this reason, alternative sources for islet transplantation, including the kidney capsule and the liver surface, are being investigated in mouse models (Fujita et al., 2018, Rajab et al., 2008). To date, the portal vein is the primary site for clinical islet transplantation, for this reason, minimising the size of the graft to avoid portal vein thrombosis is essential. Thus, it is important to consider the size of the co-transplanted MSCs to minimise unwanted inflammation. In the current study, murine BM MSCs were smaller than Is and Ad MSCs (Figure 3–17) under resting and inflammatory conditions, which suggests that infusion of BM MSCs would minimise the development of portal vein thrombosis. However, as previously mentioned, in humans, size of MSCs does not vary significantly between tissue sources and thus, it is not valid interpretation for human therapy (Thirlwell, 2018). CCR7 transcript levels were statistically significantly higher in BM MSCs compared to Is and Ad MSCs under resting and inflammatory conditions (Figure 4-3). Moreover, CXCR6 transcription levels were higher in BM MSCs compared to Is and Ad MSCs under resting conditions, while no variation was observed under inflammatory conditions (Figure 4-4). CCR7 programs naïve T cells and B cells to migrate to the spleen and lymph nodes (Bjorkdahl et al., 2003), while CXCR6 has

been reported to be involved in immune cell trafficking and retention within the liver as it is highly expressed by liver-infiltrating $CD8^+$ T cells (Sato et al., 2005b). Moreover, human BM MSCs have also been shown to express higher levels of CXCR6 than human Is and Ad MSCs (Thirlwell, 2018). These observations suggest that BM MSCs could possess enhanced migratory and retention capacity within the liver and could therefore deliver greater therapeutic effects than Is and Ad MSCs when infused with the bolus of islets into the hepatic portal vein or on the surface of the liver. In contrast, Is MSCs express higher CXCR3 transcript levels than BM and Ad MSCs; thus, Is MSCs could have better clinical outcome if co-transplanted with the islets in the kidney capsule (Figure 4-4).

Once MSCs and islets are infused into the liver, islets must engraft to restore glycaemic awareness and MSCs must therefore avoid the rejection of the allogeneic islets while promoting the re-vascularisation of the islets to promote long-term graft survival.

When co-transplanted into a patient, any cell could have the potential to trigger allospecific T cells. However, MSCs do not express the key antigens involved in immediate rejection-ABO blood group antigens (Moll et al., 2014). This is an essential factor in humans but not in mice as the murine equivalent of the ABO group gene is a *cis*-AB gene that encodes a glycosyltransferase with both A and B transferase activity (Yamamoto et al., 2001). Moreover, mismatched MSCs should not promote alloreactivity as MSCs express very low levels of MHC Class I under resting conditions and only inflammatory stimulation can upregulate the expression of this key molecule involved in graft rejection (Machado et al., 2013). This study has shown that BM, Is and Ad MSCs express very low levels of MHC Class II molecules and inflammatory stimulation only increases the expression of this marker in BM MSCs (Figure 3-18). BM MSCs expressed the highest levels of MHC Class I under resting conditions, Is MSCs expressed low levels, while Ad MSCs barely expressed this marker (Figure 3-18). Cytokine-mediated inflammatory stimulation increased the expression of MHC Class I, with Is MSCs expressing the most. This study found that Ad MSCs expressed the least MHC Class I, suggesting that this source could be better for co-transplantation than BM or Is MSCs. However, MSCs lack the expression of co-stimulatory molecules and they are therefore unlikely to promote activation of T cells based

on mismatched MHC Class I expression. For these reasons, murine MSCs isolated from the bone marrow, islets of Langerhans and adipose tissue will not trigger allospecific T cells and their role will be focused on controlling the activation and proliferation of immune cells generated by the infusion of islets.

After infusion of the islets into the hepatic portal vein the host's immune system can trigger rejection by several mechanisms. Firstly, infusion of islets can induce the activation of complement, leading to the destruction of the graft (Tjernberg et al., 2008, Ricordi and Strom, 2004). CFH is a complement regulatory protein that regulates complement mediated cell destruction by disrupting C3b and avoiding, in this manner, the formation of C3 convertases. CFH deficiency has been associated with increased graft rejection and thus, CFH secretion by MSCs would be a beneficial mechanism for islet survival (Mella et al., 2014, Dragon-Durey et al., 2010). Human MSCs have been reported to secrete CFH under resting conditions and this secretion has been shown to increase under inflammatory conditions, where human BM MSCs were secreting significantly higher amounts of CFH than Is and Ad MSCs (Thirlwell, 2018). In this study, we demonstrate that BM MSCs transcribed statistically significantly more *CFH* than Is and Ad MSCs under resting and stimulatory conditions; however, inflammatory stimulation resulted in the downregulation of *CFH* transcript levels in MSCs from every source (Figure 6-2). Despite RNA levels not always correlating with protein levels, if we assume that transcription levels of *CFH* are proportionately maintained at protein level, BM MSCs would be secreting the most CFH. Therefore, we could hypothesise that BM MSCs would have better therapeutic outcome than Is and Ad MSCs due to their increased potential to avoid complement system activation after islet transplantation.

Secondly, infusion of islets can induce the activation of pre-existing autoimmune T cells within the host as well as the proliferation and activation of allogeneic T cells. Autoreactive $CD4^+$ and $CD8^+$ T cells have the potential to recognise identical antigens in the graft, while auto-antibodies against β-cell antigens can trigger an attack on the graft leading to early graft loss (Sutherland et al., 1984, Jaeger et al., 1997). MSCs have been widely reported to inhibit T cell proliferation as well as to promote a regulatory phenotype in leukocytes via the secretion of anti-inflammatory and immunomodulatory molecules, including IL-

10, TSG-6, TGF-ß, IDO and CD274 (Choi et al., 2008, Li et al., 2019b, Niu et al., 2017, Davies et al., 2017, Chinnadurai et al., 2018). Among all these molecules, *iNOS* was transcribed at the highest levels and IDO is at least in part, involved in the suppressive effect of MSCs on cytotoxic cell proliferation, and thus, immunomodulation. For this reason, Is MSCs could have an increased immunosuppressive potential compared to BM and Ad MSCs, which would support graft tolerance (Figure 6-3). It is important to bear in mind that despite T cells triggering graft rejection, recipient cytotoxic cells are essential to initiate MSC-mediated immunosuppression; MSCs need to be targeted by cytotoxic T cells to undergo apoptosis to be engulfed by macrophages and produce IDO (Galleu et al., 2017).

In addition, to supress the host's immune system to avoid rejection, MSCs must promote islet survival. Islets in the pancreas receive 0% to 15% of arterial blood, providing a very high amount of oxygen to satisfy the metabolic demands to fulfil the physiological activities of the islets. The process of islet isolation destroys the external vasculature and the partial oxygen in the liver is much lower than that in the pancreas; thus, revascularization of the islets is essential to avoid hypoxia and to ensure survival of the graft (Komatsu et al., 2018). Intrahepatic co-transplantation of autologous MSC and islets has been shown to improve islet engraftment after transplantation in a pilot study (Wang et al., 2018). Among the three tissue sources, Is MSCs secreted the most VEGF under resting and stimulatory conditions and thus we hypothesise that Is MSCs would be the preferential tissue source of MSC to promote angiogenesis and islet re-vascularisation. However, increased concentrations of VEGF do not correlate with increased re-vascularization of islets (Carlsson and Mattsson, 2002), which suggests the essential role of other angiogenic mediators like HGF and MMP9.

BM MSCs transcribed the highest levels of *HGF* while *MMP9* was transcribed at similar levels by MSCs from the three sources, suggesting that BM MSCs could have greater re-vascularisation potential, but HGF protein secretion by MSCs from the three sources should be studied. CXCL8 secretion by contaminating duct cells within the islets are currently under study to increase islet engraftment after transplantation (Movahedi et al., 2008). Moreover, co-transplantation of islets and human MSCs into a diabetic mouse model has shown

that IL-6 and CXCL8 secretion by human MSCs enhances vascularization and basement membrane formation (Takahashi et al., 2018). IL-6 was secreted at similar levels by MSCs from the three sources while CXCL1 and CXCL2, the murine equivalents of CXCL8, were secreted at higher rates by Is MSCs, suggesting that Is MSCs could have a greater potential to promote re-vascularization and to therefore, promote graft survival. This hypothesis is not in agreement with Citro et al., as they reported that inhibition of the receptors for CXCL1 and CXCL2 (CXCR1 and CXCR2) enhanced pancreatic islet survival after transplantation (Citro et al., 2012). CXCR1 and CXCR2 blockade would lead to decreased neutrophil recruitment towards the graft. Neutrophils are considered detrimental in transplantation due to their role promoting inflammation. However, as discussed in Chapter 5, Christoffersson et al. identified a $CD11b^{+}$/ $Gr\text{-}1^{+}$/ $CXCR4^{high}$ murine neutrophil subset that was recruited by VEGF-a into the site of islet engraftment that lead to the revascularization of transplanted islets (Christoffersson et al., 2012). More importantly, this neutrophil subset produced MMP9 *and* the revascularization of transplanted islets was MMP9 *dependent and* $CD11b^{+}$/ $Gr\text{-}1^{+}$/ $CXCR4^{high}$ *dependent*. *In Chapter 5,* neutrophils were the predominant $CD45^{+}$ cell type recruited towards MSCs *in vivo*. Under resting conditions, MSCs from any source were unable to recruit neutrophils towards the air pouch. However, inflammatory stimulation of MSCs prior to infusion into the air pouch led to a statistically significant recruitment of neutrophils by MSCs from every source, where Ad MSCs were able to produce the highest recruitment of neutrophils. Therefore, I propose that MSCs could promote graft re-vascularization not only by the well-described VEGF and HGF secretion but by the extensive secretion of pro-angiogenic cytokines (IL-6), matrix metallopeptidases (MMP9) and chemokines (CXCL1 and CXCL2) that recruit neutrophils. However, this hypothesis is based on an air pouch model instead of an islet transplant model. Moreover, the flow panel used for neutrophil identification did not include specific markers for neutrophil phenotyping.

In conclusion, BM, Is and Ad MSCs express different levels of CXCR3 and CXCR6 which could impact MSC migration and retention within the liver. The current study highlights that MSCs differentially transcribed immunoregulatory mediators like CFH, IL-10, TSG-6, TGF-β, iNOS and CD274, suggesting differential potential to suppress effector T cell activation and to induce proliferation and activation

of regulatory T cells. Moreover, MSCs isolated from all sources secreted substantial amounts of CXCL1, CXCL2, VEGF, and IL-6 which could have an impact on the re-vascularisation of the graft. These differences among MSC tissue source of isolation not only have an impact on islet transplantation but in any transplantation setting. Additionally, as discussed in Chapter 5, the tissue source of MSCs leads to differences in their immune cell attraction profile, which could be detrimental or beneficial in an islet transplant setting, depending on the phenotype of the immune cells attracted.

7.2.2 The optimal tissue for MSC isolation for potential therapeutic use in psoriasis

Psoriasis is a multifactorial inflammatory disease involving the skin, joints, or both. It is mediated by both the innate and adaptive immune systems and affects around 2% of people in the UK and worldwide. Psoriasis can be triggered by several factors including injury and trauma, infection and medications; imiquimod, a TLR7 agonist, induces skin inflammation like psoriasis and is therefore used in murine models. Histologically, psoriasis is characterised by: epidermal hyperplasia, leukocyte infiltrate, and increased dermal vascularity (Casciano et al., 2018). Little is known about the initiation of psoriasis but models suggest that injury to the skin leads to AMP LL37 production by keratinocytes, which upon interaction with DNA or RNA activates plasmacytoid dendritic cells (pDCs) and myeloid DCs, leading to the secretion of IFN-α and IFN-β and promoting T cell activation (Lowes et al., 2014). Supporting this model of psoriasis initiation, neutrophil extracellular traps (NETs) and extracellular DNA have been found in the epidermis under inflammatory conditions (Kumar and Sharma, 2010). DCs secrete TNF-α and IL-23 and present self-antigens on their surface, leading to the activation of autoreactive $CD8^+$ T cells and the polarization of $CD4^+$ T cells towards a Th17 phenotype in the dermis. Activated T cells migrate to the epidermis where they recognise self-antigens, leading to the secretion of cytokines, including IL-22, which promote epidermal hyperproliferation and activation of keratinocytes, leading to the secretion of more inflammatory molecules that promote progression of the disease (Zaba et al., 2009). Once in the epidermis, activated T cells can differentiate towards tissue-resident memory T cells (Trm), where Trm with a pathogenic IFN-γ-IL-17A cytokine profile have been described as potential drivers of disease memory in

resolved psoriatic lesions (Matos et al., 2017, Cheuk et al., 2014). Moreover, Bovenschen et al. described a dysfunction of regulatory T cells in psoriasis as they differentiate towards IL-17 expressing Tregs, promoting in this manner exacerbated chronic inflammation (Bovenschen et al., 2011).

Unfortunately, there is no fully satisfactory therapy against psoriasis yet. There are multiple palliative treatment options and it often depends on patient preferences, as treatment satisfaction is very variable among patients. Primary care level is based on topical agents like corticosteroids, vitamin D3 analogues or the combination of both, which are well tolerated and effective with mild psoriasis. When psoriasis is wide-spread and topical agents are not enough, they are usually combined with systemic therapy, mainly phototherapy and methotrexate. Methotrexate is a well-known inhibitor of folate biosynthesis with a very good record of success in psoriasis. However, it is not always tolerated or suitable, as it is not compatible with pregnancy, and biologic therapy has emerged as a strong alternative (Kim et al., 2017). Biological therapy is based on the use of inhibitors that specifically target a biological mediator of a pathophysiological process, including anti-TNF-α agonists and anti-p40 agonists, where p40 is a subunit shared by both IL-12 and IL-23. The formation of antibodies against these inhibitors has been documented and affects their long-term efficacy (Sivamani et al., 2010). As explained in the previous section, the potential of MSCs to immunomodulate and suppress effector T cell activities provides a rationale for the clinical use of MSCs in diseases in which T cell hyperactivation contributes to the onset of the disease. The use of stem cells for the treatment of psoriasis started gathering strong support when, in 2009, a 35-year old man with psoriasis was diagnosed with diffuse large B-cell lymphoma. His treatment included autologous haematopoietic stem cell transplantation and unexpectedly, six months later, his psoriasis had significantly resolved, twelve months later his skin had become essentially normal and his condition remained normal for 5 years (Chen et al., 2016).

A study conducted by Lee et al. showed that psoriasis-like skin inflammation generated by imiquimod or IL-23 was ameliorated after MSC infusion, which reduced the expression of the proinflammatory cytokines IL-6 and IL-17 as well as the expression of chemokines such as CCL17, CCL20, and CCL27 (Lee et al.,

2017b). Chen et al. infused umbilical cord derived MSCs into a 26-year-old woman once a week for three weeks and another two MSC infusions three months later. As a result, the psoriasis had been in remission for four years in 2016, when the study was published (Chen et al., 2016). Intravenous infusion of stromal vascular fraction, a mixture of Ad MSCs, progenitor cells and other cells obtained from fat, into psoriasis patient demonstrated a significant alleviation of the symptoms (Comella et al., 2018). MSC conditioned medium accelerates skin wound healing *in vitro* in fibroblast and keratinocyte scratch assays (Walter et al., 2010) and the results from the first clinical report on the use of MSC conditioned medium demonstrates that the secretion of immunomodulatory molecules, cytokines and growth factors by MSCs ameliorate the symptoms of psoriasis (Seetharaman et al., 2019).

Psoriasis can appear anywhere in the skin as well as in the joints, for this reason local administration is not an option. When delivered by systemic infusion, only a small percentage of the cells home and remain in the target tissue due to MSC trapping in the lung (Fischer et al., 2009, Schrepfer et al., 2007). CCR3, CCR4 and CCR10 are highly expressed by T cells in skin (Fujimoto et al., 2008, Ma et al., 2002) while CXCR4 expression is increased on the surface of extravascular neutrophils in the lung and the bone marrow (30). *CCR3*, *CCR4* and *CCR10* transcript levels were very low in MSCs from every source but *CCR3* transcript levels were statistically significantly higher in Ad MSCs compared to BM and Is MSCs (Figure 4-3), suggesting that Ad MSCs could have an increased potential to migrate towards the skin. In contrast, *CXCR4* transcript levels were highest in Ad MSCs, which could favour lung entrapment. These observations, combined with the smaller size of BM MSCs, suggest that BM MSCs would have a better chance of avoiding lung entrapment and migrating towards target areas despite lower *CCR3* transcript levels.

Once MSCs are infused into the psoriasis patient, MSCs must supress effector T cells and activate regulatory T cells to dampen and control excessive inflammation. The mechanisms by which MSCs could inhibit T cell proliferation as well as promote a regulatory phenotype of leukocytes via the secretion of anti-inflammatory and immunomodulatory molecules has already been described in the previous sections and Is MSCs have been suggested to have increased

immunosuppressive potential compared to BM and Ad MSCs and could therefore, be more effective in regulating excessive inflammation in the skin. However, it is important to mention that deficiency of IL-10 and TGF-ß in serum and skin are important elements of psoriasis pathogenesis. MSCs have been reported to secrete IL-10 and TGF-ß, promoting the generation of immunosuppressive Treg subsets both *in vitro* and *in vivo* (Sah et al., 2016). In this study, MSCs from the three sources transcribed very low levels of *IL-10* (Figure 6-3). Under resting conditions, MSCs from the three sources expressed substantial *TGF-ß* transcript levels; inflammatory stimulation upregulated *TGF-ß* transcript level in BM and Ad MSCs and Ad MSCs reached very high levels of transcription (Figure 6-3). If protein levels correlate with transcription, we could suggest that infusion of Ad MSCs would be more beneficial than infusion of BM or Is MSCs for the treatment of psoriasis.

IL-6 has been associated with the pathogenesis of psoriasis and increased levels of this cytokine in the skin and serum is a characteristic of this disease (Arican et al., 2005, Neuner et al., 1991). Moreover, IL-6 levels are positively correlated with clinical severity and effective treatment of psoriasis results in a reduction of IL-6 levels (Mizutani et al., 1997). BM MSCs expressed the highest transcript levels of *IL-6* under resting and stimulatory conditions and inflammatory stimulation led to an upregulation of *IL-6* in MSCs from every source, especially in BM MSCs. At the protein level, MSCs isolated from the three tissues produced very little IL-6 under resting conditions (Figure 6-3); however, inflammatory stimulation resulted in the upregulation of IL-6 to similar levels in MSCs from the three sources (Figure 6-7). IL-6 promotes anti-inflammatory Th2 cell differentiation (Diehl et al., 2002) and increases IL-27 secretion by monocytes and macrophages promoting the maturation of regulatory T cells (Pyle et al., 2017). However, IL-6 has also been reported to inhibit TGF-ß-induced Treg differentiation and to promote the differentiation of pro-inflammatory Th17 cells from naïve T cells, disrupting the immunological tolerance and leading to autoimmune and chronic inflammatory diseases (Bettelli et al., 2006, Tanaka et al., 2014). Due to the dual roles of IL-6 it is difficult to rationalise the role of IL-6 secretion by MSCs and its roles in psoriasis.

In conclusion, BM, Is and Ad MSCs have different sizes and express different levels of CCR3, CCR4, CCR10 and CXCR4 which could impact lung entrapment and MSC migration towards the skin. The current study highlights that MSCs differentially transcribed IL-10 and TGF-β, suggesting differential potential to counteract the deficiency of IL-10 and TGF-β in serum and skin in psoriasis.

7.2.3 The optimal tissue for MSC isolation for potential therapeutic use in rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease characterised by the production of auto-antibodies targeting the joints and producing pain, swelling and even changing the joint's shape, which can cause bone and cartilage break down; it affects up to 1% of the worldwide population. Rheumatoid arthritis flares can be triggered by several factors including overextension related injury, trauma and infections. The pathophysiology of RA starts with synovitis, followed by subsequent cartilage destruction and bone erosion leading to loss of joint function (Milner and Day, 2003). The aetiology, timing and anatomic site of RA is currently unknown. It is well established that auto-antibodies like rheumatoid factor and antibodies to citrullinated peptides can be increased in the serum years prior to the onset of the first flare of joint inflammation (Demoruelle et al., 2014). In addition to auto-antibodies, biomarkers of systemic inflammation like cytokines, chemokines and C reactive protein can also be identified in the serum. Evaluation of the joints during this period of high auto-antibody levels and no joint symptoms shows no histologic or magnetic resonance imaging (MRI) evidence of RA (van de Sande et al., 2011), which suggests that RA related autoimmunity does not initiate in the joints. There are several findings which suggest that RA initiates in mucosal sites including oral, lungs and gut mucosal sites (Demoruelle et al., 2014). Necrosis results in the release of endogenous nuclear material, which can be recognised by TLRs leading to the generation of citrullinated endogenous proteins (Makrygiannakis et al., 2006). Moreover, recruitment of neutrophils to mucosal sites can produce tissue citrullination, generating, in this manner, antibodies to citrullinated peptides (Khandpur et al., 2013). Furthermore, mucosal inflammation has been associated with an increase in rheumatoid factor (Elkayam et al., 2006). Three immunologic mechanisms to explain the transition of autoimmunity from the mucosal sites into articular sites have been considered. First, immune complex formation could lead to

transportation and deposition in the synovium; Zhao et al. described immune complexes containing citrullinated fibrinogen in plasma as well as in the joint tissue (Zhao et al., 2008). Second, a shared antigenic target between the mucosal sites and the joints; to support this, Ytterberg et al. described the presence of a citrullinated vimentin protein both in the lungs and joint tissues of patients suffering from RA (Ytterberg et al., 2015). Lastly, epitope spreading and subsequent migration of activated T cells; Huo et al. reported that antigen-specific T cell responses can traffic to a different organ (Huo et al., 2012). Altogether, these three mechanisms would explain how auto-immunity could be generated in the mucosal sites and trigger symptoms in a completely different tissue.

As with psoriasis, there is no fully satisfactory therapy against RA. There is a large variety of drugs to reduce the symptoms and the progression of the disease to avoid the loose of joint mobility due to bone and cartilage break down. For this purpose, all the treatments aim to dampen inflammation including disease-modifying anti-rheumatic drugs like methotrexate and sulfasalazine and biologic response modifiers. Due to the anti-inflammatory, immunomodulatory and regenerative properties of MSCs, infusion of these cells could contribute to the repair of damaged cartilage as well as dampening the inflammation and avoiding, in this manner, further damage. In support of this hypothesis, allogeneic MSC infusion and transplantation of chondrocytes differentiated from allogeneic MSCs were able to suppress the responses of type-II collagen reactive T cells *in vitro* (Zheng et al., 2008). Furthermore, MSC infusion into collagen-induced arthritis mouse models reduced the severity of RA symptoms. At present, there are several trials that include MSCs for the treatment of RA. So far, the systemic infusion of BM and umbilical cord derived MSCs in a RA group that did not respond to other treatments lead to the decrease of anti-cyclic citrullinated peptide antibodies and to amelioration of symptoms (Liang et al., 2012).

RA can appear in any joint of the body and, as previously described, it is reported to initiate in mucosal sites, thus, local administration of MSCs is not an alternative. As already mentioned, when delivered by intravenous infusion, only a small percentage of the cells home and remain in the target tissue due to MSC

trapping in the lung (Fischer et al., 2009, Schrepfer et al., 2007). However, in this particular clinical scenario, as autoimmunity towards joints is initiated in mucosal sites, lung entrapment could be an advantage instead of an unfavourable circumstance. On the one hand, Is and Ad MSCs are statistically significantly bigger in size than BM MSCs. On the other hand, *CXCR4* transcript levels were highest in Ad MSCs and we could therefore hypothesise that Ad MSCs are more likely to remain in the lungs where they could dampen mucosal inflammation by regulating effector T cells and at the same time, promoting a regulatory phenotype. As explained in Section 7.2, MSCs inhibit T cell proliferation and promote a regulatory phenotype of leukocytes via the secretion of anti-inflammatory and immunomodulatory molecules. In this study Is MSCs transcribe higher levels of *iNOS* and for this reason it has been suggested that Is MSCs could have increased immunosuppressive potential compared to BM and Ad MSCs, which suggests that Is MSCs could be more effective in regulating excessive inflammation in the lungs. However, it is important to mention that TSG-6 and IL-10 have been reported to be essential for dampening the autoimmune response in RA and for this reason further discussion will follow.

TSG-6 levels have been found to be increased in the synovial fluid, in the blood vessel walls of inflamed synovium and to a lesser extent in serum (Wisniewski et al., 1993, Bayliss et al., 2001). Its presence within these locations has been associated with the roles of TSG-6 in cell proliferation and extracellular matrix remodelling (Ye et al., 1997). The use of autoimmune polyarthritis inducible mouse models has enabled an investigation of the roles of systemic recombinant TSG-6 and of TSG-6 produced locally by T cells in the arthritic joints of transgenic mice. In this way, it has been shown that TSG-6 was a potent inhibitor of inflammation which protected cartilage and bone and thus, prevented joint destruction (Mindrescu et al., 2000, Mindrescu et al., 2002). Moreover, treatment of collagen-induced arthritis in mice with TSG-6 lead to a significant reduction of antibodies against type II collagen (Mindrescu et al., 2002). BM MSCs expressed higher levels of *TSG-6* (Figure 6-3), under resting and inflammatory conditions, than Is and Ad MSCs, and 24-hour stimulatory licensing led to the downregulation of these transcripts in MSCs from all sources. If transcript and protein levels correlate, BM MSCs could have the potential to produce increased

inhibition of inflammation and in that way, promote a better clinical outcome in the context of RA.

IL-10 has been found in RA synovial membrane biopsies and spontaneous production of IL-10 in cell suspension cultures of RA synovial membranes has been described. Moreover, neutralization of IL-10 led to an increase of TNF-α and IL-1β secretion *in vitro*, which are strongly implicated in the pathology of RA. More importantly, exogenous addition of IL-10 to these cultures led to a significant decrease of TNF-α and IL-1β secretion and therefore, IL-10 could be an effective RA treatment (Katsikis et al., 1994). Systemic infusion of IL-10 had a negligible effect in both mouse models and clinical trials (Whalen et al., 1999, van Roon et al., 2003). However, efficacy increased when IL-10 was delivered locally or tagged to an antibody fragment specific to damaged arthritic cartilage (Hughes et al., 2014). Infusion of MSCs alone into mouse and rat models of inflammatory RA produced no effect, while injection of IL-10 transduced MSCs significantly decreased the severity of arthritis whilst also decreasing anti-collagen II antibodies and T cell proliferation (Hughes et al., 2014, Peruzzaro et al., 2019). These results show the therapeutic relevance of high doses of IL-10. Ad MSCs transcribed significantly higher levels of *IL-10* compared to BM and Is MSCs and for this reason, Ad MSCs could be a better source of MSCs for an IL-10 mediated RA therapy.

In conclusion, BM, Is and Ad MSCs have different sizes and express different levels of CXCR4 which could impact retention within the lung. The current study highlights that MSCs differentially transcribed TSG-6 and IL-10, suggesting differential potential to immunoregulate the exacerbated immune response in RA.

7.3 Overview

It is widely reported that MSCs have the potential to switch pro-inflammatory environments into anti-inflammatory and pro-regenerative environments by immunomodulating surrounding immune cells; for this reason, MSCs are involved in an increasing number of clinical trials. As previously mentioned, BM MSCs are

considered the gold standard MSC as they were the first MSCs isolated and used within the clinic; however, their yield within the bone marrow is very low and isolation imposes a burden on the donor. For this reason, alternative sources with higher frequency of MSCs within the tissue and that are isolated in a less invasive and harmful manner are being investigated. The variability in tissue sources, donors, isolation and culture procedures as well as differences in passage number has led to controversial results through the literature making the comparison of MSCs isolated from different sources nearly impossible. It is important to point out that there are few studies that have examined the immunogenicity of MSCs *in vivo* and many of those studies have been conducted with human MSCs (Thirlwell, 2018) which usually lack a control for mismatched major histocompatibility complex molecule expression. Infusion of MHC-mismatched MSCs into an inflammatory environment leads to both cell-mediated and humoral immune responses (Eliopoulos et al., 2005, Joswig et al., 2017). For these reasons, the standardised methodologies throughout this study allowed comparative study of the potential *in vivo* differences of MSCs isolated from the bone marrow, islets of Langerhans and adipose tissue. Through the assessment of chemokine receptors at transcript level this study highlighted that MSCs isolated from different tissue sources could have differential migration potential. This is critical when considering which tissue source of MSC to administer when MSCs are being infused systemically, where high specific chemokine receptor surface expression could enhance homing to target tissues. In contrast, when administered locally, low chemokine receptor surface expression would enhance retention within the tissue of infusion.

Through the assessment of chemokine ligands at transcript and protein level, this study highlighted that MSCs isolated from different tissue sources could have differential interactions with their surrounding environment when infused into a patient. Under resting conditions, all MSCs regardless of tissue source of origin had a similar chemokine secretion profile, where CCL2 was the top chemokine secreted by all MSC populations. Despite CCL2 and other chemokine secretion at lower levels, infusion of resting BM, Is and Ad MSCs did not lead to leukocyte recruitment above background levels. Inflammatory stimulation of MSCs resulted in the upregulation of CC and CXC chemokine secretion with an emphasis on CCL2 and CXCL1 secretion. The secretion of these chemokines was followed by

chemoattraction of neutrophils and macrophages towards MSCs isolated from all tissue sources; however, the number of cells attracted towards MSCs was tissue of origin dependent, where Ad MSCs attracted statistically significantly more leukocytes. These observations have huge clinical implications due to the described role of inflammatory chemokine secretion and the subsequent monocyte, macrophage and neutrophil recruitment in pathogenesis in several clinical settings.

Furthermore, the anti-inflammatory and immunomodulatory properties of MSCs have been studied by thorough analysis of the complement system, TLRs and multiple inflammatory and extra cellular matrix remodelling mediators. The transcription of these molecules was MSC source dependent and inflammatory-dependent patterns were not observed. Variability in the transcription and secretion of anti-inflammatory and angiogenic factors demonstrates that tissue source of origin could have a major impact in MSC mediated immunomodulation within a clinical setting.

To sum up, the stringent set of standardised methodologies established in this study to objectively compare the phenotype, migration, interaction with the environment and immunomodulation potential demonstrates the complexity of the MSC field and shows the need for further studies to fully understand the role of each of the assessed molecules in MSC *in vivo* behaviour.

7.4 Hypothesis

I therefore hypothesise that the source for MSC isolation would influence the clinical outcome of MSC infusion via the differential expression of chemokine receptors and the specific secretion of chemokine ligands and anti-inflammatory and immunomodulatory mediators. These differences lead to differential recruitment of immune cells by MSCs while also affecting their interactions with the recruited and tissue resident cells. Furthermore, I strongly believe that there are other undiscovered factors involved in the differential behaviour of MSCs isolated from different sources, which may have a critical impact on MSC clinical potential.

7.5 Conclusions

This study provides a highly standardised methodology for murine bone marrow, islet of Langerhans and adipose MSC isolation, characterisation and comparison. The findings detailed in this thesis show the variability of MSC behaviour according to the source for MSC isolation and the surrounding environment, resting or inflammatory.

Differences in the transcription and expression of chemokine receptors, chemokines and other angiogenic and immunoregulatory molecules suggest that their *in vivo* function within a clinical setting could potentially be different and this could have a major impact for the choice of MSC tissue of isolation.

7.6 Future directions

This study has generated novel data that will help to further understand the potential use of MSCs as cellular therapeutics. However, the experiments described have their own limitations and this project would benefit from further testing to gain a better understanding of the differences among MSCs isolated from different sources.

To confidently compare MSCs migratory potential, experiments on chemokine receptor expression by MSCs must be performed. Moreover, expression and functionality do not always come together, and intracellular calcium flux assays should be performed to study if receptors are able to recognise, interact with and signal in the presence of ligand. Furthermore, *in vitro* MSC migration assays would test the migratory potential of MSCs. As an example, CCL19 or CCL21, the two well characterised CCR7 ligands, could be used to assess and compare MSC migratory capacity using a transwell system with increasing concentrations of these chemokines or via a CCR7 blocker. In this manner, we could confidentially conclude which tissue source of MSCs possessed greater migratory capacity towards specific tissues, in this case, towards the lymph nodes. To further confirm this hypothesis, *in vivo* trafficking and retention of MSCs should be studied via fluorescent labelling of MSC. To rule out nonspecific migration or to

identify the involvement of other receptors in migration, genetically modified MSCs with specific chemokine receptor knock out should be used as negative controls.

As previously mentioned, chemokine secretion was not correlated with leukocyte recruitment *in vivo*. As an example, Is MSCs secreted the most CXCL1 and CXCL2 but Ad MSCs recruited the most neutrophils. These findings suggest that even if CXCL1 and CXCL2 are strong neutrophil chemoattractants, there are other molecules involved in neutrophil migration or that only a small concentration of CXCL1 and CXCL2 is needed to induce neutrophil migration and once that is reached, increased secretion will not produce an increment in neutrophil recruitment. Therefore, MSCs could recruit leukocytes via alternative mechanisms. In the case of neutrophils, the VEGF-VEGFR or GMCSF-GMCSFR axis could be involved. Therefore, transwell analysis with the appropriate chemoattractants and the appropriate receptor blockers should be performed to gain insight in the mechanisms used by MSCs to migrate towards specific tissues.

The *in vivo* air pouch model assessed the immune cell attraction profile of MSCs from the three sources. However, 24 hours might not have been enough time for the adaptive immune cells to migrate towards the air pouch. For this reason, a second time point at 72 hours would allow us to better study the infiltration of innate and adaptive immune cells mediated by the infusion of MSCs within the air pouch. Furthermore, despite the fluorescent labelling of the cells we could not find any MSC within the air pouch. MSCs could have migrated elsewhere and for this reason, fluorescent labelling could be used to try find them in blood, bone marrow or lymph nodes, among other tissues.

Lastly, this study would benefit from the study and comparison of MSCs isolated from various tissues in different clinical models such as mouse islet transplantation, psoriasis and RA models. In this way, we could clarify if differences in transcription and expression of chemokines and their receptors, as well as other immunoregulatory molecules, could have, as hypothesised, an impact in different clinical settings.

8 Appendices

8.1 Analysis of the effect of a double inflammatory stimulus over time on the transcription of chemokines and chemokine receptors

As previously discussed, pro-inflammatory cytokine-mediated MSC licensing produced a transcriptional regulation of chemokines, which could explain why MSCs are described to be more immunosuppressive upon activation. Within clinical settings, it has been reported that pre-treatment of MSCs with inflammatory cytokines prior to administration within animal models of inflammatory diseases boosts the therapeutic effect of MSCs (Duijvestein et al., 2011, Noronha et al., 2019). For this reason, we wondered if pre-licensing the MSCs prior to expose them to an inflammatory environment *in vitro* would lead to even bigger variations in chemokines transcriptional levels and therefore, in enhanced therapeutic potential. To study this, cells were pre-licensed for 48 hours, after which cells were washed twice with PBS and stimulated again for another 24 hours. The first stimulation primes the MSCs while the second stimulation would mimic the inflammatory environment MSCs would face when infused into a patient with an inflammatory disorder. Figure 8-1 illustrates the time points at which supplemented medium was added. Culture medium was removed at the time cells were harvested and was kept for experimental procedures.

Two different control conditions were used. In the first one, cells were stimulated for 48 hours, after which cells were washed twice with PBS and fresh culture medium was added; lastly, cells were harvested 24 hours later. In the second condition, cells were washed twice with PBS, the culture medium was replaced with fresh one and the cells were left growing for 48 hours. Cells were then washed twice with PBS, the culture medium was replaced with supplemented one and the cells were harvested 24 hours later. There is wide literature about how cytokine-mediated licensing enhances the potential therapeutic efficacy of MSCs however, little is known about the role of TLR ligands mediated activation on the secretion of chemotactic cytokines by MSCs. For this reason, 100 ng/ mL LPS, 100 ng/ mL LTA and 4 mg/ mL Poly I:C, as well as the previously described cytokine cocktail, was used for MSC licensing.

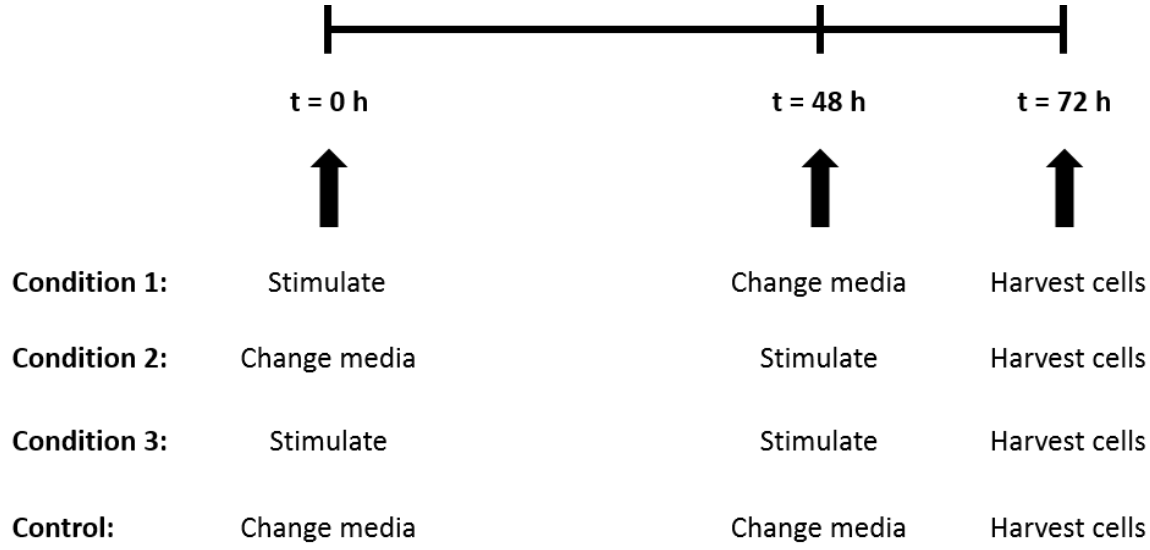


Figure 8-1. Diagrammatic illustration of the time course of the MSC licensing.

According to the results described in Section 4.2, the genes that regulated their transcript levels in a statistically significant manner upon stimulation were selected and their transcriptional levels were assessed after licensing with different inflammatory molecules and different inflammatory conditions.

8.1.1 Transcription of CC chemokines under resting and inflammatory conditions

Under resting conditions, BM, Is and Ad MSCs transcribed very little, if any, CC chemokines with the exception of *CCL2*, which was transcribed by all tissue sources of MSCs at similar levels (Figure 8-2, A), and *CCL7*, which was very highly expressed by BM MSCs (D).

A pattern of transcriptional upregulation was observed in *CCL2* (Figure 8-2, A), *CCL3* (B), *CCL5* (C), *CCL7* (D) and *CCL20* (E) genes after licensing in every condition; however, these chemokines were upregulated differentially in MSCs according to their tissue of origin and licensing agent. CC chemokine transcript levels 24 hours after stimulation increased (Condition 2) in MSCs from every tissue source; however, this upregulation was not sustained, as cells harvested 72 hours after licensing (Condition 1) showed a decrease in the transcript levels of CC chemokines. A second stimulation 48 hours after the first stimulation (Condition 3) was able to induce the transcription of CC chemokines in MSCs from the three sources; however, the second stimulation was not able to match

the transcript levels of CC chemokines found in Condition 2 in MSCs from every source with the exception of Ad MSCs after cytokine-mediated stimulation, where these cells massively increased the transcript levels of CC chemokine ligands. Fold changes of transcriptional regulation upon licensing of MSCs are specified in Table 8-1.

As previously described, BM MSCs had the highest *CCL2* transcript levels under resting conditions (Figure 8-2, A). After 72 hours of stimulation (Condition 1), cytokine-mediated licensing led to an increase of the transcript levels in BM and Is MSCs and to a decrease in Ad MSCs. 24 hours after stimulation with the cytokine cocktail (Condition 2), MSCs from the three tissues upregulated their *CCL2* transcript levels. A second stimulation with the cytokine cocktail 48 hours after the first stimulation (Condition 3) was able to induce the production of *CCL2* in MSCs from the three sources; however, the second stimulation was not able to match the transcript levels of *CCL2* found in Condition 2 in BM and Is MSCs. Ad MSCs, at the contrary, produced 202 times more *CCL2* after the second stimulation than Ad MSCs under resting conditions or 16 times more than in Condition 2. After 72 hours of stimulation (Condition 1), LPS-mediated licensing led to an increase of *CCL2* transcript levels in Is and Ad MSCs and to a decrease in BM MSCs, while LTA and Poly I:C-mediated licensing only produced an upregulation of *CCL2* in Is MSCs and a downregulation in BM and Ad MSCs. After 24 hours of stimulation (Condition 2), LPS produced no variation in *CCL2* transcript levels in BM MSCs but produced an increase of transcript levels in Is and Ad MSCs; LTA had no effect on Is MSCs but led to an upregulation of the transcript levels in BM and Ad MSCs. Lastly, Poly I:C produced an upregulation of *CCL2* transcript levels in MSCs from every source. Double stimulation (Condition 3) with any of the licensing agents led to higher transcript levels of *CCL2* than cells that had been harvested 72 hours after stimulation (Condition 1), but lower levels than those obtained by the cells harvested 24 hours after stimulation.

BM MSCs had the highest *CCL3* transcript levels under resting conditions (Figure 8-2, B). After 72 hours of stimulation (Condition 1), cytokine-mediated licensing led to a decrease in the transcript levels of *CCL3* in BM MSCs, produced no effect in Is MSCs and led to an increase in Ad MSCs; LPS licensing led to a decrease of the transcript levels in BM and Ad MSCs and produced no effect in Is MSCs and

LTA and Poly I:C licensing produced a decrease on *CCL3* transcript levels in MSCs from the three sources. After 24 hours of stimulation (Condition 2), cytokine-mediated licensing led to an increase of the transcript levels in BM and Is MSCs and led to a decrease in Ad MSCs; LPS licensing led to a decrease of the transcript levels in BM and Ad MSCs and produced an increase in Is MSCs; LTA licensing produced no effect in BM MSCs and led to a decrease of the transcript levels in Is and Ad MSCs; lastly, Poly I:C led to a decrease of *CCL3* transcript levels in MSCs from every source. Double stimulation (Condition 3) with the cytokine cocktail led to a decrease of the transcript levels in BM MSCs, produced no effect in Is MSCs and led to an increase in Ad MSCs; LPS licensing led to a decrease of the transcript levels in Is MSCs and had no effect in BM and Is MSCs; LTA licensing led to a decrease in *CCL3* transcript in MSCs from every source while Poly I:C had no effect in BM MSCs and produced a decrease in *CCL3* transcript levels in Is and Ad MSCs.

CCL5 transcript levels were very low under resting conditions, where BM MSCs had the highest levels of transcript and Ad MSCs the lowest, but inflammatory stimulation led to substantial levels in MSCs from the three sources (Figure 8-2, C). Cytokine-mediated licensing produced an upregulation of *CCL5* transcript levels in MSCs from every source under every condition. 72 hours after stimulation (Condition 1) MSCs from every tissue source had undergone at least a 7.35 fold change in *CCL5* transcript levels; 24 hours after stimulation (Condition 2) MSCs from the three tissues had higher transcript levels than in Condition 1, reaching significant amounts in the case of BM and Is MSCs. Double stimulation (Condition 3) led to the upregulation of *CCL5* transcript to levels that matched the ones achieved in Condition 1 in BM and Is MSCs; Ad MSCs, however, had 419 times higher amounts of transcript compared to resting conditions. 72 hours of LPS licensing produced an upregulation of *CCL5* transcript levels in BM and Is MSCs and barely produced any effect in Ad MSCs; LTA licensing had no effect in BM MSCs, led to a small downregulation in Is MSCs and produced a hundred times fold change in Ad MSCs. Poly I:C-mediated stimulation led to an upregulation of *CCL5* transcript levels in MSCs from the three sources, where this upregulation was the highest in BM MSCs even if Is MSCs had a higher amount of *CCL5* transcripts. Double stimulation (Condition 3) with the cytokine cocktail led to an increase of the transcript levels in MSCs from the three sources, but Ad MSCs

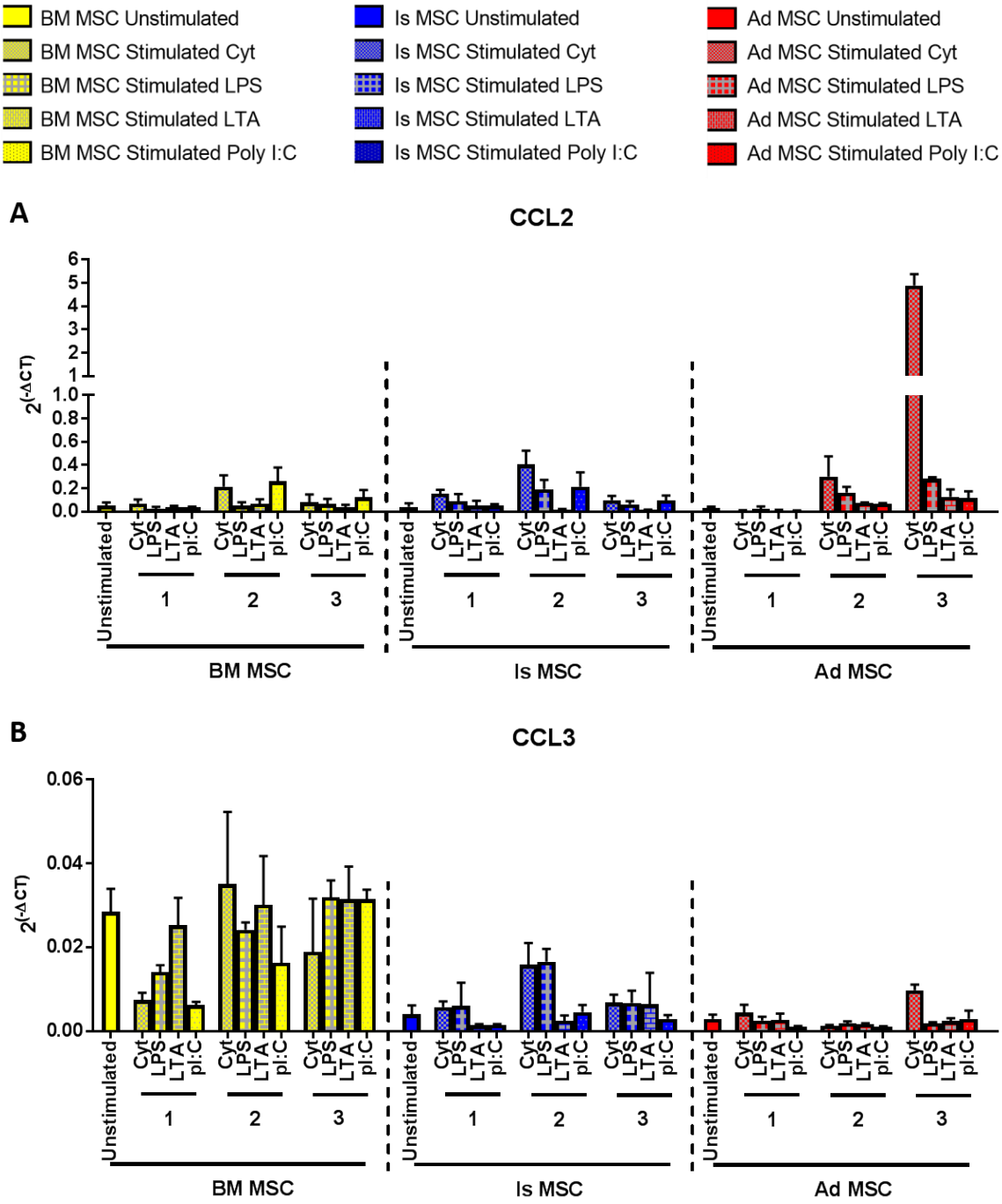
produced significantly higher amounts of transcript than BM and Is MSCs. LPS-mediated licensing led to an upregulation in MSCs from the three sources and this upregulation was higher than the one observed in Condition 1 and smaller than the one in Condition 2 for BM and Ad MSCs. Is MSCs produced the highest *CCL5* transcript levels in Condition 1 after LPS licensing. LTA-mediated stimulation produced no effect in BM and Is MSCs after double stimulation and produced a small downregulation in Ad MSCs. Poly I:C, on the contrary, produced a ≥117-fold change in BM and Is MSCs and a smaller upregulation in Ad MSCs.

As previously discussed, *CCL7* was expressed at very substantial levels under resting conditions in all MSCs; however, tissue of origin of MSCs had an effect in the transcript levels as BM MSCs expressed much higher transcript levels than Is and Ad MSCs (Figure 8-2, D). After 72 hours of stimulation (Condition 1), cytokine-mediated licensing led to a decrease of the transcript levels in BM MSCs, while it produced a small increase in Is and Ad MSCs. 24 hours after stimulation with the cytokine cocktail (Condition 2), MSCs from the three tissues upregulated their *CCL7* transcript levels. A second stimulation with the cytokine cocktail 48 hours after the first stimulation (Condition 3) was able to induce the production of *CCL7* in Is and Ad MSCs, however, the second stimulation was not able to match the transcript levels of *CCL7* observed in Condition 2. BM MSCs, on the contrary, downregulated their *CCL7* transcript levels after the double stimulation with the cytokine cocktail. After 72 hours of stimulation (Condition 1), LPS and LTA-mediated licensing led to a decrease of *CCL7* transcript levels in BM MSCs, led to an increase in Is MSCs and produce no effect in Ad MSCs. Poly I:C-mediated licensing produced a downregulation of *CCL7* transcript levels in MSCs from every source after 72 hours of stimulation (Condition 1). After 24 hours of stimulation (Condition 2), LPS produced no variation in *CCL7* transcript levels in BM MSCs but produced an increase of transcript levels in Is and Ad MSCs; LTA led to a slight decrease in BM MSCs but led to an upregulation of the transcript levels of *CCL7* in Is and Ad MSCs. Lastly, Poly I:C had no effect in BM MSCs but produced an upregulation of *CCL7* transcript levels in Is and Ad MSCs. Double stimulation (Condition 3) with any of the licensing agents led to higher transcript levels of *CCL7* in Is MSCs when compared to cells that had been harvested 72 hours after stimulation (Condition 1), but lower levels than those obtained by the cells harvested 24 hours after stimulation. Cytokine-mediated

double stimulation (Condition 3) led to a decrease of *CCL7* transcript levels in BM MSCs, produced an upregulation in Is MSCs smaller than the one obtained in Condition 2 and produced 8 times more *CCL7* transcript in Ad MSCs compared to Condition 2. LPS-mediated double stimulation led to an increase of *CCL7* transcript levels in MSCs from all sources, LTA-mediated double stimulation led to a decrease of *CCL7* transcript levels in BM and Ad MSCs while it upregulated *CCL7* transcript levels in Is MSCs. Lastly, Poly I:C-mediated double stimulation increased *CCL7* transcript levels in BM and Is MSCs while it decreased *CCL7* transcript levels in Ad MSCs.

CCL20 transcript levels were almost undetectable under resting conditions, with $2^{(-\Delta CT)} \leq 0.00059$, but inflammatory stimulation led to substantial transcript expression levels in MSCs from the three sources (Figure 8-2, E). After 72 hours of stimulation (Condition 1), cytokine-mediated licensing led to an increase of *CCL20* transcript levels in BM and Is MSCs while it produced a decrease in Ad MSCs. 24 hours after stimulation with the cytokine cocktail (Condition 2), MSCs from the three tissues massively upregulated their *CCL20* transcript levels, with fold changes of up to 630 in BM MSCs. A second stimulation with the cytokine cocktail 48 hours after the first stimulation (Condition 3) was able to induce the transcription of *CCL20* in MSCs from every source, however, the second stimulation was not able to match the transcript levels of *CCL20* found in Condition 2 in BM and Is MSC, while Ad MSCs transcribed similar amounts to Condition 2. 72 hours LPS-mediated stimulation (Condition 1) led to the upregulation of the transcript levels in Is MSCs but had barely no effect in BM and Ad MSCs; however, 24 hours LPS-mediated stimulation (Condition 2) led to the upregulation of transcript levels in MSCs from the three sources. Once again, a second stimulation with LPS 48 hours after the first stimulation (Condition 3) was able to induce the production of *CCL20* in MSCs from every source but the levels produced could not match the ones from Condition 2 except for BM MSCs. 72 hours of LTA-mediated stimulation (Condition 1) led to no effect in BM MSCs and to a reduction of the transcript levels of *CCL20* in Is and Ad MSCs. 24 hours stimulation (Condition 2), on the contrary, led to an upregulation of *CCL20* transcript in BM and Ad MSCs, while Is MSCs produced less transcript levels. Double stimulation of the cells (Condition3) with LTA produced a slight upregulation in BM MSCs, a downregulation in Is MSCs and led to no effect on the

transcript levels of *CCL20* in Ad MSCs. 72 hours of Poly I:C-mediated stimulation led to a slight upregulation on BM MSCs while it had no significant effect on *CCL20* transcript levels in Is and Ad MSCs. 24 hours stimulation (Condition 2) led to an upregulation in MSCs from every source while double stimulation with Poly I:C led to an upregulation in BM and Is MSCs that did not match the transcript levels produced by these cells after 24 hours of stimulation (Condition 2). Double stimulation with Poly I:C produced a downregulation of *CCL20* transcript levels to almost undetectable amounts.



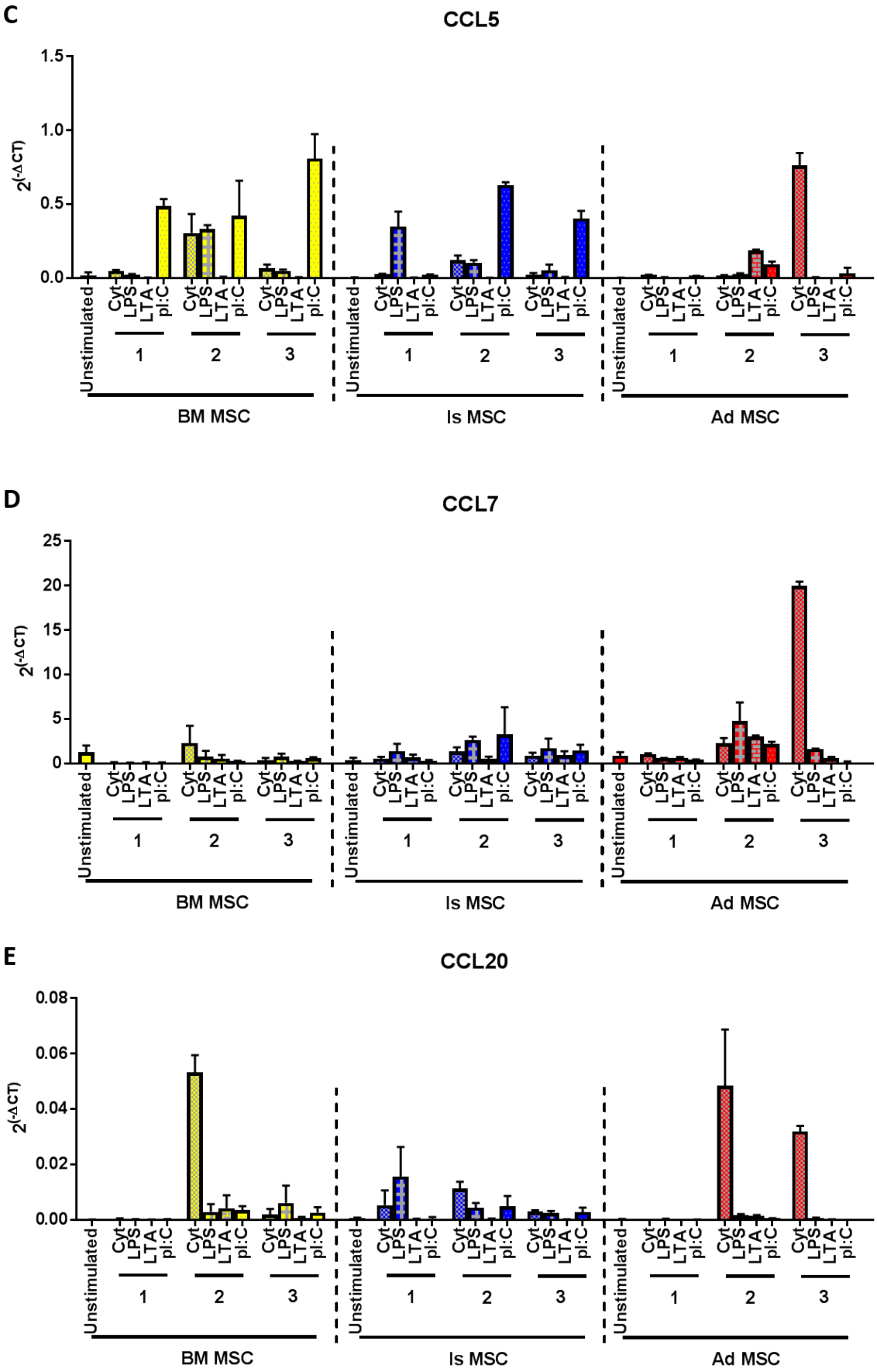


Figure 8-2. Inflammatory agent, repetitive stimulus and MSC tissue origin impacts CC chemokine transcript levels in MSCs.

MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with either a cocktail of cytokines (40 ng/ mL of

IFN-Y, TNF-α and IL-1β), 100 ng/ mL LPS, 100 ng/ mL LTA or 4 μg/ mL Poly I:C. Unstimulated cells were left growing in MSC culture medium as a control. Three different licensing conditions were tested. In the first one, cells were stimulated for 48 hours, after which cells were washed twice with PBS and fresh culture medium was added; cells were harvested 24 hours later. In the second condition, cells were washed twice with PBS, the culture medium was replaced with fresh one and the cells were left growing for 48 hours. Cells were then washed twice with PBS, the culture medium was replaced with supplemented one and the cells were harvested 24 hours later. In the last condition, cells were stimulated for 48 hours, after which cells were washed twice with PBS and were stimulated again for another 24 hours. Figure 4-7 illustrates the time points at which supplemented medium was added. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate CCL transcripts in BM, Is and Ad MSCs under resting and inflammatory conditions. Each bar represents an n of 4 independent experiments and is graphed as mean ± SEM. Data are normalised to the housekeeping gene *B2M* and expressed as $2^{(-\Delta CT)}$. Statistically significant differences are marked with a colour code in Table 4-7.

Table 8-1. Fold change in CC chemokine transcript levels of cytokine, LPS, LTA or Poly I:C-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.

Following the experimental set up explained in Figure 8-2, fold change in transcript levels of CC chemokines is represented as mean of fold change ± standard deviation. One Way ANOVA with Tukey's multiple comparisons post-test was performed to compare all MSC sources and the different conditions. p = 0.05 was considered the limit for statistical significance. Statistically significant differences are marked with a colour code, where p < 0.05 is represented by green, p < 0.01 is represented by orange, p < 0.001 is represented by blue and p < 0.0001 is represented by red.

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
CCL2	1	Cyt	1.84 ± 1.43	8.07 ± 1.77	0.51 ± 0.11
		LPS	0.71 ± 0.56	4.58 ± 2.60	1.21 ± 1.04
		LTA	0.75 ± 0.15	2.67 ± 1.79	0.56 ± 0.06
		Poly I:C	0.89 ± 0.33	2.66 ± 0.62	0.41 ± 0.03
	2	Cyt	4.24 ± 1.14	22.93 ± 5.23	6.77 ± 1.70
		LPS	0.98 ± 0.29	10.01 ± 3.32	6.71 ± 1.76
		LTA	1.32 ± 0.36	1.02 ± 0.15	3.21 ± 0.60
		Poly I:C	5.39 ± 1.85	10.97 ± 5.25	2.88 ± 0.56
	3	Cyt	1.38 ± 0.77	5.02 ± 1.31	202.76 ± 51.81
		LPS	1.26 ± 0.51	3.19 ± 1.18	11.66 ± 2.49
		LTA	0.82 ± 0.53	0.80 ± 0.09	5.01 ± 1.65
		Poly I:C	2.28 ± 0.79	4.96 ± 1.86	4.47 ± 1.76
CCL3	1	Cyt	0.27 ± 0.07	1.02 ± 0.24	1.26 ± 0.45
		LPS	0.50 ± 0.03	1.03 ± 0.75	0.76 ± 0.31
		LTA	0.89 ± 0.14	0.24 ± 0.04	0.73 ± 0.19
		Poly I:C	0.22 ± 0.03	0.27 ± 0.07	0.30 ± 0.10
	2	Cyt	1.23 ± 0.50	3.57 ± 1.01	0.36 ± 0.06
		LPS	0.88 ± 0.16	3.06 ± 0.97	0.56 ± 0.16
		LTA	1.04 ± 0.27	0.45 ± 0.21	0.51 ± 0.08
		Poly I:C	0.61 ± 0.33	0.82 ± 0.25	0.32 ± 0.06
	3	Cyt	0.64 ± 0.35	1.21 ± 0.30	2.88 ± 0.69
		LPS	1.14 ± 0.14	1.18 ± 0.43	0.57 ± 0.15
		LTA	1.16 ± 0.37	1.22 ± 1.37	0.70 ± 0.22
		Poly I:C	1.13 ± 0.13	0.52 ± 0.12	0.84 ± 0.52
CCL5	1	Cyt	11.59 ± 10.85	7.35 ± 2.09	10.95 ± 3.50

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
		LPS	4.58 ± 4.07	**96.57 ± 22.51**	2.63 ± 0.41
		LTA	0.25 ± 0.20	0.70 ± 0.30	0.46 ± 0.20
		Poly I:C	**107.27 ± 97.50**	5.82 ± 1.15	7.86 ± 2.49
	2	Cyt	**48.22 ± 45.00**	**29.01 ± 11.82**	9.58 ± 1.87
		LPS	**70.79 ± 63.95**	**29.90 ± 10.46**	16.00 ± 5.69
		LTA	1.61 ± 1.53	0.35 ± 0.07	**104.45 ± 33.37**
		Poly I:C	**138.31 ± 134.11**	**177.83 ± 38.71**	**52.32 ± 16.60**
	3	Cyt	10.25 ± 8.63	5.92 ± 1.49	**419.49 ± 93.64**
		LPS	10.17 ± 9.13	13.12 ± 6.50	2.83 ± 1.06
		LTA	1.22 ± 1.18	0.64 ± 0.55	0.56 ± 0.26
		Poly I:C	**208.49 ± 195.87**	**117.21 ± 39.74**	23.07 ± 23.86
CCL7	1	Cyt	0.39 ± 0.30	1.82 ± 2.02	1.62 ± 0.86
		LPS	0.23 ± 0.14	4.56 ± 5.13	0.90 ± 0.47
		LTA	0.22 ± 0.10	2.30 ± 2.68	0.95 ± 0.46
		Poly I:C	0.33 ± 0.22	0.73 ± 0.49	0.66 ± 0.32
	2	Cyt	1.29 ± 0.70	5.27 ± 5.15	**3.77 ± 2.14**
		LPS	1.14 ± 0.20	**7.54 ± 6.36**	**6.60 ± 2.51**
		LTA	0.87 ± 0.08	1.85 ± 2.26	**4.59 ± 2.26**
		Poly I:C	0.88 ± 0.68	13.03 ± 17.73	**3.38 ± 1.83**
	3	Cyt	0.55 ± 0.07	2.87 ± 3.03	**30.78 ± 15.81**
		LPS	1.72 ± 0.72	6.00 ± 7.25	2.53 ± 1.32
		LTA	0.86 ± 0.67	3.06 ± 3.48	0.92 ± 0.48
		Poly I:C	1.58 ± 0.91	5.10 ± 5.83	0.09 ± 0.04
CCL20	1	Cyt	4.23 ± 0.74	8.74 ± 7.80	0.18 ± 0.14
		LPS	2.45 ± 1.14	**28.10 ± 13.76**	2.13 ± 1.91
		LTA	1.17 ± 0.63	0.35 ± 0.25	0.02 ± 0.01
		Poly I:C	2.19 ± 0.50	0.68 ± 0.47	0.59 ± 0.57
	2	Cyt	**631.34 ± 148.34**	**19.27 ± 2.37**	**470.60 ± 222.05**
		LPS	42.76 ± 42.62	7.47 ± 2.27	14.68 ± 6.22
		LTA	64.25 ± 66.43	0.57 ± 0.25	13.20 ± 4.91
		Poly I:C	45.35 ± 26.57	8.32 ± 6.09	5.25 ± 2.10
	3	Cyt	27.91 ± 26.21	5.21 ± 0.89	**298.33 ± 107.66**
		LPS	87.39 ± 80.84	4.40 ± 1.63	5.93 ± 2.20
		LTA	8.18 ± 7.03	0.12 ± 0.15	1.01 ± 0.35
		Poly I:C	36.44 ± 30.10	5.21 ± 2.65	0.00 ± 0.00

8.1.2 Transcription of CXC chemokines under resting and inflammatory conditions

Under resting conditions, BM, Is and Ad MSCs transcribed very little, if any, CXC chemokines with the exception of *CXCL1*, which was transcribed by BM MSCs (Figure 8-3). A pattern of transcriptional upregulation was observed in *CXCL1* (Figure 8-3, A), *CXCL2* (B), *CXCL5* (C), *CXCL10* (D) and CXCL16 (F) after licensing

in every condition, while stimulation of MSCs led to the downregulation of *CXCL12* (E). However, these chemokines were differentially regulated in MSCs according to their tissue of origin and licensing agent.

CXCL1, *CXCL5*, *CXCL10* and *CXCL16* chemokine transcript levels increased 24 hours after stimulation (Condition 2) in MSCs from every tissue source; however, this upregulation was not sustained, as cells harvested 72 hours after licensing (Condition 1) showed a decrease in CC chemokines transcript levels. *CXCL2* chemokine transcript levels increased after 24 hours stimulation (Condition 2) in MSCs from every tissue source. However, while this upregulation was not sustained in BM and Is MSCs, Ad MSCs expressed higher amounts of *CXCL2* transcript levels 72 hours after licensing (Condition 1). A second stimulation 48 hours after the first stimulation (Condition 3) was able to induce the transcription of *CXCL1*, *CXCL2*, *CXCL5*, *CXCL10* and *CXCL16* chemokines in MSCs from the three sources, however, the second stimulation was not able to match the transcript levels of CXC chemokines found in Condition 2 in MSCs from every source with the exception of Ad MSCs after cytokine-mediated stimulation, where these cells massively increased the transcript levels of CXC chemokine ligands. *CXCL12* transcriptional levels were very dependent on MSC source and licensing agent; cytokine-mediated licensing produced a downregulation of the transcriptional levels in every condition in BM and Is MSCs, while it produced an upregulation in Ad MSCs. LPS-mediated licensing produced a downregulation of *CXCL12* levels in BM and Ad MSCs, while it was able to induce the transcription levels in Is MSCs after 72 hours stimulation (Condition 1). LTA licensing led to a trend of upregulation in BM MSCs while it produced no statistically significant effect on Is and Ad MSCs. Lastly, Poly I:C stimulation produced a downregulation in BM MSCs, no variation in Is MSCs and a downregulation in Ad MSCs which was overcome after a double stimulation (Condition 3). Fold changes of transcriptional regulation upon licensing of MSCs are specified in Table 8-2.

As previously described, BM MSCs had the highest *CXCL1* transcript levels under resting conditions (Figure 8-3, A). After 72 hours of stimulation (Condition 1), cytokine-mediated licensing led to no variation in the transcript levels in BM MSCs, an increase in Is MSCs and to a decrease in Ad MSCs. 24 hours after stimulation with the cytokine cocktail (Condition 2), MSCs from the three tissues

upregulated their *CXCL1* transcript levels, with Is MSCs expressing the highest levels. A second stimulation with the cytokine cocktail 48 hours after the first stimulation (Condition 3) was able to induce the production of *CXCL1* in MSCs from the three sources; however, the second stimulation was not able to match the transcript levels of *CXCL1* found in Condition 2 in BM and Is MSCs. Ad MSCs, at the contrary, produced 57 times more *CXCL1* after the second stimulation than Ad MSCs under resting conditions or 11 times more than in Condition 2. After 72 hours of stimulation with LPS, LTA or Poly I: C (Condition 1), *CXCL1* levels were almost undetectable in BM and Ad MSCs, while no variation was observed in Is MSCs. 24 hours stimulation with LPS produced a statistically non-significant upregulation in MSCs from every source while double stimulation (Condition 3) with LPS led to higher transcript levels of *CXCL1* in BM and Ad MSCs compared to Condition 1, but lower levels than those obtained by the cells harvested 24 hours after stimulation. On the contrary, double stimulation of Ad MSCs with LPS (Condition 3) produced an increase of *CXCL1* transcript levels.

As previously described, BM MSCs had the highest *CXCL2* transcript levels under resting conditions (Figure 8-3, B). After 72 hours of stimulation (Condition 1), cytokine-mediated licensing led to no variation in the transcript levels of *CXCL2* in BM MSCs, a decrease in Is MSCs and to 64 times increase in Ad MSCs. On the contrary, 24 hours stimulation (Condition 2) led to a transcriptional upregulation in MSCs from every tissue source; however, the upregulation in Ad MSCs is 18 times lower than in the previous condition. A second stimulation with the cytokine cocktail 48 hours after the first stimulation (Condition 3) was able to induce the production of *CXCL2* in MSCs from every source, but the transcript levels produced did not match the ones from Condition 2 for BM and Is MScs, while for Ad MSCs the *CXCL2* transcript levels were higher than in Conditions 1 and 2. 24 hours of LPS licensing (Condition 1) led to the increase of *CXCL2* levels in BM and Is MSCs, while it led to a decrease in Ad MSCs. Analysis of *CXCL2* transcript levels after 72 hours of LPS licensing showed a bigger upregulation in BM MSCs compared to the previous condition, while Is MSCs upregulated the transcription of *CXCL2* but could not match the expression levels achieved in Condition 1; Ad MSCs upregulated their *CXCL2* transcript levels but these were still very low. After 72 hours of stimulation (Condition 1), LTA-mediated licensing led to no variation in the transcript levels of *CXCL2* in BM MSCs and to a

decrease in Is and Ad MSCs, while 24 hours of licensing led to upregulation in BM and Ad MSCs and to no variation in Is MSCs. A second stimulation with the cytokine cocktail 48 hours after the first stimulation (Condition 3) led to upregulation in BM MSCs, to downregulation in Is MSCs and to no variation in Ad MSCs. 72 hours of Poly I:C stimulation (Condition 1) led to a decrease of *CXCL2* transcript levels in MSCs from every source while 24 hours of licensing (Condition 2) led to an increase of *CXCL2* transcript levels in MSCs from every source. Double stimulation of MSCs (Condition 3) led to no variation in BM MSCs and to a statistically non-significant increase in Is and Ad MSCs.

As previously described, *CXCL5* expression levels were almost undetectable under resting conditions in Is and Ad MSCs, while BM MSCs had the highest *CXCL5* transcript levels (Figure 8-3, C). After 72 hours of stimulation (Condition 1), cytokine-mediated licensing led to a decrease in BM MSCs, an increase in Is MSCs and no variation in Ad MSCs. 24 hours after stimulation with the cytokine cocktail (Condition 2), MSCs from the three tissues upregulated their *CXCL5* transcript levels, with BM MSCs expressing the highest levels. A second stimulation with the cytokine cocktail 48 hours after the first stimulation (Condition 3) was able to induce the production of *CXCL5* in MSCs from the three sources; however, the second stimulation was not able to match the transcript levels of *CXCL5* found in Condition 2 in BM and Is MSCs. Ad MSCs, at the contrary, produced 400 times more *CXCL5* after the second stimulation than Ad MSCs under resting conditions or 27 times more than in Condition 2. After 72 hours of stimulation with LPS (Condition 1), *CXCL5* levels were downregulated to almost undetectable levels in BM MSCs, while Is and Ad MSCs upregulated their levels even if they were still very low. 24 hours stimulation with LPS produced a statistically non-significant upregulation in BM and Is MSCs while Ad MSCs upregulated their *CXCL5* transcript levels in a statistically significant manner. A second stimulation of MSCs 48 hours after the first one (Condition 3) with LPS led to higher transcript levels of *CXCL5* in MSCs from every source compared to Condition 1, but lower levels than those obtained by the cells harvested 24 hours after stimulation. After 72 hours of stimulation with LTA (Condition 1), *CXCL5* levels were downregulated to almost undetectable levels in MSCs from every source, while 24 hours licensing with LTA produced no variation in BM MSCs but an upregulation of the transcript levels in Is and Ad MSCs. A second LTA

stimulation 48 hours after the first one led to a downregulation of *CXCL5* transcript levels in MSCs from every source. After 72 hours of stimulation with Poly I:C (Condition 1), *CXCL5* levels were downregulated to almost undetectable levels in MSCs from every source, with BM MSCs showing the lowest levels of *CXCL5* transcript. 24 hours licensing with Poly I:C (Condition 2) produced a similar downregulation as Condition 1 in BM MSCs, had no effect in Is MSCs but led to an upregulation of the transcript levels in Ad MSCs. A second Poly I:C stimulation 48 hours after the first one led to a downregulation of *CXCL5* transcript levels in BM MSCs while it produced an upregulation in Is and Ad MSCs bigger than the one in Condition 2.

CXCL10 levels were very low in MSCs from all the tissue sources under resting conditions, with Is MSCs having the highest transcript levels (Figure 8-3, D). After 72 hours of stimulation (Condition 1), cytokine-mediated licensing led to a decrease in the transcript levels in BM and Is MSCs, while it produced no variation in Ad MSCs. 24 hours after stimulation with the cytokine cocktail (Condition 2), MSCs from the three tissues upregulated their *CXCL10* transcript levels, with Is MSCs expressing the highest levels. A second stimulation with the cytokine cocktail 48 hours after the first stimulation (Condition 3) was able to induce the production of *CXCL10* in MSCs from the three sources, however, the second stimulation was not able to match the transcript levels of *CXCL10* found in Condition 2 in BM and Is MSCs. Ad MSCs, at the contrary, produced 900 times more *CXCL10* after the second stimulation than Ad MSCs under resting conditions or 18 times more than in Condition 2. After 72 hours of stimulation with LPS (Condition 1), *CXCL10* levels were downregulated to almost undetectable levels in BM and Ad MSCs, while Is MSCs upregulated their levels even if they were still very low. 24 hours stimulation with LPS produced an upregulation in MSCs from the three sources. A second stimulation of MSCs 48 hours after the first one (Condition 3) with LPS led to no variation in *CXCL10* transcript levels in BM and Is MSCs and to a small downregulation in Ad MSCs. After 72 hours of stimulation with LTA (Condition 1), *CXCL10* levels were downregulated to almost undetectable levels in BM and Ad MSCs, while it produced no effect on Is MSCs. 24 hours licensing (Condition 2) with LTA produced no variation in BM and Is MSCs but an upregulation of the transcript levels on Is MSCs. Double stimulation of MSCs (Condition 3) with LTA led to a downregulation in BM MSCs and to an

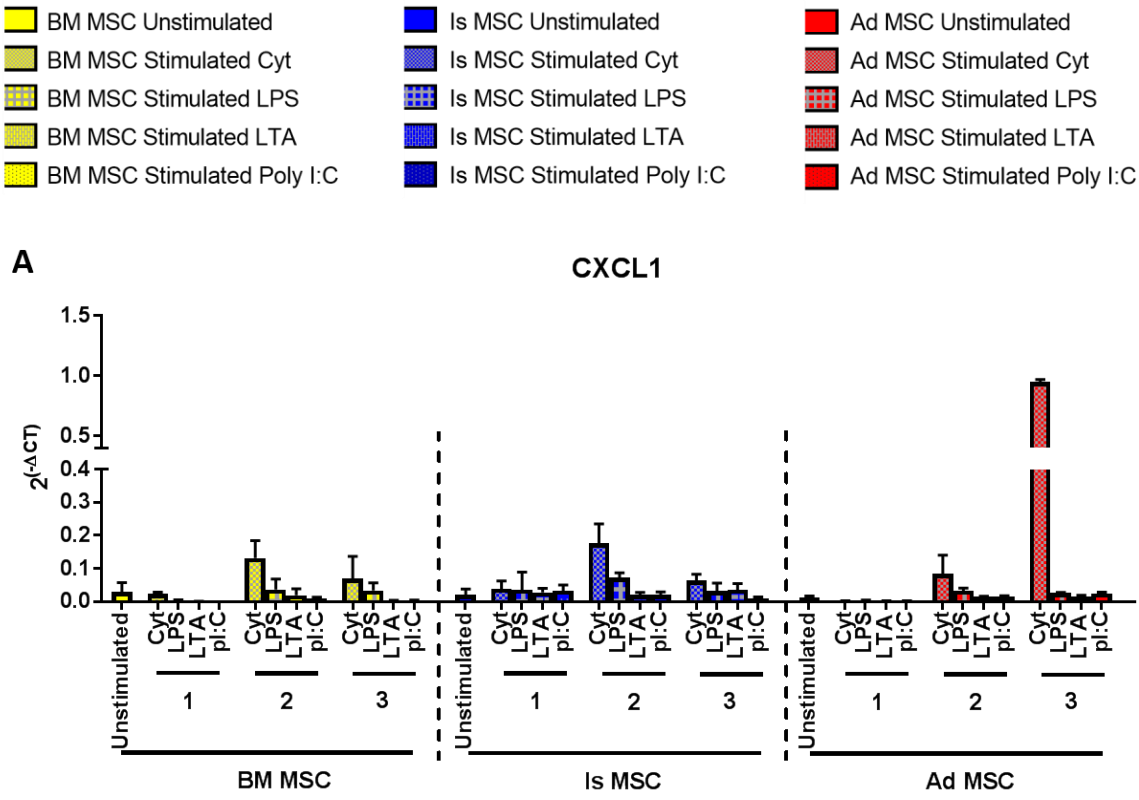
upregulation in Is and Ad MSCs. 72 hours of Poly I:C-mediated licensing (Condition 1) led to downregulation of *CXCL10* transcript levels in MSCs from every source. After 24 hours of licensing, MSCs from every source upregulated their transcript levels but this upregulation was only significant in BM MSCs. A second stimulation of MSCs 48 hours after the first one (Condition 3) with Poly I:C was able to induce the production of *CXCL10* in BM and Is MSCs; however, the double stimulation was not able to match the transcript levels of *CXCL10* found in Condition 2. On the contrary, double stimulation of Ad MSCs with Poly I:C led to a downregulation of the transcript levels.

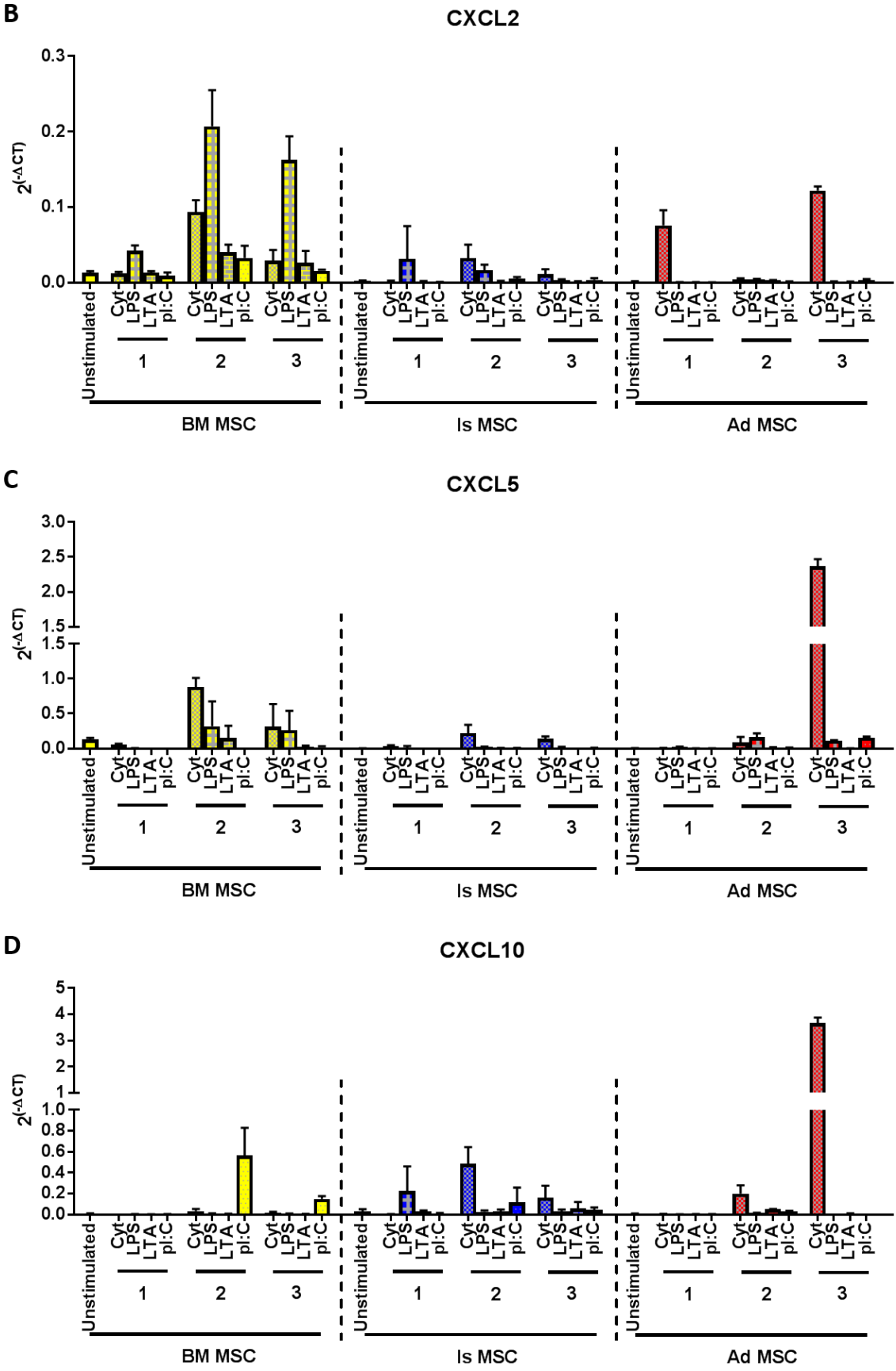
As previously mentioned, during resting conditions BM MSCs were able to produce significantly higher transcript amounts of *CXCL12* than Is and Ad MSCs, which produced very little (Figure 8-3, E). After 72 hours of stimulation (Condition 1), cytokine-mediated licensing led to a decrease of the transcript levels in BM and Is MSCs, while it produced an increase in Ad MSCs. 24 hours after stimulation with the cytokine cocktail (Condition 2), BM and Is MSCs had downregulated their transcript levels and this downregulation was more pronounced in Is MSCs compared to Condition 1. On the other hand, Ad MSCs upregulated their *CXCL12* transcript levels after 24 hours licensing with the cytokine cocktail. A second stimulation with the cytokine cocktail 48 hours after the first stimulation (Condition 3) was able to increase the transcript levels obtained after a single stimulation; however, despite being higher than in Conditions 1 and 2, the transcript levels found in BM and Is MSCs were still lower than in resting cells. 24 hours stimulation with the cytokine cocktail led to doubling the transcript levels of Ad MSCs, while the double stimulation was able to increase five times the amount of *CXCL12* transcript in Ad MSCs. After 72 hours of stimulation with LPS (Condition 1), *CXCL12* levels were downregulated in BM and Ad MSCs, while Is MSCs upregulated their levels; 24 hours stimulation produced a downregulation in BM MSCs and an upregulation in Is and Ad MSCs. A second stimulation of MSCs 48 hours after the first one (Condition 3) with LPS led to a downregulation of *CXCL12* transcript levels in BM and Ad MSCs and to a small upregulation in Is MSCs. 72 hours of licensing with LTA (Condition 1) produced no effect on *CXCL12* transcript levels in BM and Ad MSCs, while it produced an upregulation in Is MSCs. 24-hour licensing (Condition 2) with LTA produced a small upregulation in BM and Is MSCs and no variation in Ad MSCs,

while a second stimulation 48 hours after the first one (Condition 3) led to an upregulation in BM and Ad MSCs and to a downregulation in Is MSCs. 72 hours of Poly I:C-mediated licensing (Condition 1) led to downregulation of *CXCL12* transcript levels in BM and Ad MSCs while it led to an upregulation in Is MSCs. 24 hours after Poly I:C licensing (Condition 2) *CXCL12* transcript levels were downregulated in BM MSCs, but the transcript levels were not as low as the ones obtained on Condition 1; Is MSCs had upregulated their *CXCL12* transcript levels to the same level as in Condition 1 and Ad MSCs showed no variation on their transcript levels. A second stimulation of MSCs 48 hours after the first one (Condition 3) with Poly I:C was able to induce the downregulation of *CXCL12* transcript level in BM MSCs, while it produced an upregulation of *CXCL12* transcript level in Is and Ad MSCs.

Lastly, *CXCL16* transcript was barely expressed by MSCs of every source during resting conditions (Figure 8-3, F). After 72 hours of stimulation (Condition 1), cytokine-mediated licensing led to an increase of the transcript level in BM and Ad MSCs, while it produced a decrease in Is MSCs. 24 hours after stimulation with the cytokine cocktail (Condition 2), MSCs from every source had upregulated their *CXCL16* transcript level, with Ad MSCs expressing the highest transcript levels. A second stimulation with the cytokine cocktail 48 hours after the first stimulation (Condition 3) was able to increase the transcript levels of *CXCL16* in MSCs from the three sources; however, the second stimulation was not able to match the transcript levels of *CXCL16* found in Condition 2 in BM and Is MSCs. Ad MSCs, on the contrary, were able to produce even higher *CXCL16* transcript levels after a second stimulation with the cytokine cocktail. After 72 hours of stimulation with LPS (Condition 1), *CXCL16* levels were upregulated in BM MSCs, no variation was observed in Is MSCs and *CXCL16* transcript levels were downregulated in Ad MSCs. 24 hours licensing with LPS produced an upregulation in MSCs from every source while a second stimulation of MSCs 48 hours after the first one (Condition 3) with LPS led to an upregulation of *CXCL16* transcript levels in BM and Ad MSCs and to a small downregulation in Is MSCs. 72 hours of LTA-mediated stimulation (Condition 1) produced an upregulation of *CXCL16* transcript levels in BM MSCs, while it produced a downregulation of the transcript levels in Ad and Is MSCs. 24 hours licensing (Condition 2) with LTA produced an upregulation in BM and Ad MSCs and a small downregulation in Is

MSCs. A second stimulation 48 hours after the first one (Condition 3) led to an upregulation of *CXCL16* in MSCs from every source, but this upregulation was only significant in BM MSCs. 72 hours of Poly I:C-mediated licensing (Condition 1) led to no variation in BM MSCs while it led to a downregulation of *CXCL16* transcript levels in Is and Ad MSCs. 24 hours after Poly I:C licensing (Condition 2) *CXCL16* transcript levels were upregulated in a statistically non-significant manner in MSCs from every source. A second stimulation of MSCs 48 hours after the first one (Condition 3) with Poly I:C was able to induce the upregulation of *CXCL16* transcript levels in MSCs from every source in similar levels as the ones observed in Condition 2.




B
CXCL2
2(-ΔCT)
0.3
0.2
0.1
0.0
Unstimulated
Cyt
LPS
LTA
pI:C
1
2
3
BM MSC
Is MSC
Ad MSC
C
CXCL5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
D
CXCL10
5
4
3
2
1
0.8
0.6
0.4
0.2

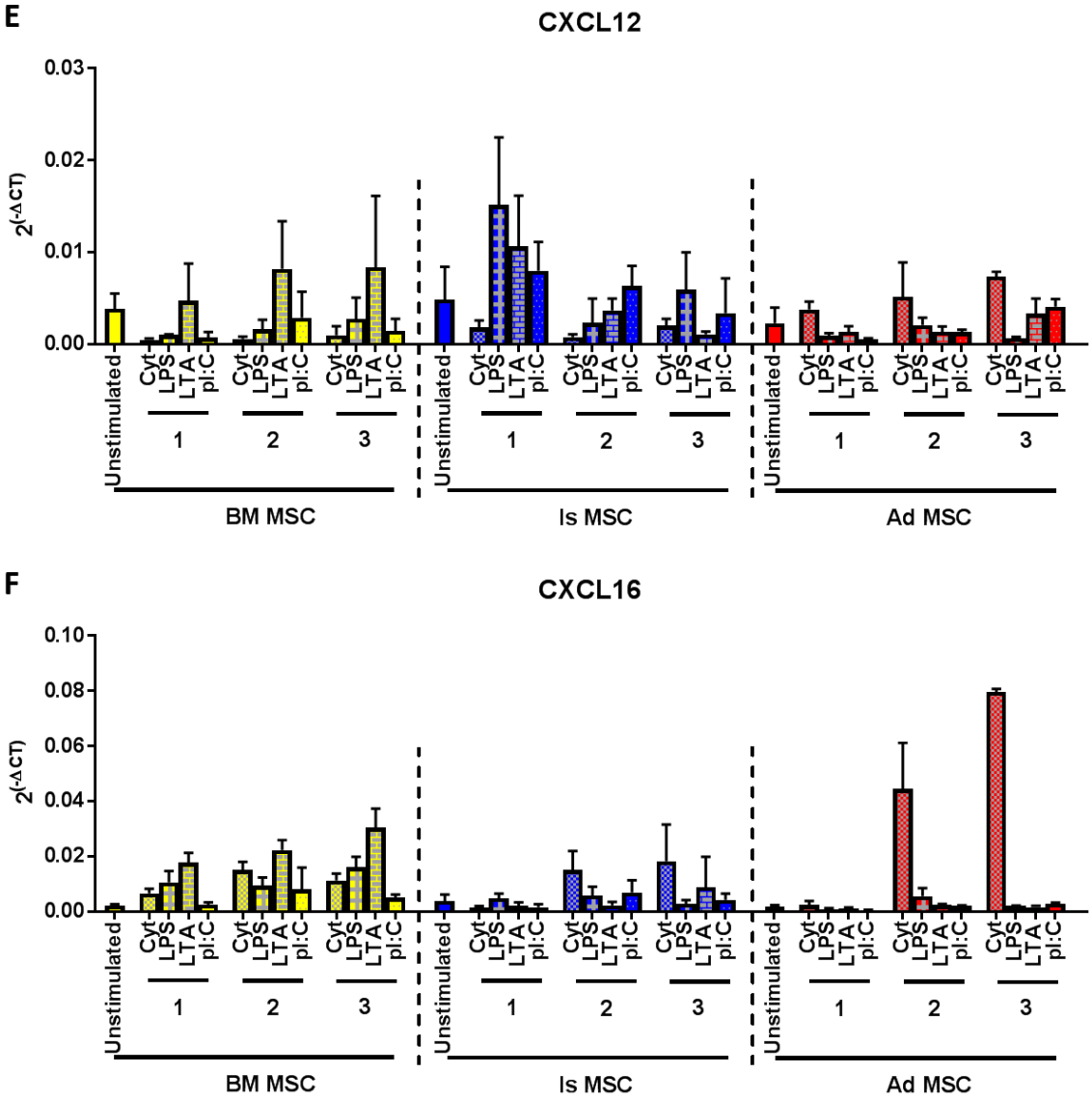


Figure 8-3. Inflammatory agent, repetitive stimulus and MSC tissue origin impacts CXCL chemokine transcript levels in MSCs.

MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with a cocktail of cytokines (40 ng/ mL of IFN-Ɣ, TNF-α and IL-1β), or 100 ng/ mL LPS, or 100 ng/ mL LTA or 4 µg/ mL Poly I:C. Unstimulated cells were left growing in MSC culture medium as a control. Three different licensing conditions were tested. In the first one, cells were stimulated for 48 hours, after which cells were washed twice with PBS and fresh culture medium was added; cells were harvested 24 hours later. In the second condition, cells were washed twice with PBS, the culture medium was replaced with fresh one and the cells were left growing for 48 hours. Cells were then washed twice with PBS, the culture medium was replaced with supplemented one and the cells were harvested 24 hours later. In the last condition, cells were stimulated for 48 hours, after which cells were washed twice with PBS and were stimulated again for another 24 hours. Figure 4-7 illustrates the time points at which supplemented medium was added. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate CXCL transcripts in BM, Is and Ad MSCs under resting and inflammatory conditions. Each bar represents an n of 4 independent experiments and is graphed as mean ± SEM. Data are normalised to the housekeeping gene *B2M* and expressed as $2^{(-\Delta CT)}$. Statistically significant differences are marked with a colour code in Table 4-8.

Table 8-2. Fold change in CXC chemokine transcript levels of cytokine, LPS, LTA or Poly I:C-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.
Following the experimental set up explained in Figure 8-3, fold change in transcript levels of CXC chemokines is represented as mean of fold change ± standard deviation. One Way ANOVA with Tukey's multiple comparisons post-test was performed to compare all MSC sources and the different conditions. p = 0.05 was considered the limit for statistical significance. Statistically significant differences are marked with a colour code, where p < 0.05 is represented by green, p < 0.01 is represented by orange, p < 0.001 is represented by blue and p < 0.0001 is represented by red.

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
CXCL1	1	Cyt	3.70 ± 3.41	3.04 ± 3.73	0.09 ± 0.06
		LPS	0.35 ± 0.25	0.78 ± 0.87	0.23 ± 0.06
		LTA	0.09 ± 0.07	1.94 ± 2.28	0.11 ± 0.04
		Poly I:C	0.06 ± 0.05	2.11 ± 2.17	0.13 ± 0.03
	2	Cyt	**13.66 ± 10.35**	**7.77 ± 7.58**	**2.56 ± 0.23**
		LPS	1.78 ± 0.56	5.05 ± 5.82	1.96 ± 0.41
		LTA	0.62 ± 0.10	0.93 ± 0.64	0.95 ± 0.06
		Poly I:C	1.01 ± 0.77	1.21 ± 1.20	0.91 ± 0.12
	3	Cyt	2.49 ± 0.30	3.19 ± 2.58	**57.55 ± 4.31**
		LPS	2.25 ± 1.25	1.20 ± 0.63	1.58 ± 0.09
		LTA	0.18 ± 0.11	2.41 ± 2.56	0.89 ± 0.19
		Poly I:C	0.16 ± 0.08	0.44 ± 0.29	1.45 ± 0.22
CXCL2	1	Cyt	0.95 ± 0.20	0.70 ± 0.41	**64.35 ± 10.64**
		LPS	3.24 ± 0.66	13.12 ± 12.57	0.52 ± 0.17
		LTA	1.01 ± 0.13	0.61 ± 0.22	0.33 ± 0.21
		Poly I:C	0.69 ± 0.28	0.40 ± 0.15	0.19 ± 0.08
	2	Cyt	**7.14 ± 1.15**	**10.04 ± 3.51**	3.18 ± 0.49
		LPS	15.99 ± 4.05	8.70 ± 2.38	3.63 ± 1.65
		LTA	3.03 ± 0.65	0.98 ± 0.71	2.70 ± 0.89
		Poly I:C	2.56 ± 1.19	2.91 ± 0.50	1.40 ± 0.46
	3	Cyt	2.21 ± 0.95	5.49 ± 1.02	**110.74 ± 34.09**
		LPS	12.61 ± 2.99	1.95 ± 0.49	1.34 ± 0.53
		LTA	2.05 ± 1.16	0.52 ± 0.50	0.97 ± 0.42
		Poly I:C	1.20 ± 0.14	1.86 ± 0.44	3.12 ± 1.31
CXCL5	1	Cyt	0.41 ± 0.15	27.08 ± 24.87	0.97 ± 0.50
		LPS	0.05 ± 0.01	5.60 ± 4.80	4.38 ± 0.98
		LTA	0.01 ± 0.00	0.40 ± 0.17	0.51 ± 0.09
		Poly I:C	0.01 ± 0.00	0.89 ± 0.62	0.51 ± 0.12
	2	Cyt	**6.86 ± 1.60**	**182.44 ± 101.70**	**26.79 ± 4.95**
		LPS	2.27 ± 2.22	13.58 ± 4.69	**28.05 ± 10.42**
		LTA	1.09 ± 1.06	1.40 ± 1.15	2.96 ± 0.48
		Poly I:C	0.02 ± 0.00	1.46 ± 1.33	2.30 ± 0.46
	3	Cyt	2.22 ± 1.94	**108.75 ± 37.36**	**402.41 ± 86.69**
		LPS	1.85 ± 1.62	14.43 ± 6.41	**19.12 ± 4.38**
		LTA	0.14 ± 0.13	0.10 ± 0.05	0.65 ± 0.20
		Poly I:C	0.13 ± 0.10	1.68 ± 1.53	**26.34 ± 6.16**
CXCL10	1	Cyt	0.11 ± 0.06	0.19 ± 0.15	1.02 ± 0.63
		LPS	0.43 ± 0.31	11.88 ± 15.22	0.31 ± 0.08

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
		LTA	0.50 ± 0.21	1.42 ± 1.21	0.48 ± 0.18
		Poly I:C	0.69 ± 0.23	0.62 ± 0.50	0.91 ± 0.46
	2	Cyt	10.16 ± 5.26	**19.21 ± 10.64**	**45.62 ± 21.79**
		LPS	1.64 ± 0.46	1.23 ± 1.06	3.65 ± 1.84
		LTA	0.99 ± 0.47	1.14 ± 0.63	12.40 ± 6.16
		Poly I:C	**97.19 ± 36.00**	3.61 ± 2.02	6.92 ± 2.85
	3	Cyt	4.09 ± 2.34	6.51 ± 5.88	**913.91 ± 470.61**
		LPS	1.07 ± 0.69	1.21 ± 0.58	0.64 ± 0.25
		LTA	0.80 ± 0.38	2.73 ± 3.09	1.62 ± 1.12
		Poly I:C	30.72 ± 14.62	2.58 ± 1.92	0.15 ± 0.04
CXCL12	1	Cyt	0.13 ± 0.09	0.87 ± 0.90	2.87 ± 1.41
		LPS	0.27 ± 0.11	**5.88 ± 1.89**	0.66 ± 0.23
		LTA	1.15 ± 0.92	5.47 ± 4.99	1.02 ± 0.46
		Poly I:C	0.17 ± 0.14	2.68 ± 1.59	0.41 ± 0.23
	2	Cyt	0.16 ± 0.12	0.36 ± 0.30	**2.09 ± 0.86**
		LPS	0.39 ± 0.18	1.48 ± 1.62	1.54 ± 0.60
		LTA	1.98 ± 1.13	1.43 ± 0.75	1.14 ± 0.83
		Poly I:C	0.61 ± 0.39	2.69 ± 2.03	1.04 ± 0.57
	3	Cyt	0.22 ± 0.19	0.81 ± 0.59	**5.50 ± 2.08**
		LPS	0.63 ± 0.38	1.70 ± 0.41	0.53 ± 0.27
		LTA	1.88 ± 1.43	0.44 ± 0.38	2.20 ± 1.04
		Poly I:C	0.32 ± 0.23	1.95 ± 1.89	3.01 ± 1.13
CXCL16	1	Cyt	2.95 ± 0.24	0.35 ± 0.23	1.81 ± 0.92
		LPS	**5.07 ± 2.20**	1.15 ± 0.47	0.70 ± 0.31
		LTA	**8.30 ± 2.33**	0.52 ± 0.42	0.81 ± 0.50
		Poly I:C	1.20 ± 0.47	0.31 ± 0.12	0.46 ± 0.23
	2	Cyt	**7.59 ± 1.34**	**5.13 ± 2.00**	**43.11 ± 16.81**
		LPS	4.20 ± 0.62	1.52 ± 0.90	4.96 ± 3.71
		LTA	**10.53 ± 3.08**	0.54 ± 0.31	1.89 ± 0.97
		Poly I:C	3.25 ± 2.57	1.87 ± 1.42	1.57 ± 0.75
	3	Cyt	**5.30 ± 1.62**	**5.01 ± 3.71**	**62.89 ± 24.29**
		LPS	**7.48 ± 2.10**	0.74 ± 0.44	1.57 ± 0.61
		LTA	**13.80 ± 1.23**	2.68 ± 3.20	1.29 ± 0.66
		Poly I:C	2.26 ± 0.14	1.04 ± 0.59	2.33 ± 1.11

8.1.3 Transcription of CX3CL1 chemokine and atypical chemokine receptor ACKR4 under resting and inflammatory conditions

Under resting conditions, BM, Is and Ad MSCs transcribed very little, if any, *CX3CL1* (Figure 8-4, A), while *ACKR4* (Figure 8-4, B) was transcribed at higher rates, but in both cases transcript level variations were not observed among the MSCs isolated from the different sources under resting conditions. However, in both cases tissue of origin and licensing agent did have an influence in the levels

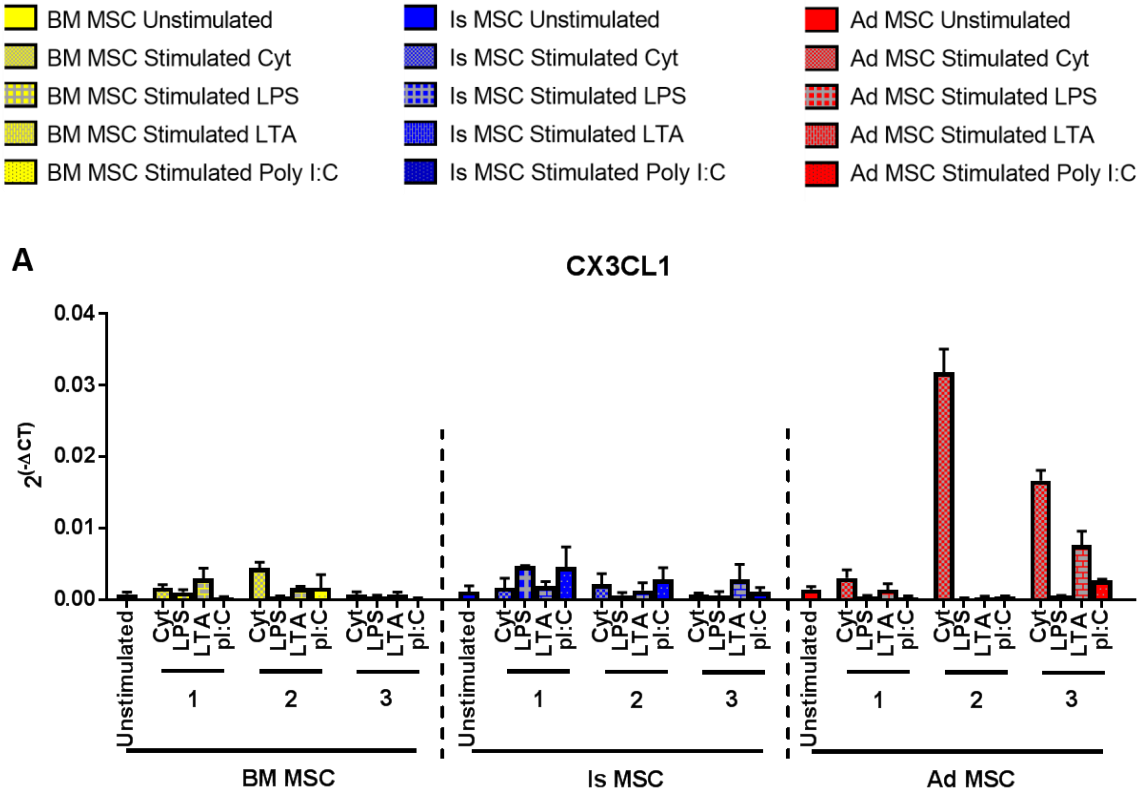
of transcript levels after inflammatory stimulation. *CX3CL1* showed a pattern of transcriptional upregulation after licensing while *ACKR4* showed a pattern of transcriptional downregulation. With a few exceptions, *CX3CL1* transcript levels increase after a 24 hours stimulation (Condition 2) in MSCs from every tissue source; however, this upregulation was not sustained, as cells harvested 72 hours after licensing (Condition 1) showed a decrease in the transcript levels of *CX3CL1* compared to Condition 2. A second stimulation 48 hours after the first stimulation (Condition 3) was able to induce the transcription of *CX3CL1* in MSCs from the three sources; however, the second stimulation was not able to match the transcript levels observed in Condition 2. Overall, *ACKR4* transcript levels decreased after a 24-hour stimulation (Condition 2) in MSCs from every tissue source; however, this downregulation was not sustained, as even if the downregulation was still notable, cells harvested 72 hours after licensing (Condition 1) showed higher transcript levels than the ones observed 24 hours after licensing. A second stimulation 48 hours after the first stimulation (Condition 3) was able to sustain the downregulation in MSCs from the three sources as *ACKR4* transcript levels were like the ones observed 24 hours after licensing. Fold changes of transcriptional regulation upon licensing of MSCs are specified in Table 8-3.

As previously mentioned, during resting conditions BM, Is and Ad MSCs transcribed very little *CX3CL1 (*Figure 8-4, A). After 72 hours of stimulation (Condition 1), cytokine-mediated licensing led to an increase in the transcript levels in MSCs from all sources, where Ad MSCs had the highest transcript levels. 24 hours after stimulation with the cytokine cocktail (Condition 2), MSCs from the three sources had upregulated their transcript levels, but it was only statistically significant in BM and Ad MSCs. A second stimulation with the cytokine cocktail 48 hours after the first stimulation (Condition 3) was able to induce the transcription of *CX3CL1* in MSCs from the three sources; however, the second stimulation was not able to match the transcript levels observed in Condition 2. 72 hours after LPS licensing BM and Is MSCs had upregulated their *CX3CL1* transcript levels, while Ad MSCs had downregulated their transcript levels. 24 hours stimulation with LPS led to a downregulation in BM and Ad MSCs while it produced no variation in Is MSCs transcript levels; a second stimulation of MSCs 48 hours after the first one (Condition 3) with LPS led to a

downregulation of *CX3CL1* transcript levels in MSCs from the three sources. 72 hours of licensing with LTA (Condition 1) produced an upregulation of *CX3CL1* transcript levels in BM and Is MSCs, while it produced no effect in Ad MSCs. 24-hour licensing (Condition 2) with LTA produced an upregulation of *CX3CL1* transcript levels in BM and Is MSCs; however, transcript levels were not as high as in Condition 1. 24 hours LTA licensing produced a downregulation in Ad MSCs. A second stimulation 48 hours after the first one (Condition 3) led to no variation in BM MSCs and to upregulation of *CX3CL1* transcript levels in Is and Ad MSCs. 72 hours of Poly I:C-mediated licensing (Condition 1) led to downregulation of *CX3CL1* transcript levels in BM and Ad MSCs while it led to an upregulation in Is MSCs. 24 hours after Poly I:C licensing (Condition 2) *CX3CL1* transcript levels were upregulated in BM and Is MSCs, while Ad MSCs had downregulated their *CX3CL1* transcript levels. A second stimulation of MSCs 48 hours after the first one (Condition 3) with Poly I:C was able to induce the downregulation of *CX3CL1* transcript levels in BM MSCs, while it produced an upregulation of *CX3CL1* transcript levels in Is and Ad MSCs.

As indicated previously, during resting conditions BM, Is and Ad MSCs transcribed very little *ACKR4 (*Figure 8-4, B). After 72 hours of stimulation (Condition 1), cytokine-mediated licensing produced a downregulation in the transcript levels of BM and Is MSCs, while it produced a small upregulation in Ad MSCs. 24 hours after stimulation with the cytokine cocktail (Condition 2), MSCs from the three sources had downregulated their transcript levels. After the double stimulation with the cytokine cocktail (Condition 3), *ACKR4* transcript levels remained downregulated at the same level as in Condition 2 in MSCs from the three sources. 72 hours after LPS licensing, BM and Is MSCs had downregulated their transcript levels, while Ad MSCs showed no variation in their *ACKR4* transcript levels. 24 hours stimulation with LPS led to a downregulation in MSCs from the three sources, but this downregulation was only significant in BM and Is MSCs. After the double stimulation with LPS (Condition 3), *ACKR4* transcript levels remained downregulated at the same level as in Condition 2 in MSCs from the three sources. 72 hours of licensing with LTA (Condition 1) produced a downregulation of *ACKR4* transcript levels in BM and Is MSCs, while it produced no effect in Ad MSCs. 24 hours licensing (Condition 2) with LTA produced a downregulation in MSCs from the three sources, but this downregulation was

only significant in BM and Is MSCs. A second stimulation 48 hours after the first one (Condition 3) led to the downregulation of transcript levels in BM and Is MSCs, while Ad MSCs upregulated their *ACKR4* transcript levels. 72 hours of Poly I:C-mediated licensing (Condition 1) led to a downregulation of *ACKR4* transcript levels in MSCs from the three sources, while 24 hours after Poly I:C licensing (Condition 2) *ACKR4* transcript levels were upregulated in MSCs from the three sources. Lastly, after the double stimulation with Poly I:C (Condition 3), *ACKR4* transcript levels remained downregulated at the same level as in Condition 2 in BM and Is MSCs, while they remained downregulated, but slightly higher, in Ad MSCs.



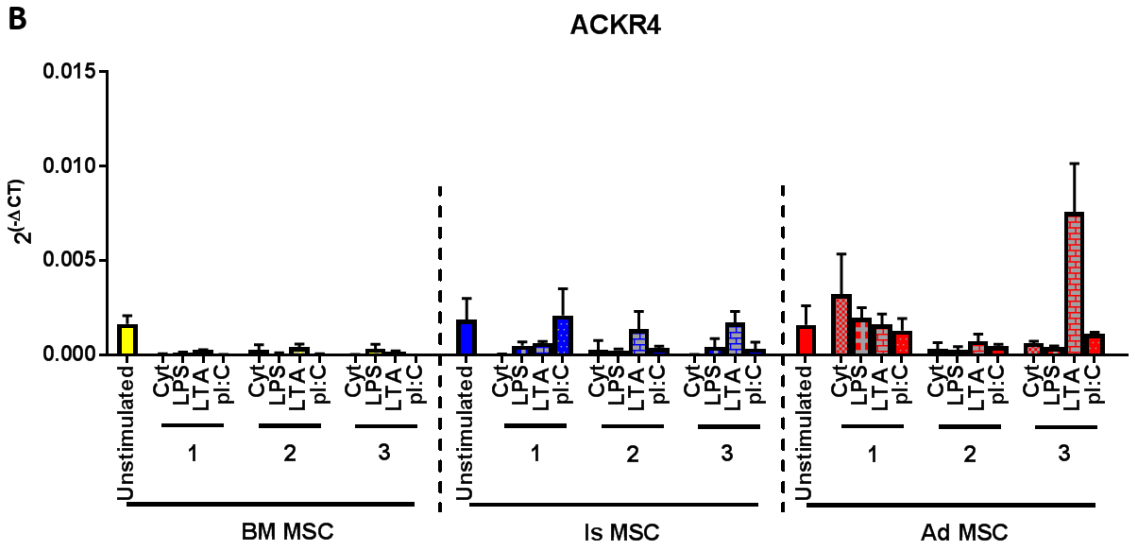


Figure 8-4. Inflammatory agent, repetitive stimulus and MSC tissue origin impacts CX3CL1 and ACKR4 chemokine transcript levels in MSCs.
MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with either a cocktail of cytokines (40 ng/ mL of IFN-ϒ, TNF-α and IL-1β), 100 ng/ mL LPS, 100 ng/ mL LTA or 4 µg/ mL Poly I:C. Unstimulated cells were left growing in MSC culture medium as a control. Three different licensing conditions were tested. In the first one, cells were stimulated for 48 hours, after which cells were washed twice with PBS and fresh culture medium was added; cells were harvested 24 hours later. In the second condition, cells were washed twice with PBS, the culture medium was replaced with fresh one and the cells were left growing for 48 hours. Cells were then washed twice with PBS, the culture medium was replaced with supplemented one and the cells were harvested 24 hours later. In the last condition, cells were stimulated for 48 hours, after which cells were washed twice with PBS and were stimulated again for another 24 hours. Figure 4-7 illustrates the time points at which supplemented medium was added. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate transcripts in BM, Is and Ad MSCs under resting and inflammatory conditions. Each bar represents an n of 4 independent experiments and is graphed as mean ± SEM. Data are normalised to the housekeeping gene *B2M* and expressed as $2^{(-\Delta CT)}$. Statistically significant differences are marked with a colour code in Table 4-9.

Table 8-3. Fold change in CX3CL1 and ACKR4 transcript levels of cytokine, LPS, LTA or Poly I:C-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.
Following the experimental set up explained in Figure 8-4, fold change in transcript levels of CX3CL1 and ACKR4 is represented as mean of fold change ± standard deviation. One Way ANOVA with Tukey's multiple comparisons post-test was performed to compare all MSC sources and the different conditions. p = 0.05 was considered the limit for statistical significance. Statistically significant differences are marked with a colour code, where p < 0.05 is represented by green, p < 0.01 is represented by orange, p < 0.001 is represented by blue and p < 0.0001 is represented by red.

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
CX3CL1	1	Cyt	2.39 ± 0.51	2.34 ± 2.02	2.19 ± 0.73
		LPS	1.40 ± 0.10	**7.44 ± 3.38**	0.29 ± 0.12
		LTA	**5.13 ± 3.48**	2.84 ± 1.46	1.09 ± 0.75
		Poly I:C	0.49 ± 0.32	**5.71 ± 1.25**	0.28 ± 0.14
	2	Cyt	**7.07 ± 3.78**	4.82 ± 4.06	**23.71 ± 5.55**
		LPS	0.61 ± 0.16	1.01 ± 0.88	0.15 ± 0.04
		LTA	2.40 ± 0.65	2.38 ± 2.75	0.24 ± 0.05

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
		Poly I:C	1.74 ± 1.62	4.29 ± 3.14	0.30 ± 0.09
	3	Cyt	1.24 ± 0.96	1.17 ± 0.80	**12.29 ± 2.38**
		LPS	0.73 ± 0.27	0.77 ± 0.75	0.42 ± 0.11
		LTA	1.20 ± 0.79	4.24 ± 2.95	**5.63 ± 1.68**
		Poly I:C	0.32 ± 0.20	1.92 ± 1.60	1.99 ± 0.43
ACKR4	1	Cyt	**0.02 ± 0.02**	**0.01 ± 0.00**	1.66 ± 1.30
		LPS	**0.07 ± 0.01**	0.18 ± 0.09	0.85 ± 0.30
		LTA	**0.16 ± 0.05**	0.23 ± 0.04	0.80 ± 0.49
		Poly I:C	**0.01 ± 0.00**	0.82 ± 0.57	0.52 ± 0.16
	2	Cyt	**0.02 ± 0.01**	**0.01 ± 0.00**	0.26 ± 0.14
		LPS	**0.05 ± 0.02**	**0.08 ± 0.02**	0.12 ± 0.09
		LTA	**0.26 ± 0.11**	0.47 ± 0.23	0.31 ± 0.18
		Poly I:C	**0.03 ± 0.01**	**0.13 ± 0.03**	0.19 ± 0.05
	3	Cyt	**0.01 ± 0.00**	**0.01 ± 0.00**	0.29 ± 0.12
		LPS	**0.18 ± 0.08**	0.17 ± 0.18	0.18 ± 0.04
		LTA	**0.11 ± 0.04**	0.60 ± 0.11	**3.15 ± 0.66**
		Poly I:C	**0.00 ± 0.00**	**0.13 ± 0.14**	0.50 ± 0.16

8.1.4 Transcription of the receptors of the stimulatory agents under resting and inflammatory conditions

Under resting conditions, BM, Is and Ad MSCs transcribed very little, if any, *IL1R1* (Figure 8-5, A), *IL1R2* (B), *IFNGR2* (F), *TLR2* (G), *TLR3* (H) and *TLR4* (I), while *TNFR1a* (C), *TNFR1b* (D) and *IFNGR1* (E) were transcribed at higher rates. In all the cases, transcript level variations were not observed among the MSCs isolated from the different sources under resting conditions. However, tissue of origin did influence the transcriptional regulation upon exposure to the different licensing agents and the different conditions. Fold changes of transcriptional regulation upon licensing of MSCs are summarised in Table 8-4.

Briefly, cytokine-mediated stimulation led to the downregulation of the cytokine receptors (*IL1R1* [Figure 8-5, A], *IL1R2* [B], *TNFR1a* [C], *TNFR1b* [D], *IFNGR1* [E] and *IFNGR2* [F]) in BM and Is MSCs under all conditions. Cytokine-mediated stimulation downregulated the cytokine receptors in Ad MSCs too; however, this downregulation was not sustained 72 hours after stimulation and, in some cases, even led to the upregulation of the receptors. LPS and LTA were able to produce the upregulation of the cytokine receptors transcript levels in certain conditions in MSCs from the three sources. The effects of Poly I:C in the regulation of the cytokine receptors transcription levels was tissue specific; Poly I:C stimulation of

BM MSCs led to the downregulation of all the cytokine receptors but *IFNGR2* in all the conditions tested. Poly I:C was able to downregulate the transcription of all the cytokine receptors but *IFNGR2* in Is MSCs; however, 24 hours after the stimulation the downregulation was not sustained, and the transcript levels started increasing. The effect of Poly I:C stimulation in Ad MSCs was gene dependent and the effect it produced in each of the genes at the established time points will be described later in detail.

LPS-mediated stimulation downregulated its receptor, *TLR4*, after 24 hours licensing in BM MSCs; however, 24 hours after the stimulation the downregulation was not sustained and the transcript levels started increasing (Figure 8-5, I). LPS produced no variation on *TLR4* transcript levels after a single stimulation in Is MSCs; however, a second stimulation 48 hours after the first one downregulated the receptor transcript levels. Overall, LPS-mediated licensing produced no variation on *TLR4* transcript levels in Ad MSCs under any condition. Cytokine and Poly I:C-mediated licensing were able to downregulate the transcription of *TLR4* in BM and Is MSCs; while cytokines upregulated its transcription in every condition and Poly I:C was able to upregulate the transcription of *TLR4* after a double stimulation. LTA-mediated stimulation produced an upregulation of *TLR4* transcript levels in BM MSCs, a downregulation that was not sustained 72 hours after in Is MSCs and was only able to upregulate the transcription in Ad MSCs after a double stimulation.

LTA-mediated stimulation upregulated its receptor, *TLR2*, in BM MSCs under every condition, while it produced no variation in Is MSCs and a small downregulation in Ad MSCs after a single stimulation but no variation after a double stimulation (Figure 8-5, G). All the licensing agents were able to increase the transcript levels of *TLR2* in BM MSCs. LPS and Poly I:C produced no variation on *TLR2* transcript levels in Is and Ad MSCs while cytokine-mediated licensing produced an upregulation after 24 hours in Is MSCs which was not sustained and Ad MSCs required a double stimulation to upregulate *TLR2* transcript levels.

Poly I:C-mediated stimulation downregulated its receptor, *TLR3*, in MSCs from the three sources under every condition tested. All the licensing agents were able to decrease the transcript levels of *TLR3* in BM MSCs (Figure 8-5, H). Cytokine-mediated licensing downregulated *TLR3* transcript levels in Is MSCs

under every condition while it produced an upregulation in Ad MSCs. LPS or LTA-mediated stimulation produced a decrease on the transcript levels 24 hours after the first or second stimulation, while they produced an upregulation 72 hours later in Is MSCs. On the contrary, Ad MSCs responded to LPS or LTA licensing by downregulating their *TLR3* receptor, but this downregulation was not sustained as transcription levels started increasing after 24 hours.

As indicated previously, during resting conditions BM, Is and Ad MSCs transcribed very little *IL1R1* and no variation in the transcript levels were observed among MSCs from different sources (Figure 8-5, A). After 72 hours of stimulation (Condition 1), cytokine-mediated licensing produced a downregulation in the transcript levels of BM and Is MSCs, while it produced a statistically significant upregulation in Ad MSCs. 24 hours after stimulation with the cytokine cocktail (Condition 2), BM and Is MSCs had downregulated their transcript levels, while Ad MSCs had upregulated their transcript levels in a smaller amount than in Condition 1. After the double stimulation with the cytokine cocktail (Condition 3), *IL1R1* transcript levels were downregulated in MSCs from the three sources. 72 hours after LPS licensing, BM MSCs had downregulated their transcript levels, while Is and Ad MSCs had upregulated their *IL1R1* transcript levels. 24-hour stimulation with LPS led to a downregulation of transcript levels in MSCs from the three sources. After the double stimulation with LPS (Condition 3) *IL1R1* transcript levels remained downregulated at the same level as in Condition 2 in MSCs from the three sources. 72 hours of licensing with LTA (Condition 1) produced a downregulation of *IL1R1* transcript levels in BM MSCs, while it produced an upregulation in Is and Ad MSCs. 24 hours licensing (Condition 2) with LTA produced an upregulation in BM MSCs while it produced a downregulation in Is and Ad MSCs. A second stimulation 48 hours after the first one (Condition 3) produced no variation in BM MSCs and an upregulation in Is and Ad MSCs. 72 hours of Poly I:C-mediated licensing (Condition 1) led to a downregulation of *IL1R1* transcript levels in BM MSCs while it led to an upregulation in Is MSCs and to no variation in Ad MSCs. 24 hours after Poly I:C licensing (Condition 2) *IL1R1* transcript levels were downregulated in MSCs from the three sources. Lastly, after a double stimulation with Poly I:C (Condition 3), *IL1R1* transcript levels were downregulated more than in Condition 2 in MSCs from the three sources.

During resting conditions BM, Is and Ad MSCs transcribed very little, if any, *IL1R2* and no variation in the transcript levels were observed among MSCs from different sources (Figure 8-5, B). After 72 hours of stimulation (Condition 1), cytokine-mediated licensing produced a downregulation in the transcript levels of BM and Is MSCs, while it produced an upregulation in Ad MSCs. 24 hours after stimulation with the cytokine cocktail (Condition 2), BM and Is MSCs had downregulated their transcript levels, while Ad MSCs had upregulated their transcript levels. After a double stimulation with the cytokine cocktail (Condition 3), *IL1R2* transcript levels were downregulated in BM and Is MSCs, while they were upregulated in Ad MSCs. 72 hours after LPS licensing, MScs from the three sources had upregulated their *IL1R2* transcript. 24 hours stimulation with LPS led to no variation in BM MSCs, a small downregulation in Is MSCs and to an upregulation in Ad MSCs. After the double stimulation with LPS (Condition 3) *IL1R2* transcript levels showed no change in BM MSCs, Is MSCs had downregulated their transcript levels while Ad MSCs had upregulated them. 72 hours of licensing with LTA (Condition 1) produced an upregulation of *IL1R2* transcript levels in MSCs from the three sources. 24-hour licensing (Condition 2) with LTA produced an upregulation in MSCs from the three sources; in the case of BM MSCs, this upregulation was higher than in Condition 1, while it was smaller in Is and Ad MSCs. A second stimulation 48 hours after the first one (Condition 3) produced no variation in BM MSCs but it led to a significant upregulation in Is and Ad MSCs. 72 hours of Poly I:C-mediated licensing (Condition 1) led to a downregulation of *IL1R2* transcript levels in BM MSCs while it led to an upregulation in Is and Ad MSCs. 24 hours after Poly I:C licensing (Condition 2) *IL1R2* transcript levels were downregulated in BM and Is MSCs and upregulated in Ad MSCs. After the double stimulation with Poly I:C (Condition 3), *IL1R2* transcript levels were downregulated in BM MSCs and upregulated in Is and Ad MSCs.

During resting conditions BM, Is and Ad MSCs transcribed substantial levels of *TNFR1a* and no variation in the transcript levels were observed among MSCs from different sources (Figure 8-5, C). After 72 hours of stimulation (Condition 1), cytokine-mediated licensing produced a downregulation in the transcript levels of MSCs from the three sources. 24 hours after stimulation with the cytokine cocktail (Condition 2), MSCs from the three sources had downregulated their *TNFR1a* transcript levels in similar levels as in the previous condition. After the

double stimulation with the cytokine cocktail (Condition 3), *TNFR1a* transcript levels were downregulated in BM and Is MSCs, while they were upregulated in Ad MSCs. 72 hours after LPS licensing, BM and Ad MSCs downregulated their *TNFR1a* transcript levels while Is MSCs upregulated them. 24 hours stimulation with LPS led to a downregulation in MSCs from the three sources while a second stimulation 48 hours after the first one (Condition 3) led to a downregulation of *TNFR1a* transcript levels in BM and Is MSCs and to an upregulation in Ad MSCs. 72 hours of LTA licensing (Condition 1) produced an upregulation of *TNFR1a* transcript levels in BM and Is MSCs, while it produced a downregulation in Ad MSCs. 24 hours licensing (Condition 2) with LTA produced a smaller upregulation in BM MSCs compared to Condition 1 and a downregulation in Is and Ad MSCs; this downregulation in Ad MSCs was bigger than in Condition 1. A second stimulation 48 hours after the first one (Condition 3) produced an upregulation in BM MSCs and Ad MSCs, and a downregulation in Is MSCs. 72 hours of Poly I:C-mediated licensing (Condition 1) led to a downregulation of *TNFR1a* transcript levels in MSCs from the three sources, as well as after 24 hours stimulation (Condition 2). A second stimulation with Poly I:C 48 hours after the first one (Condition 3) led to a downregulation of *TNFR1a* transcript levels in BM and Is MSCs, where these downregulations were increased compared to previous conditions. On the contrary, a second stimulation with Poly I:C produced a significant upregulation of *TNFR1a* transcript levels in Ad MSCs.

During resting conditions BM, Is and Ad MSCs transcribed *TNFR1b* at substantial levels and no variation in the transcript levels were observed among MSCs from different sources (Figure 8-5, D). After 72 hours of stimulation (Condition 1), cytokine-mediated licensing produced a downregulation in the transcript levels of MSCs from the three sources. 24 hours after stimulation with the cytokine cocktail (Condition 2), MSCs from the three sources had downregulated their *TNFR1b* transcript levels in similar levels as in the previous condition. After the double stimulation with the cytokine cocktail (Condition 3), *TNFR1b* transcript levels were downregulated in BM and Is MSCs, while they were upregulated in Ad MSCs. 72 hours after LPS licensing, MSCs isolated from the three tissues had downregulated their *TNFR1b* transcript levels. 24 hours stimulation with LPS led to a smaller downregulation in MSCs from the three sources while a second stimulation 48 hours after the first one (Condition 3) led to no effect in BM and

Ad MSCs, while it led to a downregulation of *TNFR1b* transcript levels in Is MSCs. 72 hours of LTA licensing (Condition 1) produced no variation in *TNFR1b* transcript levels in BM and Ad MSCs, while it produced a downregulation in Is MSCs. 24 hours licensing (Condition 2) with LTA produced no variation in BM MSCs and a downregulation in Is and Ad MSCs. A second stimulation 48 hours after the first one (Condition 3) produced an upregulation in BM MSCs, a downregulation in Is MSCs and no effect in Ad MSCs. 72 hours of Poly I:C-mediated licensing (Condition 1) led to a downregulation of *TNFR1b* transcript levels in MSCs from the three sources, as well as after 24 hours stimulation (Condition 2). A second stimulation with Poly I:C 48 hours after the first one (Condition 3) led to a downregulation of *TNFR1a* transcript levels in BM and Is MSCs, where the downregulation was increased compared to previous conditions in BM MSCs. On the contrary, a second stimulation with Poly I:C produced a significant upregulation of *TNFR1b* transcript levels in Ad MSCs.

During resting conditions BM MSCs transcribed the highest *IFNGR1* transcript levels but MSCs from the three sources transcribed *IFNGR1* at substantial levels (Figure 8-5, E). After 72 hours of stimulation (Condition 1), cytokine-mediated licensing produced a downregulation in the transcript levels of BM and Is MSCs, while it produced no effect in Ad MSCs. 24 hours after stimulation with the cytokine cocktail (Condition 2) MSCs from the three sources had downregulated their *IFNGR1* transcript levels and in the case of the BM and Is MSCs, this downregulation was in similar levels as in the previous condition. After the double stimulation with the cytokine cocktail (Condition 3), *IFNGR1* transcript levels were downregulated in BM and Is MSCs, while there was no variation in Ad MSCs. 72 hours after LPS licensing, MSCs isolated from the three tissues had downregulated their *IFNGR1* transcript levels. 24 hours stimulation with LPS led to downregulation in MSCs from the three sources while a second stimulation 48 hours after the first one (Condition 3), led to a downregulation in BM and Is MSCs while it produced no effect in Ad MSCs. 72 hours of LTA licensing (Condition 1) produced no variation in the *IFNGR1* transcript levels in BM and Is MSCs, while it produced a downregulation in Ad MSCs. 24 hours licensing (Condition 2) with LTA produced no variation in BM MSCs and a downregulation in Is and Ad MSCs. A second stimulation 48 hours after the first one (Condition 3) produced no variation in BM MSCs, a downregulation in Is MSCs and an upregulation in Ad

MSCs. 72 hours of Poly I:C-mediated licensing (Condition 1) led to a downregulation of *IFNGR1* transcript levels in MSCs from the three sources, as well as after 24 hours stimulation (Condition 2). A second stimulation with Poly I:C 48 hours after the first one (Condition 3) led to a downregulation of *IFNGR1* transcript levels in MSCs from the three sources and the downregulation was bigger than in previous conditions.

During resting conditions BM, Is and Ad MSCs transcribed very little *IFNGR2* transcript levels and no variation in the transcript levels were observed among MSCs from different sources (Figure 8-5, F). After 72 hours of stimulation (Condition 1), cytokine-mediated licensing produced no effect in BM MSCs, a downregulation in the transcript levels of Is MSCs and an upregulation in Ad MSCs. 24 hours after stimulation with the cytokine cocktail (Condition 2), BM and Is MSCs had downregulated their *IFNGR2* transcript levels, while Ad MSCs had upregulated them. After the double stimulation with the cytokine cocktail (Condition 3), *IFNGR2* transcript levels were downregulated in BM and Is MSCs, while they were upregulated in Ad MSCs. 72 hours after LPS licensing, MSCs isolated from the three tissues had upregulated their *IFNGR2* transcript levels. 24-hour stimulation with LPS led to a downregulation in BM MSCs and to an upregulation in Is and Ad MSCs. A second stimulation 48 hours after the first one (Condition 3) led to a downregulation in BM and Is MSCs while it produced an upregulation in Ad MSCs. 72 hours of LTA licensing (Condition 1) produced an upregulation of the *IFNGR2* transcript levels in MSCs from the three sources. 24-hour licensing (Condition 2) with LTA produced an upregulation of the transcript levels in BM MSCs, a downregulation in Is MSCs and no variation in Ad MSCs. A second stimulation 48 hours after the first one (Condition 3) produced an upregulation in MSCs isolated from the three sources. 72 hours of Poly I:C-mediated licensing (Condition 1) led to no effect in BM MSCs, an upregulation in Is MSCs and to a downregulation of *IFNGR2* transcript levels in Ad MSCs; while 24 hours of Poly I:C licensing led to an upregulation of the transcript levels in BM and Is MSCs while it produced no variation in Ad MSCs. A second stimulation with Poly I:C 48 hours after the first one (Condition 3) led to a downregulation of *IFNGR2* transcript levels in BM MSCs and an upregulation in Is and Ad MSCs.

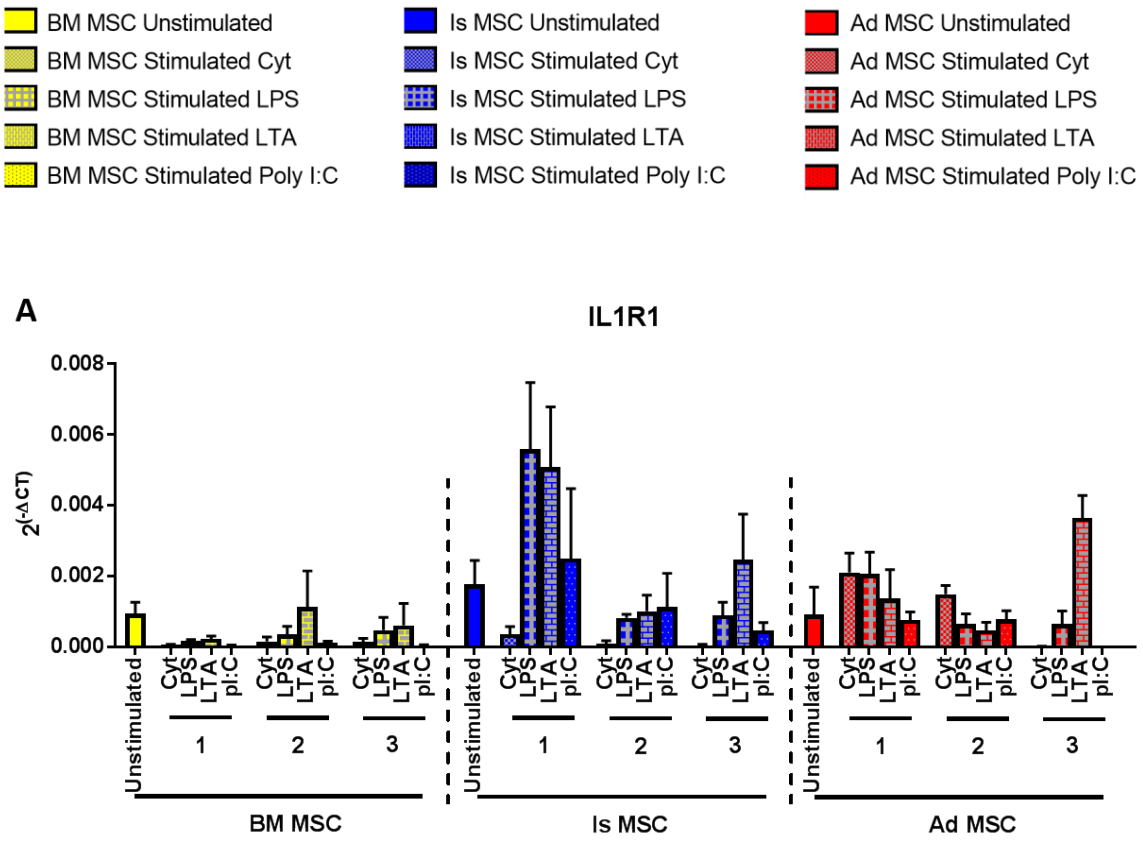
During resting conditions BM, Is and Ad MSCs transcribed very little *TLR2* transcript levels and no variation in the transcript levels were observed among MSCs from different sources (Figure 8-5, G). After 72 hours of stimulation (Condition 1), cytokine-mediated licensing produced an upregulation in BM MSCs, a downregulation in Is MSCs and no effect in Ad MSCs. 24 hours after stimulation with the cytokine cocktail (Condition 2), BM and Is MSCs had upregulated their *TLR2* transcript levels, while Ad MSCs had downregulated them. After the double stimulation with the cytokine cocktail (Condition 3), *TLR2* transcript levels were upregulated in BM and Ad MSCs, while they were downregulated in Is MSCs. 72 hours after LPS licensing, BM MSCs had upregulated their transcript levels, no variation was observed in Is MSCs and Ad MSCs had downregulated their *TLR2* transcript levels. 24 hours stimulation with LPS led to an upregulation in BM MSCs, to no variation in Is MSCs and to a downregulation in Ad MSCs. A second stimulation with LPS 48 hours after the first one (Condition 3) led to an upregulation of *TLR2* transcript levels in MSCs isolated from the three sources. 72 hours of LTA licensing (Condition 1) produced an upregulation of the *TLR2* transcript levels in BM and Is MSCs and a downregulation in Ad MSCs. 24-hour licensing (Condition 2) with LTA produced a bigger upregulation of the transcript levels in BM and Is MSCs and a bigger downregulation in Ad MSCs. A second stimulation 48 hours after the first one (Condition 3) produced an even bigger upregulation in BM and Is MSCs and no variation in Ad MSCs. 72 hours of Poly I:C-mediated licensing (Condition 1) led to an upregulation in BM MSCs and a downregulation of *TLR2* transcript levels in Is and Ad MSCs; while 24 hours of Poly I:C licensing led to an upregulation of the transcript levels in BM and Is MSCs while it produced a downregulation in Ad MSCs. A second stimulation with Poly I:C 48 hours after the first one (Condition 3) led to an upregulation of *TLR2* transcript levels in MSCs from the three sources.

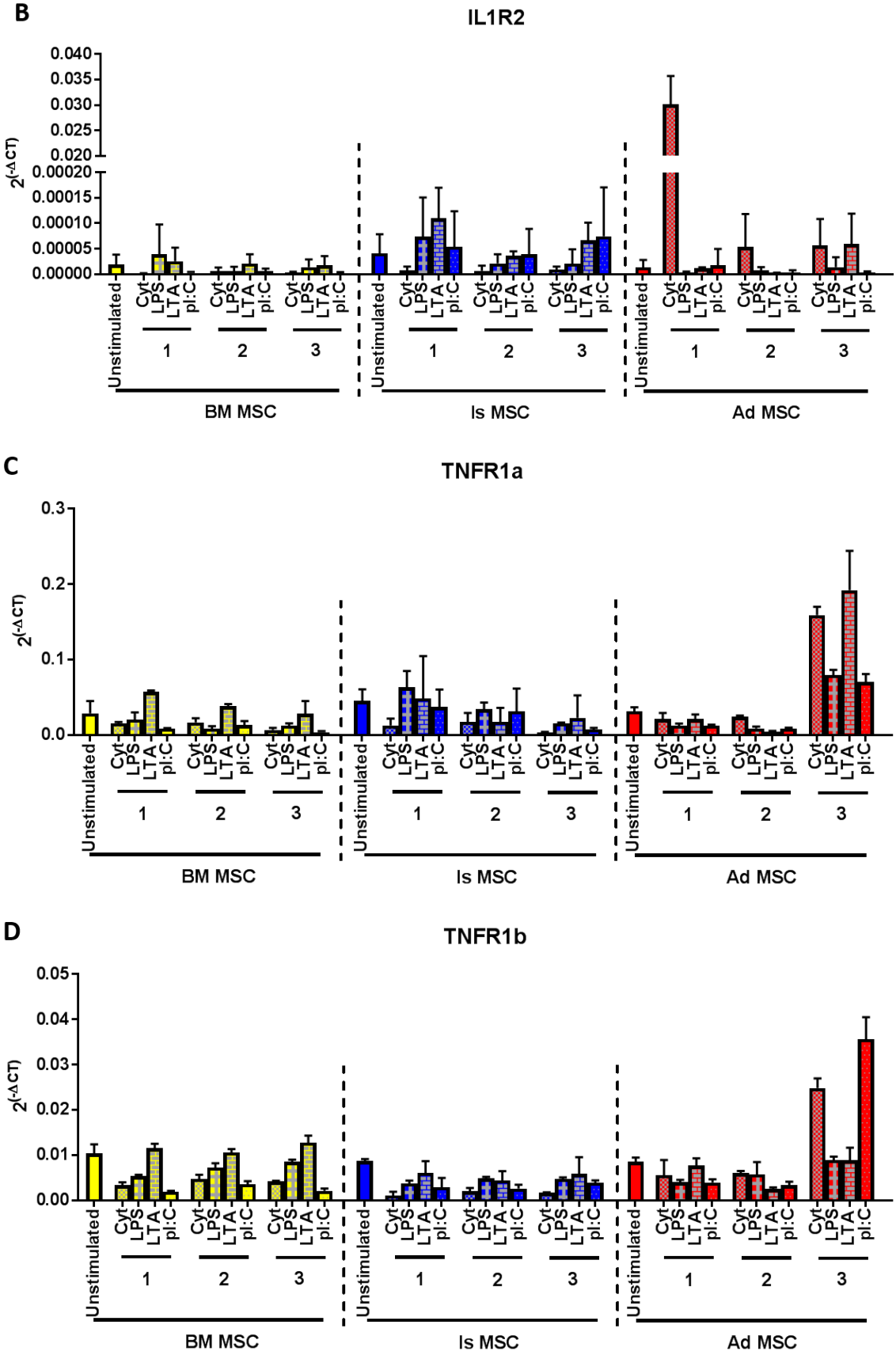
Under resting conditions BM, Is and Ad MSCs transcribed very little *TLR3* transcript levels and no variation in the transcript levels were observed among BM, Is and Ad MSCs (Figure 8-5, H). After 72 hours of stimulation (Condition 1), cytokine-mediated licensing produced a downregulation in BM and Is MSCs and an upregulation in Ad MSCs. 24 hours after stimulation with the cytokine cocktail (Condition 2), BM and Is MSCs had upregulated their *TLR3* transcript levels, while Ad MSCs were not affected by the licensing. After the double stimulation with

the cytokine cocktail (Condition 3), *TLR3* transcript levels were downregulated in BM and Is MSCs, while they were upregulated in Ad MSCs. 72 hours after LPS licensing, BM MSCs showed no variation in their transcript levels, while Is and Ad MSCs had upregulated their *TLR2* transcript levels. 24-hour stimulation with LPS led to no variations in BM and Is MSCs, while it produced a downregulation in Ad MSCs. A second stimulation with LPS 48 hours after the first one (Condition 3) led to no variation of *TLR2* transcript levels in BM and Ad MSCs while it produced a downregulation in Ad MSCs. 72 hours of LTA licensing (Condition 1) produced an upregulation of the *TLR2* transcript levels in MSCs from the three tissues. 24 hours licensing (Condition 2) with LTA produced an upregulation of the transcript levels in BM and Is MSCs and a downregulation in Ad MSCs. A second stimulation 48 hours after the first one (Condition 3) produced an upregulation in MSCs from the three sources, but this upregulation of TLR3 transcript levels was smaller than the one observed 72 hours after a single stimulation. 72 hours of Poly I:C-mediated licensing (Condition 1) led to an upregulation in BM and Is MSCs and a downregulation of *TLR3* transcript levels in Ad MSCs. 24 hours licensing with Poly I:C led to a bigger upregulation of transcript levels in BM and Is MSCs than the one observed in the previous conditions, while the downregulation observed in Ad MSCs was smaller than the one in Condition 1. A second stimulation with Poly I:C 48 hours after the first one (Condition 3) led to an upregulation of *TLR3* transcript levels in MSCs from the three sources.

During resting conditions BM, Is and Ad MSCs transcribed very little *TLR4* and no variation in the transcript levels were observed among MSCs from different sources (Figure 8-5, I). After 72 hours of stimulation (Condition 1), cytokine-mediated licensing produced a downregulation in BM and Is MSCs and an upregulation in Ad MSCs. 24 hours after stimulation with the cytokine cocktail (Condition 2), BM and Is MSCs had downregulated their *TLR4* transcript levels in smaller amounts than in Condition 1, while Ad MSCs had upregulated their transcript levels in smaller amounts compared to Condition 1. After the double stimulation with the cytokine cocktail (Condition 3), *TLR4* transcript levels were downregulated in BM and Is MSCs at the same level as Condition 1, while Ad MSCs had doubled their transcript levels compared to Condition 1. 72 hours after LPS licensing, BM MSCs had downregulated their transcript levels, Is MSCs showed no variation in their transcript levels and Ad MSCs had upregulated their *TLR4*

transcript levels. 24-hour stimulation with LPS led to a downregulation in MSCs from the three sources. A second stimulation with LPS 48 hours after the first one (Condition 3) led to a downregulation of *TLR4* transcript levels in BM and Is MSCs while it produced no effect in Ad MSCs. 72 hours of LTA licensing (Condition 1) produced an upregulation of the *TLR4* transcript levels in MSCs from the three tissues. 24-hour LTA licensing (Condition 2) produced an upregulation of the transcript levels in BM MSCs and a downregulation in Is and Ad MSCs. A second stimulation 48 hours after the first one (Condition 3) produced an upregulation in BM and Ad MSCs, while it produced a downregulation of *TLR4* transcript levels in Is MSCs. 72 hours of Poly I:C-mediated licensing (Condition 1) led to a downregulation of MSCs from the three sources. 24 hours licensing with Poly I:C led to a downregulation of *TLR4* transcript levels in BM and Ad MSCs, while it produced an upregulation in Is MSCs. A second stimulation with Poly I:C 48 hours after the first one (Condition 3) led to a downregulation of *TLR4* transcript levels in BM and Is MSCs, while it produced an upregulation in Ad MSCs.

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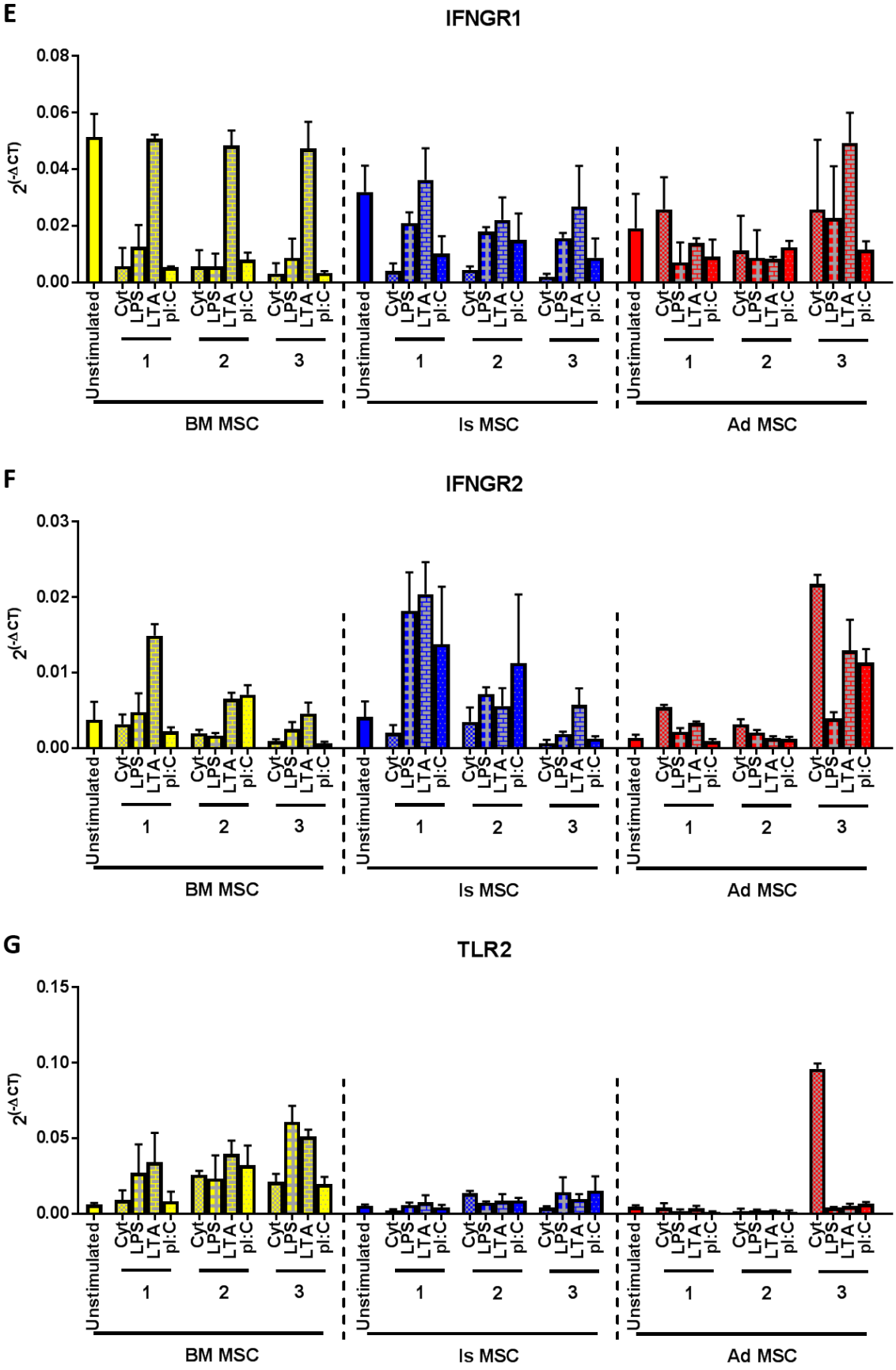
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LTA
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2
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BM MSC
Is MSC
Ad MSC
F
IFNGR2
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TLR2
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0.05
0.00

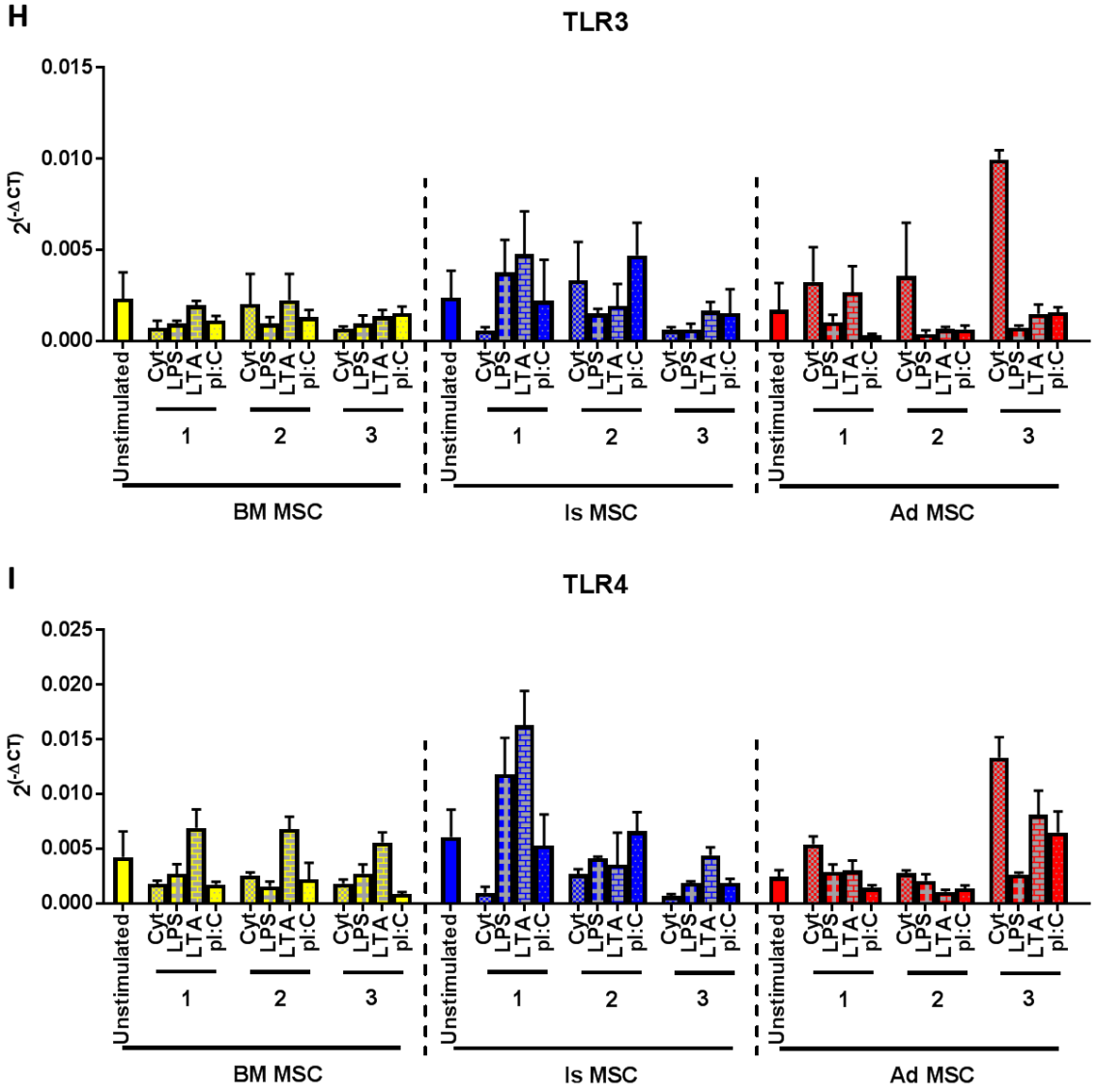


Figure 8-5. Comparison of the transcriptional expression of the receptors of the stimulatory agents among MSC tissue source under resting and inflammatory conditions.
MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with either a cocktail of cytokines (40 ng/ mL of IFN-ϒ, TNF-α and IL-1β), 100 ng/ mL LPS, 100 ng/ mL LTA or 4 µg/ mL Poly I:C. Unstimulated cells were left growing in MSC culture medium as a control. Three different licensing conditions were tested. In the first one, cells were stimulated for 48 hours, after which cells were washed twice with PBS and fresh culture medium was added; cells were harvested 24 hours later. In the second condition, cells were washed twice with PBS, the culture medium was replaced with fresh one and the cells were left growing for 48 hours. Cells were then washed twice with PBS, the culture medium was replaced with supplemented one and the cells were harvested 24 hours later. In the last condition, cells were stimulated for 48 hours, after which cells were washed twice with PBS and were stimulated again for another 24 hours. Figure 4-7 illustrates the time points at which supplemented medium was added. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate transcripts in BM, Is and Ad MSCs under resting and inflammatory conditions. Each bar represents an n of 4 independent experiments and is graphed as mean ± SEM. Data are normalised to the housekeeping gene *B2M* and expressed as $2^{(-\Delta CT)}$. Statistically significant differences are marked with a colour code in Table 4-10.

Table 8-4. Fold change in the receptors of the stimulatory agents' transcript levels of cytokine, LPS, LTA or Poly I:C-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.
Following the experimental set up explained in Figure 8-5, fold change in transcript levels of the receptors of the stimulatory agents is represented as mean of fold change ± standard deviation. One Way ANOVA with Tukey's multiple comparisons post-test was performed to compare all MSC sources and the different conditions. p = 0.05 was considered the limit for statistical significance. Statistically significant differences are marked with a colour code, where p < 0.05 is represented by green, p < 0.01 is represented by orange, p < 0.001 is represented by blue and p < 0.0001 is represented by red.

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
IL1R1	1	Cyt	0.07 ± 0.04	0.22 ± 0.13	3.20 ± 2.13
		LPS	0.20 ± 0.07	3.47 ± 1.35	3.27 ± 2.77
		LTA	0.25 ± 0.06	3.62 ± 2.31	2.03 ± 1.11
		Poly I:C	0.05 ± 0.02	1.88 ± 1.82	1.04 ± 0.58
	2	Cyt	0.18 ± 0.11	0.04 ± 0.02	1.99 ± 1.00
		LPS	0.48 ± 0.39	0.54 ± 0.25	0.73 ± 0.35
		LTA	1.61 ± 1.53	0.64 ± 0.29	0.73 ± 0.48
		Poly I:C	0.14 ± 0.09	0.56 ± 0.28	0.93 ± 0.39
	3	Cyt	0.19 ± 0.14	0.03 ± 0.01	0.01 ± 0.01
		LPS	0.59 ± 0.41	0.53 ± 0.13	0.83 ± 0.61
		LTA	0.92 ± 1.01	1.38 ± 0.40	5.59 ± 3.74
		Poly I:C	0.05 ± 0.04	0.33 ± 0.26	0.00 ± 0.00
IL1R2	1	Cyt	0.07 ± 0.07	0.86 ± 1.27	13476.79 ± 13682.54
		LPS	2.30 ± 1.21	8.66 ± 12.92	1.72 ± 1.91
		LTA	1.95 ± 1.48	4.04 ± 1.76	5.11 ± 4.66
		Poly I:C	0.52 ± 0.46	3.34 ± 3.13	4.72 ± 7.66
	2	Cyt	0.19 ± 0.17	0.10 ± 0.09	25.20 ± 38.61
		LPS	0.99 ± 1.11	0.71 ± 0.48	1.79 ± 0.79
		LTA	2.88 ± 3.17	2.50 ± 2.97	1.39 ± 1.48
		Poly I:C	0.53 ± 0.28	0.86 ± 0.31	1.70 ± 2.57
	3	Cyt	0.57 ± 0.64	0.47 ± 0.43	17.83 ± 18.07
		LPS	0.96 ± 0.31	0.77 ± 0.76	4.54 ± 6.34
		LTA	0.96 ± 0.46	5.51 ± 6.58	34.53 ± 53.66
		Poly I:C	0.17 ± 0.14	9.71 ± 14.87	2.13 ± 2.64
TNFR1a	1	Cyt	0.69 ± 0.30	0.29 ± 0.18	0.80 ± 0.31
		LPS	0.76 ± 0.10	1.52 ± 0.60	0.47 ± 0.11
		LTA	2.65 ± 1.39	1.53 ± 1.58	0.78 ± 0.24
		Poly I:C	0.39 ± 0.20	0.95 ± 0.65	0.47 ± 0.06
	2	Cyt	0.74 ± 0.49	0.44 ± 0.31	0.89 ± 0.09
		LPS	0.32 ± 0.07	0.84 ± 0.30	0.32 ± 0.06
		LTA	1.71 ± 0.81	0.54 ± 0.54	0.17 ± 0.03
		Poly I:C	0.48 ± 0.08	0.82 ± 0.78	0.32 ± 0.04
	3	Cyt	0.36 ± 0.26	0.08 ± 0.05	5.92 ± 0.68
		LPS	0.62 ± 0.38	0.38 ± 0.14	2.95 ± 0.32
		LTA	1.64 ± 1.36	0.69 ± 0.89	7.05 ± 1.26
		Poly I:C	0.20 ± 0.15	0.17 ± 0.02	2.63 ± 0.50
TNFR1b	1	Cyt	0.32 ± 0.04	0.12 ± 0.08	0.65 ± 0.35
		LPS	0.53 ± 0.11	0.43 ± 0.05	0.45 ± 0.06

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
		LTA	1.13 ± 0.16	0.69 ± 0.25	0.88 ± 0.19
		Poly I:C	**0.19 ± 0.05**	**0.34 ± 0.21**	**0.44 ± 0.06**
	2	Cyt	**0.47 ± 0.11**	**0.24 ± 0.08**	0.68 ± 0.09
		LPS	**0.71 ± 0.07**	0.55 ± 0.04	0.63 ± 0.19
		LTA	1.06 ± 0.25	**0.50 ± 0.19**	**0.28 ± 0.03**
		Poly I:C	**0.34 ± 0.03**	**0.30 ± 0.09**	**0.38 ± 0.09**
	3	Cyt	**0.41 ± 0.06**	**0.19 ± 0.01**	**2.81 ± 0.17**
		LPS	0.84 ± 0.16	**0.55 ± 0.06**	1.01 ± 0.03
		LTA	**1.25 ± 0.18**	0.67 ± 0.36	0.99 ± 0.19
		Poly I:C	**0.20 ± 0.01**	**0.45 ± 0.04**	**4.07 ± 0.66**
IFNGR1	1	Cyt	**0.10 ± 0.10**	**0.12 ± 0.07**	1.18 ± 0.44
		LPS	**0.23 ± 0.12**	0.68 ± 0.14	0.32 ± 0.28
		LTA	1.01 ± 0.17	1.13 ± 0.12	0.63 ± 0.07
		Poly I:C	**0.10 ± 0.01**	**0.37 ± 0.26**	0.42 ± 0.24
	2	Cyt	**0.10 ± 0.09**	**0.15 ± 0.06**	0.52 ± 0.49
		LPS	**0.10 ± 0.07**	0.60 ± 0.19	0.40 ± 0.38
		LTA	0.97 ± 0.22	0.72 ± 0.24	0.38 ± 0.03
		Poly I:C	**0.16 ± 0.05**	0.48 ± 0.27	0.57 ± 0.10
	3	Cyt	**0.06 ± 0.05**	**0.07 ± 0.03**	1.18 ± 0.98
		LPS	**0.16 ± 0.10**	0.52 ± 0.11	1.04 ± 0.73
		LTA	0.95 ± 0.26	0.88 ± 0.40	**2.25 ± 0.40**
		Poly I:C	**0.07 ± 0.02**	**0.26 ± 0.12**	0.53 ± 0.13
IFNGR2	1	Cyt	0.99 ± 0.27	0.69 ± 0.61	4.84 ± 1.61
		LPS	1.37 ± 0.19	5.73 ± 2.99	1.90 ± 0.70
		LTA	**5.81 ± 3.38**	**6.09 ± 2.63**	2.92 ± 0.88
		Poly I:C	0.93 ± 0.63	4.10 ± 2.21	0.85 ± 0.34
	2	Cyt	0.68 ± 0.29	0.84 ± 0.11	**2.71 ± 0.81**
		LPS	0.59 ± 0.27	**2.12 ± 0.90**	1.73 ± 0.40
		LTA	2.43 ± 1.26	0.84 ± 0.11	1.11 ± 0.21
		Poly I:C	**2.81 ± 1.70**	2.38 ± 0.88	1.02 ± 0.21
	3	Cyt	0.39 ± 0.28	0.26 ± 0.29	**19.05 ± 5.19**
		LPS	0.78 ± 0.22	0.58 ± 0.30	3.51 ± 1.54
		LTA	2.04 ± 1.50	2.08 ± 1.77	**10.72 ± 2.57**
		Poly I:C	0.28 ± 0.21	2.12 ± 0.90	**9.77 ± 2.59**
TLR2	1	Cyt	1.44 ± 0.83	0.42 ± 0.19	0.97 ± 0.62
		LPS	4.34 ± 2.29	1.16 ± 0.44	0.41 ± 0.13
		LTA	**5.60 ± 2.63**	1.56 ± 1.05	0.79 ± 0.21
		Poly I:C	1.30 ± 0.83	0.79 ± 0.19	0.32 ± 0.09
	2	Cyt	4.42 ± 0.83	2.68 ± 0.32	0.32 ± 0.28
		LPS	4.16 ± 2.60	1.41 ± 0.13	0.47 ± 0.10
		LTA	**6.85 ± 1.86**	1.71 ± 0.84	0.46 ± 0.11
		Poly I:C	5.60 ± 2.41	1.69 ± 0.26	**0.20 ± 0.20**
	3	Cyt	3.63 ± 1.06	0.82 ± 0.22	**22.83 ± 4.59**
		LPS	**10.22 ± 0.53**	2.64 ± 1.42	0.97 ± 0.15
		LTA	**8.72 ± 1.01**	2.03 ± 0.89	1.13 ± 0.34
		Poly I:C	3.40 ± 0.98	3.07 ± 1.70	1.51 ± 0.14

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
TLR3	1	Cyt	0.87 ± 0.74	0.45 ± 0.25	5.50 ± 5.82
		LPS	1.00 ± 0.38	3.16 ± 2.25	1.40 ± 1.06
		LTA	2.07 ± 0.92	3.61 ± 2.06	2.73 ± 1.04
		Poly I:C	1.14 ± 0.39	2.01 ± 2.33	0.37 ± 0.23
	2	Cyt	2.23 ± 1.21	1.77 ± 0.67	1.01 ± 0.44
		LPS	0.92 ± 0.29	1.07 ± 0.45	0.49 ± 0.31
		LTA	2.09 ± 0.82	1.24 ± 0.47	0.83 ± 0.51
		Poly I:C	1.48 ± 0.95	3.16 ± 1.01	0.82 ± 0.62
	3	Cyt	0.73 ± 0.40	0.41 ± 0.12	**13.84 ± 9.93**
		LPS	0.97 ± 0.45	0.40 ± 0.11	1.01 ± 0.72
		LTA	1.42 ± 0.62	1.22 ± 0.57	1.75 ± 0.93
		Poly I:C	1.70 ± 0.99	1.36 ± 1.44	2.19 ± 1.65
TLR4	1	Cyt	0.53 ± 0.22	0.20 ± 0.16	**2.31 ± 0.61**
		LPS	0.73 ± 0.20	**2.20 ± 0.77**	1.19 ± 0.21
		LTA	**2.01 ± 0.81**	**3.11 ± 1.21**	1.27 ± 0.43
		Poly I:C	0.51 ± 0.22	0.98 ± 0.44	0.60 ± 0.12
	2	Cyt	0.80 ± 0.43	0.52 ± 0.25	1.17 ± 0.26
		LPS	0.43 ± 0.14	0.82 ± 0.41	0.91 ± 0.38
		LTA	**2.23 ± 1.29**	0.52 ± 0.25	0.44 ± 0.08
		Poly I:C	0.49 ± 0.08	1.19 ± 0.30	0.60 ± 0.20
	3	Cyt	0.60 ± 0.36	0.13 ± 0.06	**5.64 ± 1.31**
		LPS	**0.76 ± 0.25**	0.36 ± 0.15	1.12 ± 0.26
		LTA	**1.86 ± 1.15**	0.82 ± 0.26	**3.54 ± 1.36**
		Poly I:C	0.29 ± 0.18	0.37 ± 0.20	**2.82 ± 1.17**

8.2 Analysis of the effect of a double inflammatory stimulus over time on the transcription of the complement system and other immunoregulatory and anti-inflammatory molecules

As described in Chapter 6, pro-inflammatory cytokine-mediated MSC licensing produced a transcriptional regulation of genes involved in the regulation of the immune response and immunomodulatory mediators, which could explain why MSCs are more immunosuppressive upon activation. Within clinical settings, it has been reported that pre-treatment of MSCs with inflammatory cytokines prior to administration within animal models of inflammatory diseases boosts the therapeutic effect of MSCs (Duijvestein et al., 2011, Noronha et al., 2019). For this reason, we wondered if pre-licensing the MSCs prior to expose them to an inflammatory environment *in vitro* would lead to even bigger variations in chemokines transcriptional levels and therefore, in enhanced therapeutic potential. For this aim, cells were pre-licensed for 48 hours, after which cells were washed twice with PBS and stimulated again for another 24 hours. The first stimulation primes the MSCs, while the second stimulation would mimic the inflammatory environment MSCs would face when infused into a patient with an inflammatory disorder. Figure 8-6 illustrates the time points at which supplemented medium was added. Culture medium was removed at the time cells were harvested and was kept for experimental procedures.

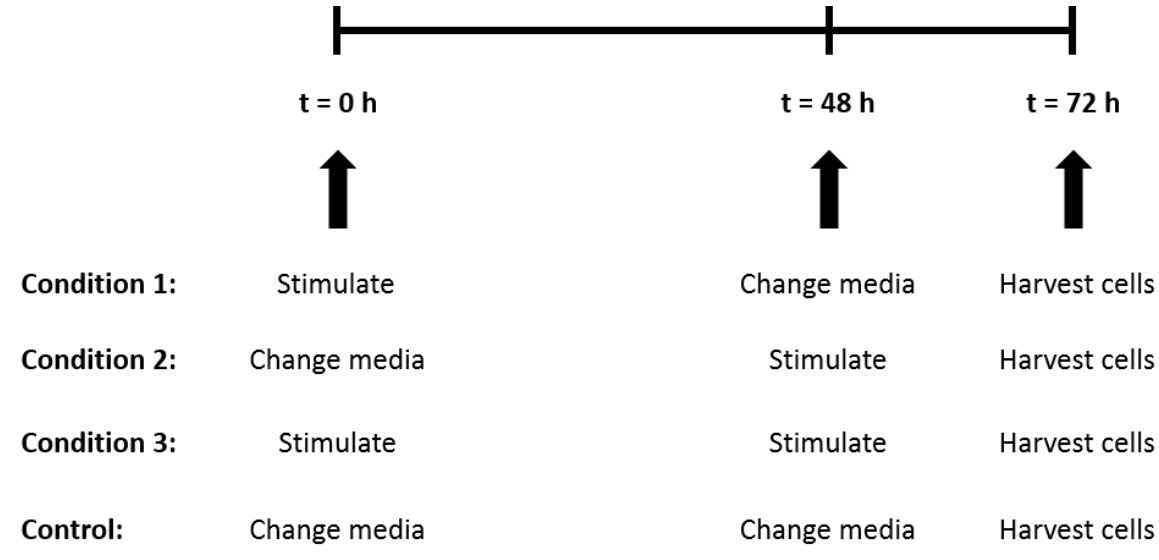


Figure 8-6. Diagrammatic illustration of the time course of the MSC licensing.

Two different control conditions were used. In the first one, cells were stimulated for 48 hours, after which, cells were washed twice with PBS and fresh culture medium was added; cells were harvested 24 hours later. In the second condition, cells were washed twice with PBS, the culture medium was replaced with fresh medium and cells were left growing for 48 hours. Cells were then washed twice with PBS, the culture medium was replaced with supplemented medium and the cells were harvested 24 hours later. There is wide literature about how cytokine-mediated licensing enhances the potential therapeutic efficacy of MSCs, however, little is known about the role of TLR ligands-mediated activation on the secretion of chemotactic cytokines by MSCs. For this reason, 100 ng/ mL LPS, 100 ng/ mL LTA and 4 mg/ mL poly I:C, as well as the previously described cytokine cocktail, was used for MSC licensing.

According to the results described in Section 6.2, the genes regulating their transcript levels in a statistically significant manner upon stimulation were selected and their transcriptional levels were assessed after licensing with different inflammatory molecules and different inflammatory conditions.

8.2.1 Transcription of the complement system under homeostatic and inflammatory conditions

Under resting conditions, BM, Is and Ad MSCs transcribed very little, if any, of the elements of the complement system and only the transcription of *CFH* (Figure 8-7, A), *C1Qc* (B) and *C5AR1* (C) was affected by MSC licensing in a statistically significant manner. A pattern of transcriptional upregulation was observed in *CFH* (E), *C1Qc* (B) and *C5AR1* (C) after cytokine, LPS and Poly I:C-mediated licensing, while LTA licensing led to a pattern of transcriptional upregulation; however, these genes were upregulated differentially in MSCs according to their tissue of origin. Fold changes of transcriptional regulation upon licensing of MSCs are specified in Table 8-5.

As previously described, *CFH* was expressed at very low levels by MSCs from all sources and Ad MSCs had the highest *CFH* transcript levels under resting conditions (Figure 8-7, A). 24 hours after stimulation with the cytokine cocktail (Condition 2), MSCs from the three tissues downregulated their *CFH* transcript levels and this downregulation increased after 72 hours of stimulation (Condition

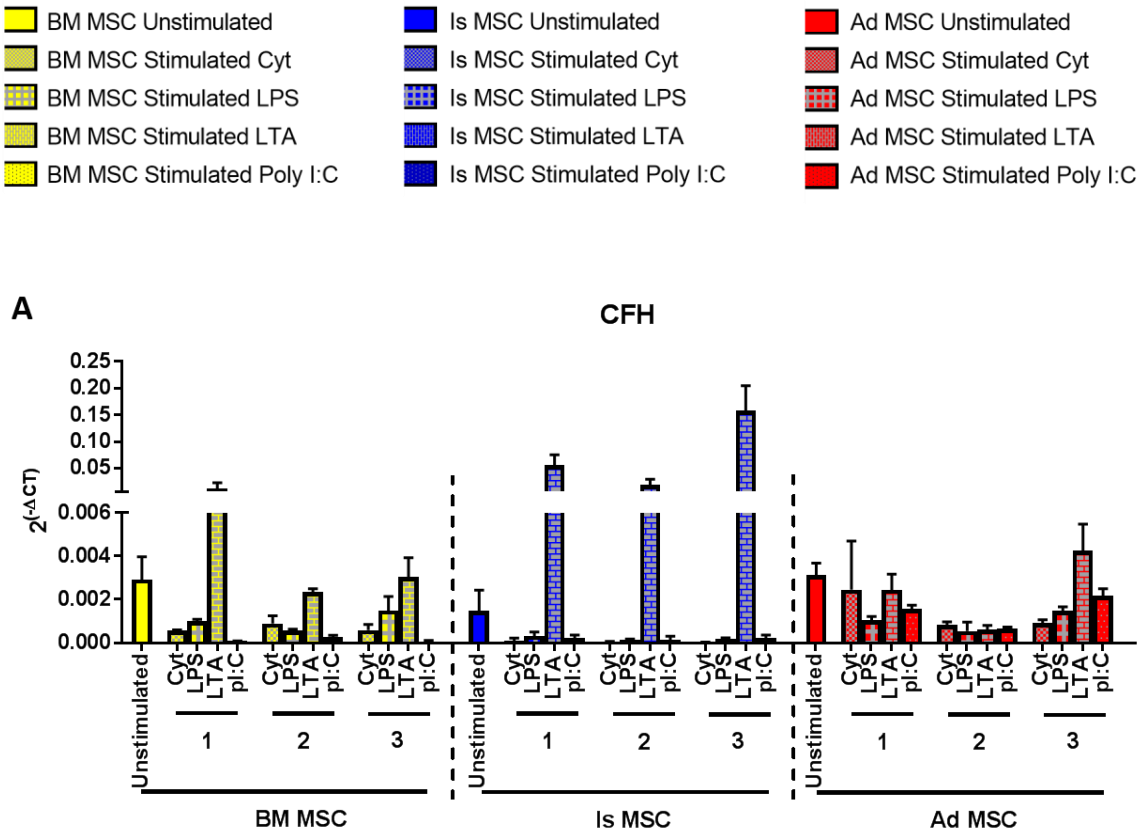
1) in BM MSCs, while it was maintained in Is and Ad MSCs. A second stimulation with the cytokine cocktail 48 hours after the first stimulation (Condition 3) produced no variation compared to Condition 2 in MSCs from every source. 72 hours after LPS-mediated stimulation (Condition 1) MSCs from every source had downregulated their *CFH* transcript levels and this downregulation was smaller after 24 hours of stimulation (Condition 2). Double stimulation of MSCs with LPS (Condition 3) led to an increase in the transcript levels compared to Condition 2 in MSCs from every source. 72 hours LTA-mediated licensing led to an increase in *CFH* transcript levels in BM and Is MSCs while it produced no effect in Ad MSCs. 24 hours LTA-mediated licensing led to no variation in BM MSCs, led to an upregulation smaller than the one achieved after 72 hours in Is MSCs and to a downregulation in Ad MSCs. A second stimulation 48 hours after the first stimulation (Condition 3) led to no variation in BM MSC and led to the biggest upregulation in Is and Ad MSCs compared to the previous conditions. 24-hour Poly I:C-mediated stimulation (Condition 2) led to the downregulation of *CFH* transcript levels in BM MSCs and this downregulation was increased 72 hours after stimulation (Condition 1) and a second stimulation (Condition 3). Poly I:C-mediated stimulation led to a decrease in *CFH* transcript levels in Is and Ad MSCs under every condition however, this downregulation was bigger after 24 hours of stimulation (Condition 2) compared to 72 hours after stimulation (Condition 1) and a second stimulation (Condition 3).

C1Qc was expressed at low levels by MSCs from the three sources but was expressed the highest by Is MSCs under resting conditions (Figure 8-7, B). 24-hour cytokine and LPS-mediated stimulation (Condition 2) led to the decrease of *C1Qc* transcript levels in MSCs from all sources; 72 hours after stimulation (Condition 1) BM and Is MSCs had still downregulated *C1Qc* transcript levels but less than in Condition 2 and a second stimulation 48 hours after the first one (Condition 3) led to the same transcript levels as in Condition 1 in these cells. However, 72 hours after cytokine-mediated stimulation or after a double stimulation Ad MSCs had upregulated their *C1Qc* transcript levels while LPS-mediated stimulation led to a smaller downregulation 72 hours after licensing and to an increased downregulation after a double licensing with LPS in Ad MSCs. LTA-mediated licensing produced an upregulation in BM MSCs in the three conditions. 24-hour LTA-mediated licensing (Condition 2) led to the

downregulation of *C1Qc* transcript levels in Is and Ad MSCs and this downregulation started to reverse 72 hours after stimulation (Condition 1). A second stimulation 48 hours after the first one (Condition 3), led to an upregulation of *C1Qc* transcript levels in Is MSCs while it produced a bigger downregulation than in Conditions 1 and 2 in Ad MSCs. Regarding Poly I:C-mediated licensing, 24-hour stimulation (Condition 2) led to the downregulation of *C1Qc* transcript levels in MSCs from all sources, this downregulation started to reverse 72 hours after stimulation (Condition 1) in Is and Ad MSCs while it was bigger in BM MSCs. A second stimulation 48 hours after the first one (Condition 3) did not increase the downregulation in BM and Is MSCs but it did lead to an increased downregulation in Ad MSCs.

C5AR1 was expressed at low levels by MSCs from the three sources but was expressed the highest by Is MSCs under resting conditions (Figure 8-7, C). 24-hour cytokine-mediated licensing (Condition 2) led to an upregulation of *C5AR1* transcript levels in BM MSCs, a downregulation in Is MSCs and to no effect in Ad MSCs, these effects were slightly more pronounced 72 hours after the cytokine-mediated stimulation (Condition 1) as the upregulation and downregulation in BM and Is MSCs respectively was increased in both MSCs while it produced no effect in Ad MSCs. A second stimulation 48 hours after the first one (Condition 3) did not make a difference in BM and Is MSCs when compared to Condition 1; however, it led to the downregulation of *C5AR1* transcript levels in Ad MSCs. 24-hour LPS-mediated licensing (Condition 2) led to the upregulation of *C5AR1* transcript levels in BM MSCs and to downregulation in Is and Ad MSCs; these effects were slightly more pronounced 72 hours after the cytokine-mediated stimulation (Condition 1) in BM MSCs, while Is and Ad MSCs had started to reverse the downregulation. A 24-hour stimulation 48 hours after the first one (Condition 3) led to the same levels of upregulation in BM MSCs, while it increased *C5AR1* transcript levels in Is and Ad MSCs without reaching control unstimulated levels. 24-hour LTA-mediated licensing (Condition 2) led to the upregulation of *C5AR1* transcript levels in BM MSCs and to downregulation in Is and Ad MSCs. This regulation started to reverse 48 hours later (Condition 1) in BM and Ad MSCs as the upregulation in BM MSCs and the downregulation of *C5AR1* transcript levels in Ad MSCs was less pronounced; however, *C5AR1* transcript levels remained as downregulated as 48 hours before in Is MSCs. A

second stimulation 48 hours after the first one (Condition 3) led to the same levels of upregulation in BM MSCs, while it slightly increased *C5AR1* transcript levels in Is and Ad MSCs compared to Conditions 1 and 2. Lastly, 24-hour Poly I:C-mediated licensing (Condition 2) led to a small upregulation in BM MSCs while it led to the downregulation of *C5AR1* transcript levels in Is and Ad MSCs. This transcriptional regulation was not sustained as 72 hours after a single stimulation (Condition 1) BM MSCs transcript levels had returned to the same level as the control ones and Is and Ad MSCs had a less pronounced downregulation of their *C5AR1* transcript levels. A second stimulation 48 hours after the first one (Condition 3) led to the downregulation of *C5AR1* transcript levels in MSCs from every source.



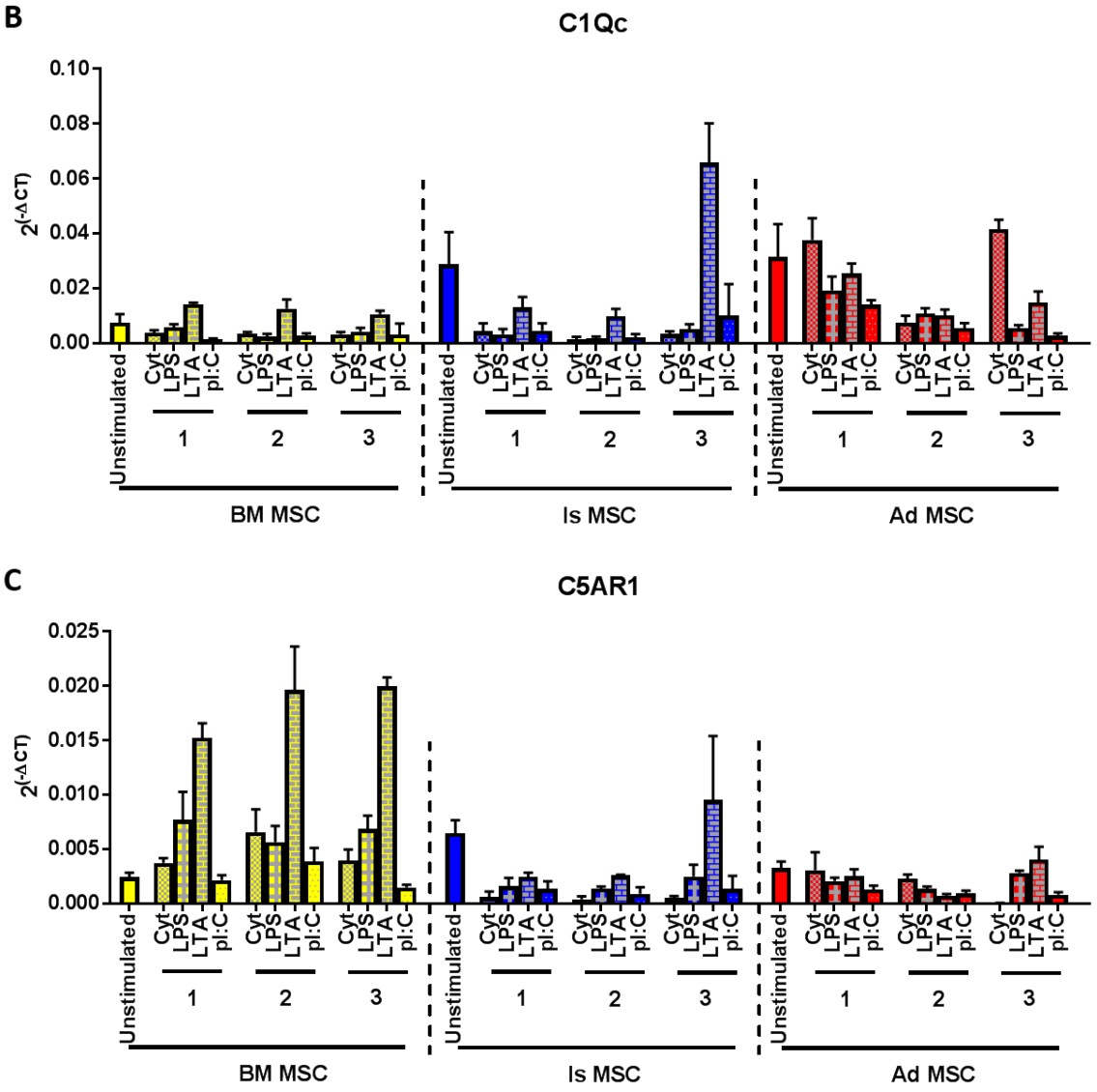


Figure 8-7. Inflammatory agent, repetitive stimulus and MSC tissue origin impacts complement system molecules transcript levels in MSCs.
MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with either a cocktail of cytokines (40 ng/ mL of IFN-Ɣ, TNF-α and IL-1β), 100 ng/ mL LPS, 100 ng/ mL LTA or 4 µg/ mL Poly I:C. Unstimulated cells were left growing in MSC culture medium as a control. Three different licensing conditions were tested. In the first one, cells were stimulated for 48 hours, after which cells were washed twice with PBS and fresh culture medium was added; cells were harvested 24 hours later. In the second condition, cells were washed twice with PBS, the culture medium was replaced with fresh one and the cells were left growing for 48 hours. Cells were then washed twice with PBS, the culture medium was replaced with supplemented one and the cells were harvested 24 hours later. In the last condition, cells were stimulated for 48 hours, after which cells were washed twice with PBS and were stimulated again for another 24 hours. Figure 6-4 illustrates the time points at which supplemented medium was added. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate complement system molecules transcripts in BM, Is and Ad MSCs under resting and inflammatory conditions. Each bar represents an n of 4 independent experiments and is graphed as mean ± SEM. Data are normalised to the housekeeping gene *B2M* and expressed as $2^{(-\Delta CT)}$. Statistically significant differences are marked with a colour code in Table 4-7.

Table 8-5. Fold change in complement system molecules transcript levels of cytokine, LPS, LTA or Poly I:C-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.
Following the experimental set up explained in Figure 8-2, fold change in transcript levels is represented as mean of fold change ± standard deviation. One Way ANOVA with Tukey's multiple

comparisons post-test was performed to compare all MSC sources and the different conditions. p = 0.05 was considered the limit for statistical significance. Statistically significant differences are marked with a colour code, where p < 0.05 is represented by green, p < 0.01 is represented by orange, p < 0.001 is represented by blue and p < 0.0001 is represented by red.

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
CFH	1	Cyt	0.21 ± 0.07	0.10 ± 0.12	0.69 ± 0.50
		LPS	0.37 ± 0.10	0.32 ± 0.26	0.30 ± 0.04
		LTA	3.40 ± 2.12	46.04 ± 25.45	0.72 ± 0.24
		Poly I:C	0.03 ± 0.01	0.24 ± 0.23	0.46 ± 0.10
	2	Cyt	0.38 ± 0.26	0.03 ± 0.00	0.24 ± 0.04
		LPS	0.22 ± 0.07	0.15 ± 0.11	0.17 ± 0.12
		LTA	0.91 ± 0.34	15.74 ± 7.32	0.18 ± 0.06
		Poly I:C	0.09 ± 0.01	0.08 ± 0.04	0.19 ± 0.03
	3	Cyt	0.26 ± 0.17	0.03 ± 0.02	0.27 ± 0.07
		LPS	0.63 ± 0.37	0.20 ± 0.14	0.43 ± 0.09
		LTA	1.26 ± 0.71	149.39 ± 91.04	1.24 ± 0.35
		Poly I:C	0.03 ± 0.02	0.28 ± 0.24	0.64 ± 0.13
C1Qc	1	Cyt	0.59 ± 0.25	0.15 ± 0.07	0.94 ± 0.23
		LPS	0.92 ± 0.42	0.12 ± 0.07	0.47 ± 0.05
		LTA	2.19 ± 0.86	0.50 ± 0.18	0.63 ± 0.10
		Poly I:C	0.22 ± 0.08	0.21 ± 0.18	0.35 ± 0.05
	2	Cyt	0.52 ± 0.19	0.04 ± 0.02	0.18 ± 0.03
		LPS	0.32 ± 0.04	0.08 ± 0.05	0.27 ± 0.07
		LTA	2.04 ± 1.26	0.40 ± 0.17	0.25 ± 0.05
		Poly I:C	0.40 ± 0.17	0.08 ± 0.03	0.14 ± 0.04
	3	Cyt	0.52 ± 0.26	0.13 ± 0.05	1.04 ± 0.18
		LPS	0.67 ± 0.33	0.21 ± 0.13	0.14 ± 0.02
		LTA	1.59 ± 0.51	2.52 ± 0.75	0.36 ± 0.08
		Poly I:C	0.39 ± 0.35	0.52 ± 0.65	0.07 ± 0.01
C5AR1	1	Cyt	1.59 ± 0.26	0.09 ± 0.06	1.06 ± 0.44
		LPS	3.14 ± 0.44	0.24 ± 0.09	0.74 ± 0.14
		LTA	6.45 ± 0.57	0.40 ± 0.10	0.88 ± 0.16
		Poly I:C	0.89 ± 0.07	0.22 ± 0.12	0.46 ± 0.13
	2	Cyt	2.71 ± 0.51	0.06 ± 0.03	0.81 ± 0.13
		LPS	2.49 ± 0.92	0.22 ± 0.04	0.48 ± 0.06
		LTA	8.17 ± 0.42	0.41 ± 0.06	0.25 ± 0.07
		Poly I:C	1.72 ± 0.70	0.13 ± 0.07	0.34 ± 0.09
	3	Cyt	1.63 ± 0.12	0.08 ± 0.03	0.01 ± 0.01
		LPS	2.87 ± 0.24	0.40 ± 0.17	1.00 ± 0.11
		LTA	8.49 ± 1.17	1.52 ± 0.82	1.44 ± 0.38
		Poly I:C	0.61 ± 0.02	0.22 ± 0.19	0.29 ± 0.08

8.2.2 Transcription of other immunoregulatory and anti-inflammatory molecules under homeostatic and inflammatory conditions

Under resting conditions BM, Is and Ad MSCs transcribed very little, if any, of the immunoregulatory and anti-inflammatory genes with the exception of *IL-6* (Figure 8-8, A), *VEGFa* (C), *VEGFd* (F) and *CD142* (J). The effect of inflammatory licensing in these genes was tissue of origin and stimulatory agent dependent and fold changes of transcriptional regulation upon licensing of MSCs are specified in Table 8-6.

IL-6 was expressed at substantial levels by MSCs from the three sources but was expressed the highest by Ad MSCs under resting conditions (Figure 8-8, A). 24-hour cytokine-mediated stimulation (Condition 2) led to the upregulation of *IL-6* transcript levels in BM and Is MSCs, while it produced no effect in Ad MSCs. This upregulation was not sustained as 72 hours after stimulation (Condition 1) BM and Is MSCs had downregulated their *IL-6* transcript levels to control levels while 72 hours cytokine-mediated licensing produced the downregulation of *IL-6* transcript levels in Ad MSCs. A second stimulation 48 hours after the first one (Condition 3) led to the upregulation of *IL-6* transcript levels in MSCs from all sources; an increased upregulation of *IL-6* transcript levels in BM MSCs, a smaller upregulation in Is MSCs compared to Condition 2 and >50 times upregulation in Ad MSCs. 24-hour LPS-mediated licensing (Condition 2) led to an upregulation of *IL-6* transcript levels in BM and Is MSCs, while it produced no effect in Ad MSCs. This upregulation was not sustained in BM MSCs as 72 hours after LPS licensing (Condition 1) BM MSCs had downregulated their *IL-6* transcript levels compared to the control; the upregulation had started to reverse in Is MSCs but these cells were still transcribing higher levels of *IL-6* than the unstimulated control cells and 72 hours licensing led to the downregulation of *IL-6* transcript levels in Ad MSCs. LTA and Poly I:C-mediated 24-hour licensing (Condition 2) led to the downregulation of *IL-6* transcript levels in BM MSCs. This downregulation was increased 72 hours after the licensing (Condition 1) and was maintained when the cells were stimulated 48 hours after the first stimulation with LTA or Poly I:C (Condition 3). LTA and Poly I:C licensing produced no effect in the transcription of *IL-6* in Is MSCs under any of the conditions tested while LTA and Poly I:C-mediated licensing produced a downregulation in *IL-6* transcript levels 72 hours

after licensing (Condition 1) in Ad MSCs, but they made no effect in *IL-6* transcript levels 24 hours after a single stimulation (Condition 2) or after a double stimulation (Condition 3).

As previously described, *TSG-6* was expressed at very low levels by MSCs from all sources and BM MSCs had the highest *TSG-6* transcript levels under resting conditions (Figure 8-8, B). 24-hour cytokine-mediated licensing (Condition 2) led to the downregulation of *TSG-6* transcript levels in MSCs from the three sources; this downregulation was not sustained in MSCs from the three sources as 72 hours after the stimulation (Condition 1) BM MSCs were transcribing slightly more *TSG-6* than 48 hours before, Is MSCs were transcribing almost the same amount of *TSG-6* as unstimulated cells and Ad MSCs had upregulated the transcription of *TSG-6*. A second stimulation 48 hours after the first one (Condition 3) maintained the downregulation in BM and Is MSCs while it produced no effect in Ad MSCs as these cells were generating the same *TSG-6* levels as under resting conditions. 24-hour LPS-mediated licensing (Condition 2) produced the downregulation of *TSG-6* transcript levels in BM and Ad MSCs while it produced an upregulation in Is MSCs. This regulation was maintained as 72 hours after LPS licensing (Condition 1) MSCs from the three sources were expressing similar levels of *TSG-6* transcript levels as 48 hours before. A second stimulation 48 hours after the first one (Condition 3) increased *TSG-6* transcript levels to almost the control levels in BM MSCs, decreased the upregulation to almost control levels in Is MSCs and maintained the downregulation obtained 24 and 72 hours after stimulation in Ad MSCs. 24-hour LTA-mediated licensing produced the downregulation of *TSG-6* transcript levels in BM and Ad MSCs while it produced a small increase in Is MSCs; these transcription levels were maintained 72 hours after stimulation (Condition 1) in Is and Ad MSCs while the downregulation was bigger in BM MSCs. A second stimulation with LTA 48 hours after the first one (Condition 3) led to a small downregulation in BM and Ad MSCs compared to control and a bigger upregulation in Is MSCs. Poly I:C licensing of BM MSCs led to the downregulation of *TSG-6* transcript levels under every condition tested, while it produced no effect in Is MSCs. 24 hours Poly I:C licensing (Condition 2) led to the downregulation of *TSG-6* transcript levels in Ad MSCs; this downregulation was smaller 72 hours after stimulation (Condition 1). A second stimulation 48 hours

after the first one (Condition 3) led to no effect in Ad MSCs as these cells had the same transcript levels as control unstimulated Ad MSCs.

VEGFa was expressed at substantial levels by MSCs from the three sources but was expressed the highest by Is MSCs under resting conditions (Figure 8-8, C). 24-hour cytokine-mediated stimulation (Condition 2) led to the downregulation of *VEGFa* transcript levels in BM and Is MSCs, while it produced a small upregulation in Ad MSCs. This regulation was increased over time as 72 hours after stimulation (Condition 1) BM and Is MSCs had downregulated more their *VEGFa* transcript levels while 72 hours cytokine-mediated licensing produced a bigger upregulation of *VEGFa* transcript levels in Ad MSCs. A second stimulation 48 hours after the first one (Condition 3) led to similar levels of downregulation of *VEGFa* transcript levels in BM and Is MSCs as after 24 hours of stimulation, while a second stimulation enhanced the upregulation in Ad MSCs. 24-hour LPS-mediated stimulation (Condition 2) led to the downregulation of *VEGFa* transcript levels in BM and Is MSCs, while it produced no effect in Ad MSCs. 72 hours after stimulation (Condition 1) BM and Is MSCs had started to reverse the downregulation while 72 hours LPS-mediated licensing produced a downregulation of *VEGFa* transcript levels in Ad MSCs. A second stimulation 48 hours after the first one (Condition 3) led to a small downregulation of *VEGFa* transcript levels in Is and Ad MSCs, while a second LPS stimulation had no effect in BM MSCs. 72-hour LTA-mediated stimulation (Condition 1) led to no effect in MSCs from the three sources while 24-hour LTA-mediated stimulation (Condition 2) led to a small upregulation of *VEGFa* transcript levels in MSCs from the three sources. A second stimulation 48 hours after the first one (Condition 3) led to a small downregulation of *VEGFa* transcript levels in BM and Ad MSCs while it produced a small upregulation in Is MSCs. 24-hour Poly I:C-mediated stimulation (Condition 2) led to the downregulation of *VEGFa* transcript levels in MSCs from the three sources and 72 hours Poly I:C-mediated licensing produced a bigger downregulation of *VEGFa* transcript levels in MSCs from the three sources. A second stimulation 48 hours after the first one (Condition 3) led to a downregulation of *VEGFa* transcript levels in BM MSCs of the same level as 24-hour licensing while a second Poly I:C stimulation had no effect in Is and Ad MSCs.

VEGFb was expressed at very low levels by MSCs from all sources and Is MSCs had the highest *VEGFb* transcript levels under resting conditions (Figure 8-8, D). 24-hour cytokine-mediated stimulation (Condition 2) led to the downregulation of *VEGFb* transcript levels in MSCs from the three sources and this downregulation was maintained 72 hours after the licensing (Condition 1) in MSCs from the three sources. A second stimulation 48 hours after the first one (Condition 3) produced the biggest downregulation of *VEGFb* transcript levels in BM and Is MSCs, while it produced an upregulation in Ad MSCs. 24-hour LPS and LTA-mediated stimulation (Condition 2) led to the downregulation of *VEGFb* transcript levels in MSCs from the three sources and this downregulation started to reverse 72 hours after the licensing (Condition 1) in MSCs from the three sources at different levels; BM MSCs had upregulated their *VEGFb* transcript levels while Is and Ad MSCs had not yet reached the control unstimulated *VEGFb* transcript levels. A second LPS stimulation 48 hours after the first one (Condition 3) led to the downregulation of *VEGFb* transcript levels in MSCs from the three sources, however this downregulation was not as strong as the one generated after 24-hour LPS stimulation. A second LTA stimulation 48 hours after the first one (Condition 3) led to no variation in *VEGFb* transcript levels compared to the control unstimulated MSCs in the three sources. 24-hour Poly I:C-mediated stimulation (Condition 2) led to the downregulation of *VEGFb* transcript levels in MSCs from the three sources and this downregulation was maintained 72 hours after licensing (Condition 1) in MSCs from the three sources. A second LPS stimulation 48 hours after the first one (Condition 3) led to the downregulation of *VEGFb* transcript levels in MSCs from the three sources; however, this downregulation was not as strong as the one generated after 24-hour Poly I:C stimulation in Is and Ad MSCs.

VEGFc was expressed at very low levels by MSCs from all sources and Is MSCs had the highest *VEGFc* transcript levels under resting conditions (Figure 8-8, E). 24-hour cytokine-mediated stimulation (Condition 2) led to the downregulation of *VEGFc* transcript levels in BM MSCs, while it had no effect in Is and Ad MSCs; however, 72-hour cytokine-mediated stimulation (Condition 1) led to the downregulation of *VEGFc* transcript levels in MSCs from the three sources. A second stimulation 48 hours after the first one (Condition 3) produced a small downregulation of *VEGFc* transcript levels in BM and Is MSCs, while it produced

an upregulation in Ad MSCs. 24-hour LPS-mediated stimulation (Condition 2) led to the downregulation of *VEGFc* transcript levels in BM and Ad MSCs, while it had no effect in Is MSCs. 72 hours after LPS licensing (Condition 1) BM and Ad MSCs had still downregulated their *VEGFc* transcript levels, while 72 hours after the licensing Is MSCs had their *VEGFc* transcript levels upregulated. A second stimulation 48 hours after the first one (Condition 3) produced a small downregulation in BM and Ad MSCs, while it had no effect in Is MSCs. 24-hour LTA and Poly I:C-mediated stimulation (Condition 2) led to the downregulation of *VEGFc* transcript levels in BM and Ad MSCs, while it upregulated *VEGFc* transcript levels in Is MSCs. This downregulation was sustained in BM and Ad MSCs 72 hours after LTA and Poly I:C licensing (Condition 1), while Is MSCs slightly increased their *VEGFc* transcript levels 72 hours after licensing. A second stimulation 48 hours after the first one (Condition 3) led to the downregulation of *VEGFc* transcript levels in BM and Is MSCs, while it had no effect in Ad MSCs.

As previously described, *VEGFd* was expressed at substantial levels by MSCs from the three sources but was expressed the highest by BM MSCs under resting conditions (Figure 8-8, F). 24-hour cytokine and LPS-mediated stimulation (Condition 2) led to the downregulation of *VEGFd* transcript levels in MSCs from the three sources; these downregulation was maintained 72 hours after licensing (Condition 1) in BM and Is MSCs, while Ad MSCs had already recovered from the licensing. A second stimulation 48 hours after the first one (Condition 3) led to the downregulation of *VEGFd* transcript levels in MSCs from the three sources, where MSCs from the three sources had similar *VEGFd* transcript levels as after a single 24-hour cytokine or LPS licensing. 24-hour LTA-mediated licensing led to a small non-significant upregulation of *VEGFd* transcript levels in BM MSCs, while it produced the downregulation of *VEGFd* transcript levels in Is and Ad MSCs. 72 hours after licensing (Condition 1), BM MSCs had downregulated their *VEGFd* transcript levels, the downregulation in Is and Ad MSCs was not sustained and transcript levels had reached resting levels in Ad MSCs. A second stimulation 48 hours after the first one (Condition 3) led to a small downregulation of *VEGFd* transcript levels in BM and Is MSCs, while it led to a small upregulation of *VEGFd* transcript levels in Ad MSCs. 24-hour Poly I:C-mediated stimulation (Condition 2) led to the downregulation of *VEGFd* transcript levels in MSCs from the three sources and this downregulation was maintained 72 hours after Poly I:C licensing

(Condition 1) in MSCs from the three sources. A second stimulation 48 hours after the first one (Condition 3) led to similar levels of downregulation of *VEGFd* transcript levels in MSCs of every source as after 24 hours of Poly I:C stimulation.

iNOS was expressed at very low levels by MSCs from all sources and BM MSCs had the highest *iNOS* transcript levels under resting conditions (Figure 8-8, G). 24-hour cytokine-mediated stimulation (Condition 2) led to the upregulation of *iNOS* transcript levels in MSCs from the three sources; however, this upregulation was not sustained as 72 hours after stimulation MSCs from the three sources were expressing similar *iNOS* transcript levels as control unstimulated MSCs. A second cytokine-mediated stimulation 48 hours after the first one (Condition 3) led to an upregulation of *iNOS* transcript levels in MSCs from the three sources; however, this upregulation did not match the one after 24-hour cytokine-mediated licensing in BM and Is MSCs while it was bigger in Ad MSCs. LPS, LTA and Poly I:C-mediated licensing in every condition led to statistically non-significant variations and to very little, if any, *iNOS* transcript levels.

COX2 was expressed at low levels by MSCs from all sources and Ad MSCs had the highest *COX2* transcript levels under resting conditions (Figure 8-8, H). 24-hour cytokine-mediated stimulation (Condition 2) led to the upregulation of *COX2* transcript levels in MSCs from the three sources; however, this upregulation started to reverse 48 hours later (Condition 1) in BM and Ad MSC as despite showing upregulated *COX2* transcript levels, they were lower than 48 hours before. Is MSCs, on the contrary, were able to maintain the upregulation 72 hours after the cytokine-mediated stimulation. A second stimulation 48 hours after the first one (Condition 3) increased the *COX2* transcript levels in BM MSCs compared to 72 hours after stimulation, but did not match the transcript levels recorded 24 hours after stimulation; while a second cytokine-mediated stimulation increased *COX2* transcript levels in Is and Ad MSCs. 24-hour LPS-mediated stimulation (Condition 2) led to the upregulation of *COX2* transcript levels in MSCs from the three sources and this upregulation was not sustained as 72 hours after licensing BM MSCs had reached control transcript levels and Is and Ad MSCs were closer to control levels. A second stimulation 48 hours after the first one (Condition 3) increased the *COX2* transcript levels in MSCs from every source but this upregulation could not match the *COX2* transcript levels observed

after 24-hour LPS-mediated stimulation in BM and Ad MSCs. 24-hour LTA-mediated stimulation (Condition 2) led to the upregulation of *COX2* transcript levels in MSCs from the three sources and this upregulation was not sustained in MSCs from every tissue as MSCs had lower *COX2* transcript levels than the control unstimulated MSCs. A second stimulation 48 hours after the first one (Condition 3) led to the downregulation of *COX2* transcript levels to similar levels as observed 72 hours after stimulation. 24-hour Poly I:C-mediated stimulation (Condition 2) led to the downregulation of *COX2* transcript levels in BM MSCs, while it produced an upregulation in Is and Ad MSCs. 72 hours after Poly I:C-mediated stimulation (Condition 1) MSCs from the three sources had downregulated their *COX2* transcript levels. A second stimulation 48 hours after the first one (Condition 3) led to a smaller downregulation of *COX2* transcript levels in BM MSCs, while it led to the upregulation of *COX2* transcript levels in Is and Ad MSCs to higher levels than the ones observed in Condition 1 or 2.

HGF was expressed at low levels by MSCs from all sources and Is MSCs had the highest *HGF* transcript levels under resting conditions (Figure 8-8, I). 24-hour cytokine-mediated stimulation (Condition 2) led to the downregulation of *HGF* transcript levels in MSCs from the three sources; this downregulation was more intense 48 hours later (Condition 1) in BM and Is MSC, while Ad MSCs had upregulated their *HGF* transcript levels compared to control unstimulated levels. A second stimulation 48 hours after the first one (Condition 3) maintained the downregulation in BM MSCs, it was not sustained in Is MSCs as transcript levels were getting back to control conditions and maintained the upregulation observed 72 hours after cytokine-mediated licensing in Ad MSCs. 24-hour LPS-mediated stimulation (Condition 2) led to the downregulation of *HGF* transcript levels in BM MSCs and to a small non-significant upregulation in Is and Ad MSCs; however, 72 hours after LPS-mediated licensing BM and Is MSCs had downregulated their *HGF* transcript levels, while Ad MSCs was expressing the same level of *HGF* transcript levels as under control unstimulated conditions. A second stimulation 48 hours after the first one led to the downregulation of BM MSCs, to no effect in Is MSCs and to the upregulation of *HGF* transcript levels in Ad MSCS. 24-hour LTA-mediated stimulation (Condition 2) led to no effect in BM MSCs, to the upregulation of *HGF* transcript levels in Is MSCs and to a small non-significant downregulation in Ad MSCs; however, 72 hours after LTA-mediated

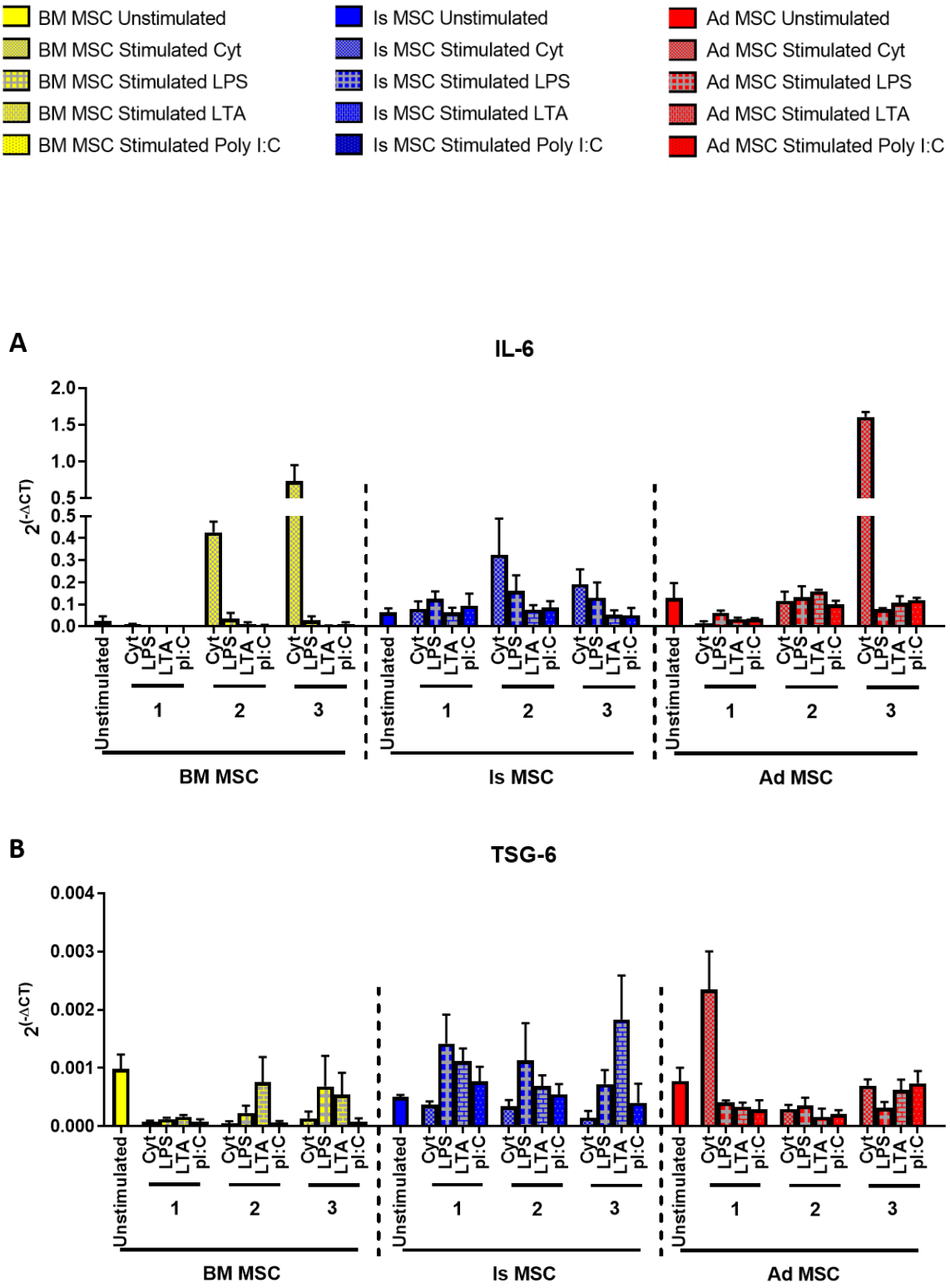
licensing BM MSCs had downregulated their *HGF* transcript levels in a statistically significant manner, the upregulation in Is MSC was not sustained, while Ad MSCs had upregulated their *HGF* transcript levels. A second stimulation 48 hours after the first one led to control-like transcript levels in BM MSCs, to a small non-significant upregulation in Is MSCs and to a small non-significant downregulation in Ad MSCs. 24-hour Poly I:C-mediated stimulation (Condition 2) led to the downregulation of *HGF* transcript levels in MSCs from the three sources; this downregulation was maintained in BM MSCs 72 hours after stimulation (Condition 1), while Is and Ad MSCs reversed the downregulation. A second stimulation 48 hours after the first one was able to mimic the *HGF* transcript levels observed after 24 hours of stimulation as it led to the downregulation of *HGF* transcript levels in MSCs from every source.

CD142 was expressed at substantial levels by MSCs from the three sources but was expressed the highest by BM MSCs under resting conditions (Figure 8-8, J). 24-hour cytokine-mediated stimulation (Condition 2) led to the downregulation of *CD142* transcript levels in MSCs from the three sources and this downregulation was maintained 72 hours after stimulation (Condition 1) in BM and Is MSCs, while it was not sustained in Ad MSCs. A second stimulation 48 hours after the first one (Condition 3) led to similar levels of downregulation of *CD142* transcript levels in BM and Is MSCs as after 24 and 72 hours of stimulation, while a second stimulation led to the upregulation of *CD142* transcript levels in Ad MSCs. 24-hour LPS-mediated stimulation (Condition 2) led to the downregulation of *CD142* transcript levels in MSCs from the three sources and this downregulation was maintained 72 hours after stimulation (Condition 1) in MSCs from the three sources. A second stimulation 48 hours after the first one (Condition 3) was not able to mimic the downregulation achieved in Conditions 1 and 2 but still led to the downregulation of *CD142* transcript levels in MSCs from the three sources. 24-hour LTA-mediated stimulation (Condition 2) led to a small upregulation of *CD142* transcript levels in BM MSCs and to a downregulation in Is and Ad MSCs; however, 72 hours after LTA licensing BM, Is and Ad MSCs had downregulated their *CD142* transcript levels. A second stimulation 48 hours after the first one (Condition 3) led to small non-significant downregulation of *CD142* transcript levels in BM and Is MSCs, while it produced a small non-significant upregulation of *CD142* transcript levels in Ad MSCs. 24-hour Poly I:C-mediated

stimulation (Condition 2) led to the downregulation of *CD142* transcript levels in MSCs from the three sources and this downregulation was maintained 72 hours after stimulation (Condition 1) in MSCs from the three sources. A second stimulation 48 hours after the first one (Condition 3) was not able to mimic the downregulation achieved in Conditions 1 and 2 but still led to the downregulation of *CD142* transcript levels in MSCs from the three sources.

GMCSF was expressed at low levels by MSCs from all sources and Ad MSCs had the highest *GMCSF* transcript levels under resting conditions (Figure 8-8, K). 24-hour cytokine-mediated stimulation (Condition 2) led to the upregulation of *GMCSF* transcript levels in MSCs from the three sources and this upregulation was maintained, at lower level, 72 hours after stimulation (Condition 1). A second stimulation 48 hours after the first one (Condition 3) led to the upregulation of *GMCSF* transcript levels but none of these upregulations was statistically significant due to the big variability between samples. 24-hour LPS-mediated stimulation (Condition 2) led to the downregulation of *GMCSF* transcript levels in BM MSCs while it upregulated *GMCSF* transcript levels in Is and Ad MSCs; this transcriptional regulation was maintained 72-hours after stimulation (Condition 1). A second stimulation 48 hours after the first one (Condition 3) led to a statistically non-significant upregulation of *GMCSF* transcript levels in MSCs from the three sources. 24-hour LTA-mediated stimulation (Condition 2) led to the upregulation of *GMCSF* transcript levels in MSCs from the three sources; this transcriptional upregulation was increased 72-hours after stimulation (Condition 1). A second stimulation 48 hours after the first one (Condition 3) led to the downregulation of *GMCSF* transcript levels in BM MSCs, while it led to the upregulation of *GMCSF* transcript levels in Is and Ad MSCs.

24-hour Poly I:C-mediated stimulation (Condition 2) led to the downregulation of *GMCSF* transcript levels in MSCs from the three sources; however, 72 hours after stimulation with Poly I:C BM and Is MSCs had upregulated their transcript levels, while Ad MSCs had reach similar transcript levels as under control unstimulated conditions. A second stimulation 48 hours after the first one (Condition 3) led to the downregulation of *GMCSF* transcript levels in BM MSCs, while led to the upregulation of *GMCSF* transcript levels in Is and Ad MSCs.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BM MSC Stimulated Cyt
BM MSC Stimulated LPS
BM MSC Stimulated LTA
BM MSC Stimulated Poly I:C
Is MSC Unstimulated
Is MSC Stimulated Cyt
Is MSC Stimulated LPS
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Is MSC Stimulated Poly I:C
Ad MSC Unstimulated
Ad MSC Stimulated Cyt
Ad MSC Stimulated LPS
Ad MSC Stimulated LTA
Ad MSC Stimulated Poly I:C
A
IL-6
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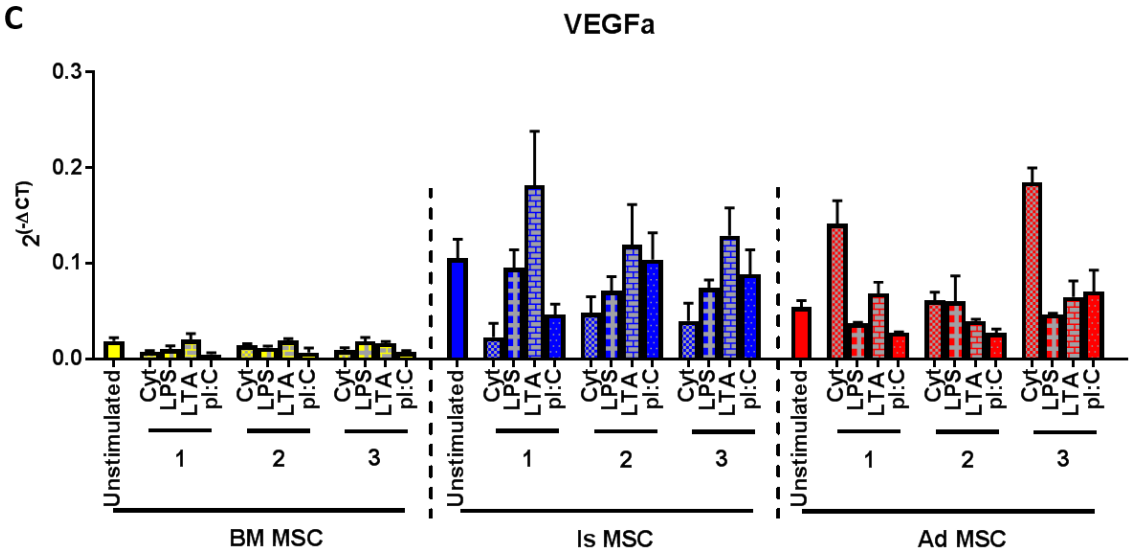
B
TSG-6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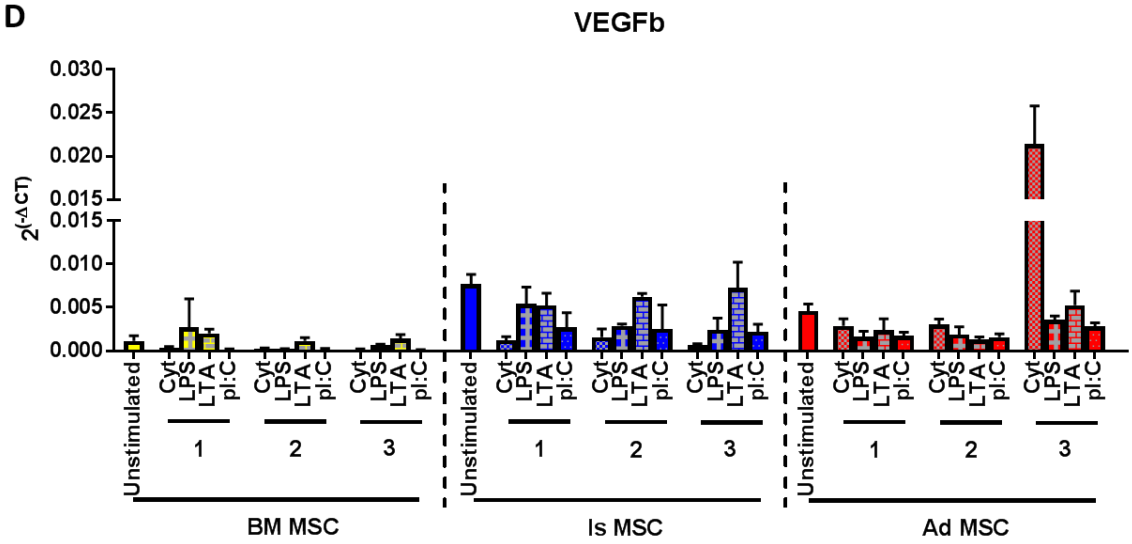
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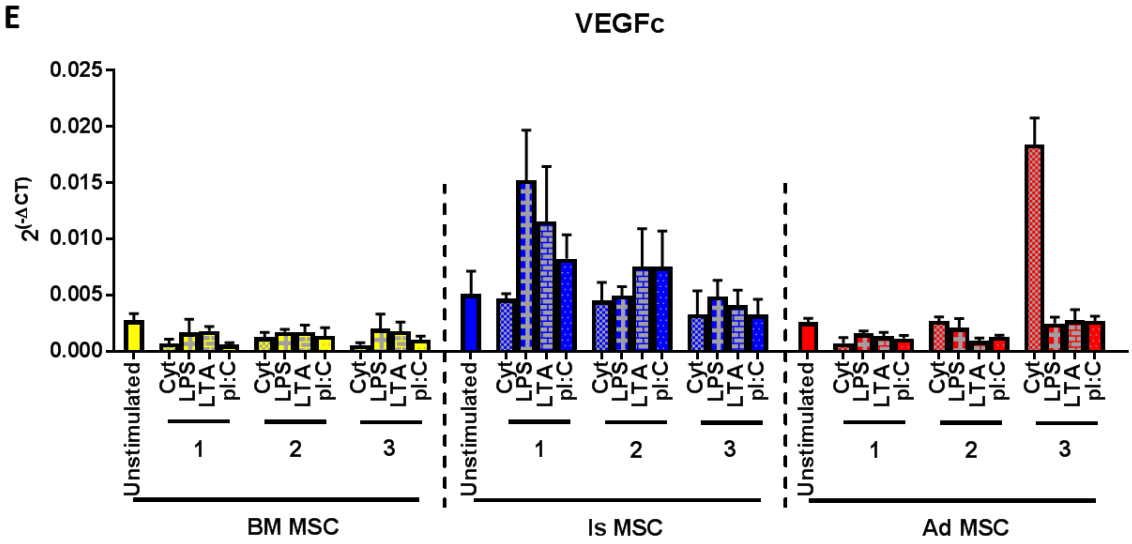
Ad MSC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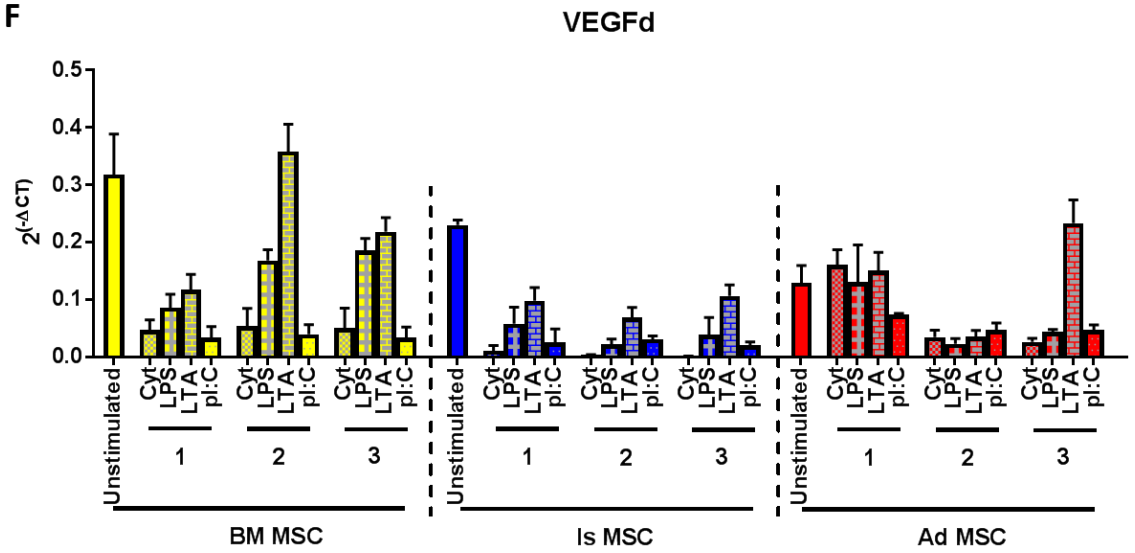
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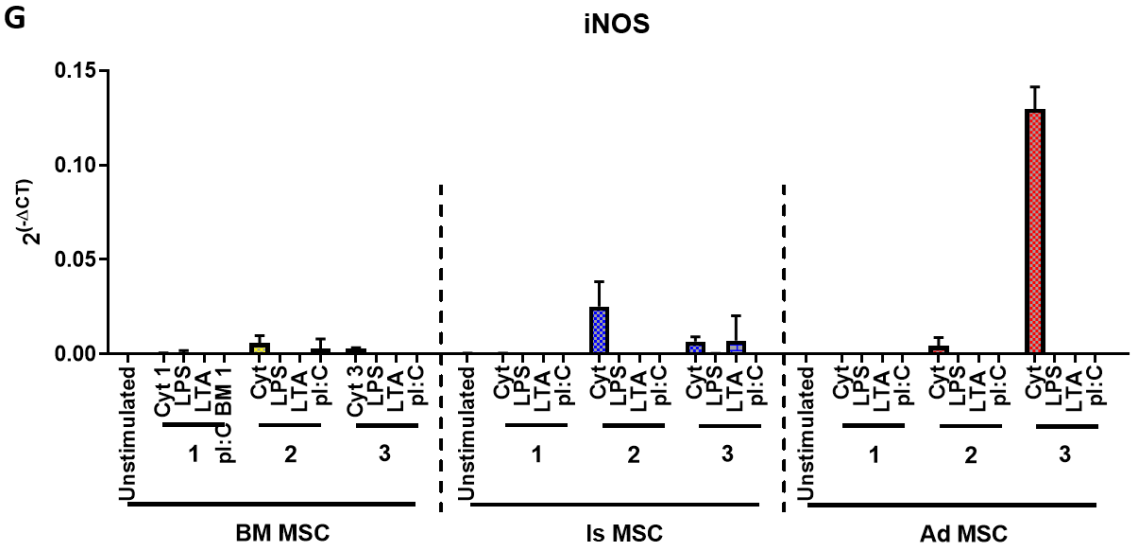
Is MSC
Ad MSC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1
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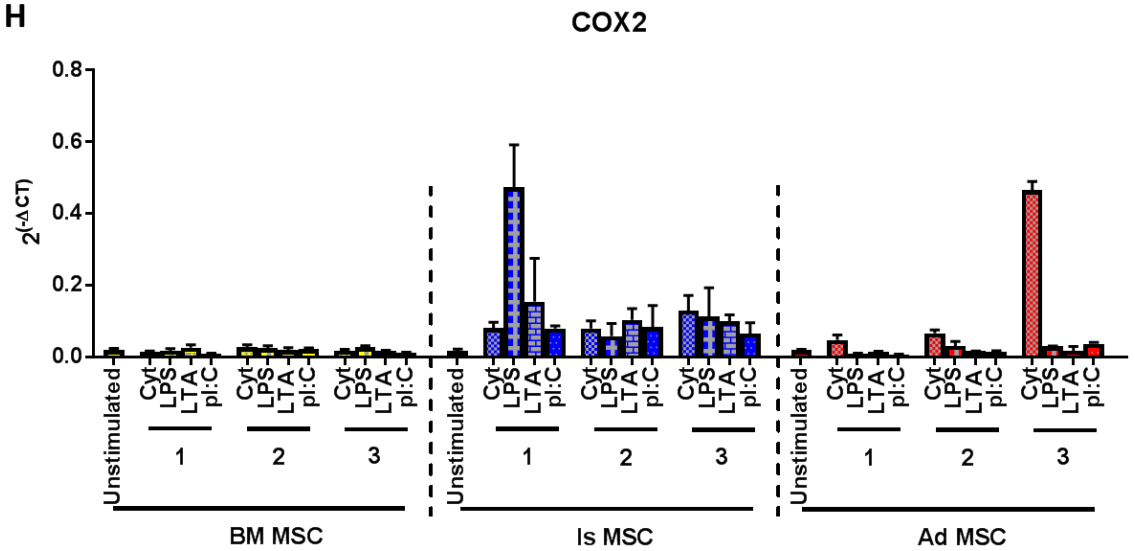
3
BM MSC
Is MSC
Ad MSC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BM MSC
Is MSC
Ad MSC

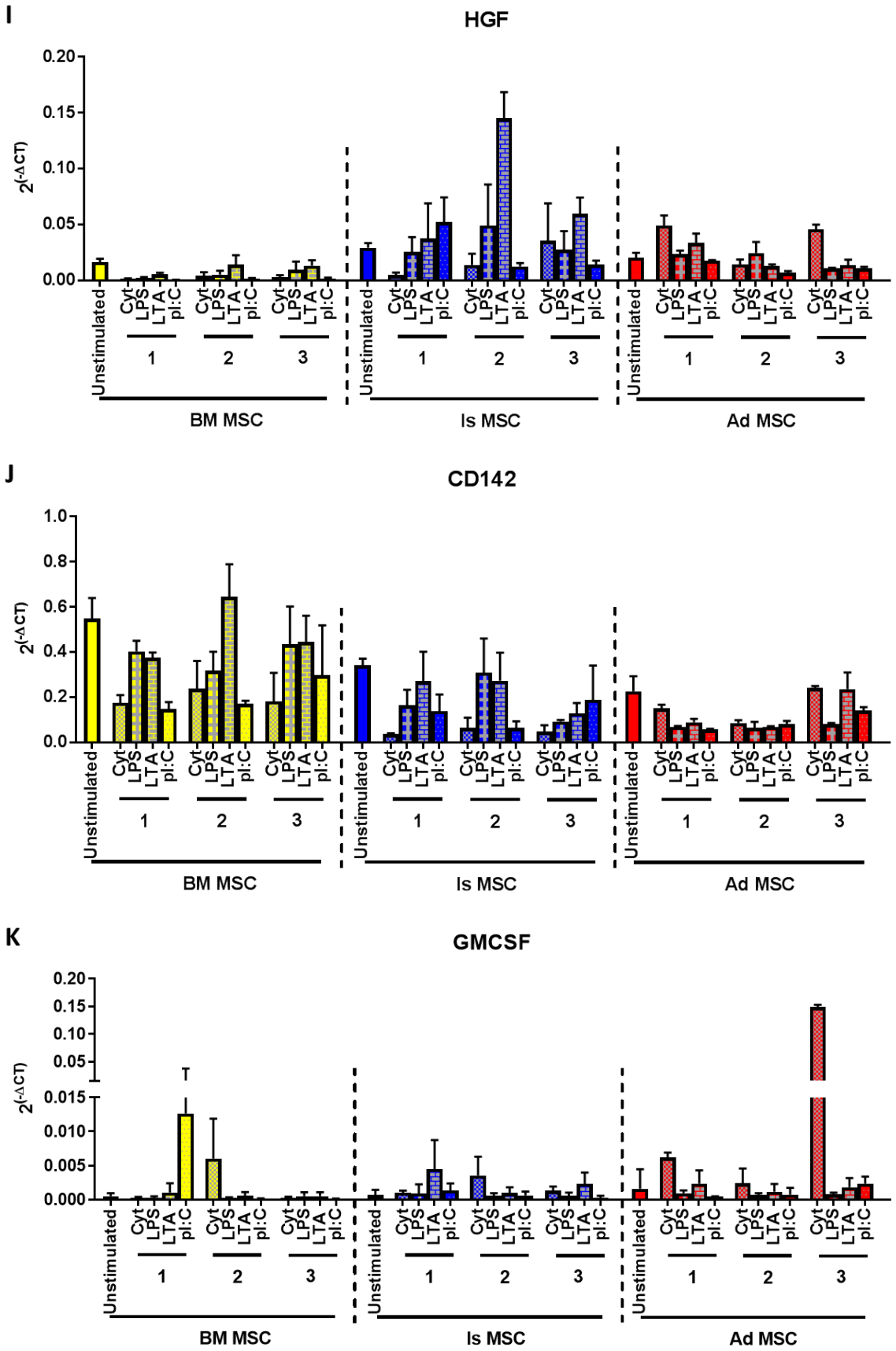

Figure 8-8. Inflammatory agent, repetitive stimulus and MSC tissue origin impacts immunomodulatory molecules transcript levels in MSCs.

MSCs isolated from BM, Is and Ad tissues were grown in culture until passage 3. Once MSCs had reached 80% confluence, cells were stimulated with either a cocktail of cytokines (40 ng/ mL of IFN-Ɣ, TNF-α and IL-1β), 100 ng/ mL LPS, 100 ng/ mL LTA or 4 µg/ mL Poly I:C. Unstimulated

cells were left growing in MSC culture medium as a control. Three different licensing conditions were tested. In the first one, cells were stimulated for 48 hours, after which, cells were washed twice with PBS and fresh culture medium was added; cells were harvested 24 hours later. In the second condition, cells were washed twice with PBS, the culture medium was replaced with fresh one and the cells were left growing for 48 hours. Cells were then washed twice with PBS, the culture medium was replaced with supplemented one and cells were harvested 24 hours later. In the last condition, cells were stimulated for 48 hours, after which cells were washed twice with PBS and were stimulated again for another 24 hours. Figure 6-4 illustrates the time points at which supplemented medium was added. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate immunomodulatory molecule transcripts in BM, Is and Ad MSCs under homeostatic and inflammatory conditions. Each bar represents an n of 4 independent experiments and is graphed as mean ± SEM. Data are normalised to the housekeeping gene *B2M* and expressed as $2^{(-\Delta CT)}$. Statistically significant differences are marked with a colour code in Table 6-5.

Table 8-6. Fold change in immunomodulatory molecules transcript levels of cytokine, LPS, LTA or Poly I:C-mediated licensed BM, Is and Ad MSCs compared to unstimulated cells from the same source.
Following the experimental set up explained in Figure 8-8, fold change in transcript levels is represented as mean of fold change ± standard deviation. One Way ANOVA with Tukey's multiple comparisons post-test was performed to compare all MSC sources and the different conditions. p = 0.05 was considered the limit for statistical significance. Statistically significant differences are marked with a colour code, where p < 0.05 is represented by green, p < 0.01 is represented by orange, p < 0.001 is represented by blue and p < 0.0001 is represented by red.

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
IL-6	1	Cyt	1.15 ± 0.90	1.56 ± 0.97	0.11 ± 0.05
		LPS	0.26 ± 0.22	2.35 ± 1.05	0.42 ± 0.06
		LTA	0.17 ± 0.12	1.24 ± 0.62	0.22 ± 0.02
		Poly I:C	0.15 ± 0.11	1.92 ± 1.31	0.23 ± 0.03
	2	Cyt	53.97 ± 44.65	8.04 ± 4.83	0.65 ± 0.06
		LPS	2.02 ± 0.64	3.09 ± 1.63	0.89 ± 0.29
		LTA	0.49 ± 0.06	1.38 ± 0.51	1.07 ± 0.13
		Poly I:C	0.78 ± 0.72	1.46 ± 0.36	0.66 ± 0.11
	3	Cyt	76.21 ± 61.41	3.41 ± 1.35	10.74 ± 1.08
		LPS	1.72 ± 0.72	2.26 ± 1.29	0.51 ± 0.02
		LTA	0.30 ± 0.20	0.88 ± 0.21	0.70 ± 0.18
		Poly I:C	0.86 ± 0.43	1.04 ± 0.79	0.80 ± 0.13
TSG-6	1	Cyt	0.08 ± 0.03	0.74 ± 0.14	3.98 ± 2.09
		LPS	0.13 ± 0.01	2.86 ± 0.90	0.66 ± 0.21
		LTA	0.17 ± 0.02	2.24 ± 0.34	0.54 ± 0.18
		Poly I:C	0.07 ± 0.03	1.53 ± 0.40	0.42 ± 0.21
	2	Cyt	0.06 ± 0.03	0.62 ± 0.17	0.51 ± 0.28
		LPS	0.23 ± 0.09	2.34 ± 1.30	0.56 ± 0.17
		LTA	0.76 ± 0.36	1.40 ± 0.39	0.31 ± 0.30
		Poly I:C	0.07 ± 0.03	1.09 ± 0.25	0.37 ± 0.21
	3	Cyt	0.13 ± 0.09	0.29 ± 0.19	1.12 ± 0.40
		LPS	0.66 ± 0.35	1.44 ± 0.36	0.51 ± 0.20
		LTA	0.54 ± 0.31	4.18 ± 1.52	1.06 ± 0.53
		Poly I:C	0.08 ± 0.05	0.82 ± 0.58	1.27 ± 0.66
VEGFa	1	Cyt	0.40 ± 0.05	0.22 ± 0.10	2.43 ± 0.51
		LPS	0.54 ± 0.17	0.93 ± 0.19	0.63 ± 0.06
		LTA	1.04 ± 0.14	1.77 ± 0.57	1.16 ± 0.10

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
		Poly I:C	**0.24 ± 0.04**	0.46 ± 0.14	0.47 ± 0.05
	2	Cyt	0.76 ± 0.12	0.37 ± 0.27	1.04 ± 0.17
		LPS	0.65 ± 0.19	0.68 ± 0.05	1.01 ± 0.35
		LTA	1.07 ± 0.26	1.14 ± 0.29	0.67 ± 0.05
		Poly I:C	**0.41 ± 0.29**	1.02 ± 0.31	0.46 ± 0.07
	3	Cyt	**0.49 ± 0.04**	0.38 ± 0.13	**3.17 ± 0.47**
		LPS	0.98 ± 0.07	0.73 ± 0.17	0.80 ± 0.08
		LTA	0.92 ± 0.21	1.25 ± 0.17	1.10 ± 0.26
		Poly I:C	**0.37 ± 0.02**	0.83 ± 0.08	1.19 ± 0.29
VEGFb	**1**	Cyt	0.35 ± 0.10	**0.16 ± 0.06**	0.61 ± 0.14
		LPS	3.30 ± 4.00	0.69 ± 0.17	0.34 ± 0.13
		LTA	2.10 ± 0.97	0.71 ± 0.22	0.48 ± 0.18
		Poly I:C	0.19 ± 0.10	**0.36 ± 0.21**	0.35 ± 0.04
	2	Cyt	0.25 ± 0.11	**0.21 ± 0.13**	0.66 ± 0.19
		LPS	0.21 ± 0.07	**0.38 ± 0.03**	0.38 ± 0.11
		LTA	1.21 ± 0.66	0.82 ± 0.07	**0.27 ± 0.04**
		Poly I:C	0.14 ± 0.04	**0.31 ± 0.29**	0.34 ± 0.07
	3	Cyt	0.19 ± 0.11	**0.09 ± 0.02**	**4.45 ± 0.52**
		LPS	0.71 ± 0.31	**0.30 ± 0.12**	0.75 ± 0.11
		LTA	1.45 ± 0.76	0.94 ± 0.34	1.10 ± 0.27
		Poly I:C	0.11 ± 0.06	**0.28 ± 0.07**	0.60 ± 0.10
VEGFc	**1**	Cyt	**0.27 ± 0.15**	1.00 ± 0.28	**0.29 ± 0.18**
		LPS	0.56 ± 0.23	**3.07 ± 0.34**	0.67 ± 0.13
		LTA	0.67 ± 0.17	2.50 ± 1.14	0.56 ± 0.13
		Poly I:C	**0.22 ± 0.06**	1.83 ± 0.68	0.47 ± 0.10
	2	Cyt	0.45 ± 0.05	0.96 ± 0.36	1.12 ± 0.07
		LPS	0.63 ± 0.08	1.05 ± 0.26	0.86 ± 0.31
		LTA	0.62 ± 0.22	1.64 ± 0.92	**0.38 ± 0.12**
		Poly I:C	0.53 ± 0.28	1.60 ± 0.71	0.54 ± 0.11
	3	Cyt	**0.20 ± 0.08**	0.61 ± 0.21	**7.80 ± 1.71**
		LPS	0.74 ± 0.46	1.09 ± 0.51	1.05 ± 0.28
		LTA	0.65 ± 0.25	0.91 ± 0.39	1.14 ± 0.36
		Poly I:C	**0.38 ± 0.10**	0.65 ± 0.19	1.14 ± 0.23
VEGFd	**1**	Cyt	**0.15 ± 0.04**	**0.05 ± 0.03**	1.44 ± 0.35
		LPS	**0.27 ± 0.03**	**0.25 ± 0.10**	1.10 ± 0.41
		LTA	**0.37 ± 0.07**	**0.42 ± 0.07**	1.30 ± 0.13
		Poly I:C	**0.10 ± 0.03**	**0.11 ± 0.10**	0.66 ± 0.11
	2	Cyt	**0.16 ± 0.07**	**0.01 ± 0.00**	**0.29 ± 0.07**
		LPS	**0.54 ± 0.10**	**0.09 ± 0.03**	**0.20 ± 0.07**
		LTA	1.15 ± 0.37	**0.30 ± 0.07**	**0.31 ± 0.08**
		Poly I:C	**0.12 ± 0.03**	**0.13 ± 0.02**	**0.40 ± 0.07**
	3	Cyt	**0.15 ± 0.08**	**0.00 ± 0.00**	**0.23 ± 0.03**
		LPS	**0.61 ± 0.08**	**0.16 ± 0.11**	**0.38 ± 0.05**
		LTA	**0.72 ± 0.19**	**0.46 ± 0.08**	**2.06 ± 0.41**
		Poly I:C	**0.10 ± 0.04**	**0.09 ± 0.02**	**0.41 ± 0.08**
iNOS	**1**	Cyt	18.43 ± 14.56	4.52 ± 3.84	4.06 ± 3.80

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
		LPS	5.04 ± 7.56	0.54 ± 0.40	1.99 ± 1.86
		LTA	0.09 ± 0.09	0.23 ± 0.24	1.01 ± 0.93
		Poly I:C	0.57 ± 0.40	0.16 ± 0.15	1.88 ± 1.73
	2	Cyt	191.3 ± 171.7	**170.15 ± 178.95**	49.31 ± 47.41
		LPS	2.25 ± 1.86	0.20 ± 0.21	271.90 ± 239.85
		LTA	0.25 ± 0.22	0.25 ± 0.04	1.04 ± 1.13
		Poly I:C	135.1 ± 222.2	0.14 ± 0.05	22.15 ± 31.04
	3	Cyt	77.75 ± 55.07	83.97 ± 86.59	**40476 ± 37122**
		LPS	4.61 ± 3.70	1.99 ± 1.83	32.53 ± 51.05
		LTA	0.22 ± 0.09	45.06 ± 53.10	28.72 ± 37.91
		Poly I:C	4.38 ± 3.46	0.15 ± 0.16	147.15 ± 153.43
COX2	**1**	Cyt	0.85 ± 0.16	5.46 ± 1.93	**2.45 ± 0.86**
		LPS	1.00 ± 0.46	**29.93 ± 4.88**	0.46 ± 0.05
		LTA	1.39 ± 0.19	11.11 ± 7.88	0.69 ± 0.17
		Poly I:C	0.50 ± 0.07	5.41 ± 1.65	0.37 ± 0.05
	2	Cyt	1.65 ± 0.67	5.16 ± 1.41	**3.34 ± 0.19**
		LPS	1.48 ± 0.67	3.96 ± 2.32	1.52 ± 0.53
		LTA	1.19 ± 0.59	6.46 ± 0.44	0.79 ± 0.06
		Poly I:C	1.31 ± 0.47	6.15 ± 4.01	0.75 ± 0.08
	3	Cyt	0.93 ± 0.09	8.71 ± 3.77	**23.83 ± 2.96**
		LPS	1.57 ± 0.59	7.92 ± 5.36	1.52 ± 0.15
		LTA	0.97 ± 0.35	6.73 ± 2.58	0.89 ± 0.54
		Poly I:C	0.57 ± 0.06	4.33 ± 1.81	1.88 ± 0.27
HGF	**1**	Cyt	**0.09 ± 0.01**	0.17 ± 0.06	**2.48 ± 0.99**
		LPS	**0.17 ± 0.05**	0.87 ± 0.36	1.13 ± 0.25
		LTA	**0.37 ± 0.07**	1.40 ± 1.24	**1.68 ± 0.56**
		Poly I:C	**0.04 ± 0.01**	1.75 ± 0.53	0.87 ± 0.19
	2	Cyt	**0.32 ± 0.24**	0.48 ± 0.28	0.67 ± 0.16
		LPS	**0.39 ± 0.28**	1.68 ± 1.01	1.27 ± 0.74
		LTA	1.06 ± 0.69	**5.03 ± 0.70**	0.61 ± 0.11
		Poly I:C	**0.11 ± 0.05**	0.43 ± 0.08	0.33 ± 0.08
	3	Cyt	**0.22 ± 0.13**	1.24 ± 1.02	**2.20 ± 0.38**
		LPS	0.74 ± 0.56	1.01 ± 0.63	0.52 ± 0.10
		LTA	0.97 ± 0.45	2.12 ± 0.69	0.65 ± 0.24
		Poly I:C	**0.12 ± 0.07**	0.35 ± 0.22	**0.53 ± 0.14**
CD142	**1**	Cyt	**0.32 ± 0.03**	**0.11 ± 0.00**	0.93 ± 0.11
		LPS	0.74 ± 0.12	0.47 ± 0.14	**0.42 ± 0.05**
		LTA	0.70 ± 0.12	0.79 ± 0.32	**0.55 ± 0.06**
		Poly I:C	**0.27 ± 0.06**	0.40 ± 0.18	**0.35 ± 0.03**
	2	Cyt	0.45 ± 0.26	**0.18 ± 0.11**	**0.53 ± 0.06**
		LPS	0.59 ± 0.17	0.90 ± 0.37	**0.39 ± 0.14**
		LTA	1.19 ± 0.25	0.78 ± 0.27	**0.42 ± 0.04**
		Poly I:C	**0.32 ± 0.05**	**0.18 ± 0.07**	**0.49 ± 0.08**
	3	Cyt	**0.33 ± 0.20**	**0.13 ± 0.06**	1.48 ± 0.15
		LPS	0.79 ± 0.21	**0.27 ± 0.03**	**0.50 ± 0.06**
		LTA	0.83 ± 0.25	0.37 ± 0.11	1.44 ± 0.40

Gene	Condition	Licensing	BM MSCs	Is MSCs	Ad MSCs
		Poly I:C	0.55 ± 0.37	0.53 ± 0.36	**0.87 ± 0.13**
GMCSF	**1**	Cyt	1.56 ± 1.30	6.07 ± 8.72	14.53 ± 12.02
		LPS	0.66 ± 0.30	13.17 ± 22.47	1.65 ± 1.05
		LTA	14.41 ± 24.03	**10.08 ± 9.00**	**2.62 ± 1.26**
		Poly I:C	145.6 ± 250.5	7.51 ± 11.31	1.07 ± 0.86
	2	Cyt	8.85 ± 8.64	2.75 ± 2.17	1.22 ± 0.60
		LPS	0.74 ± 0.40	1.59 ± 1.77	1.98 ± 2.17
		LTA	1.31 ± 0.84	10.35 ± 16.41	1.31 ± 1.05
		Poly I:C	0.87 ± 0.75	0.78 ± 0.27	0.55 ± 0.28
	3	Cyt	1.26 ± 0.95	8.57 ± 13.07	365.9 ± 300.6
		LPS	1.25 ± 0.67	1.59 ± 1.99	1.53 ± 1.14
		LTA	0.72 ± 0.40	8.85 ± 11.71	**2.35 ± 1.37**
		Poly I:C	0.74 ± 0.40	3.32 ± 5.56	4.37 ± 3.01

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