Tissue Origin Dictates Mesenchymal Stromal Cell Chemokine and Chemokine Receptor Repertoire and Predicts *in vitro* Chemotactic Activity under Homeostatic and Inflammatory Conditions

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Abstract

Due to their anti-inflammatory and immunomodulatory properties, mesenchymal stromal cells (MSCs) are under intense investigation in many pre-clinical and clinical trials as a potential cellular therapy to be used in an array of clinical settings. The majority of the literature surrounding MSC phenotype and function is derived from studies focusing on bone marrow (BM) derived MSCs. Recently however, it has become apparent that MSCs can be isolated in a less invasive manner, from the majority of tissues in the human body. In light of this, many studies have been published promoting the use of alternative tissue sources for MSC isolation with no thorough standardised comparison of the phenotype or potential *in vivo* function of these MSCs. The advanced therapeutics department within the Scottish National Blood Transfusion Service (SNBTS) is involved in the development and optimisation of several cellular therapies including the use of MSCs within various clinical settings. SNBTS has access to fully consented human tissues rich in MSCs including; pancreatic islets, visceral adipose tissue, liposuction aspirate, bone marrow and umbilical cord. Therefore this study aimed to objectively compare the phenotype and potential *in vivo* function of MSCs isolated from the aforementioned tissues in a stringent, standardised manner in order to assess if MSCs isolated from one specific tissue source might be optimal for use within the clinic. The beneficial therapeutic effect of MSCs often depends on their ability to migrate to target tissues and interact with residing or migratory immune and non-immune cells, frequently within an inflammatory environment. Therefore this study focussed on how MSCs might migrate *in vivo* by assessing and comparing MSC chemokine receptor expression, whilst also assessing and comparing MSC chemokine secretion profiles to understand which immune cells MSCs might attract, and therefore potentially interact with, *in vivo*. This study found that chemokine receptor expression by MSCs isolated from islet, visceral adipose, adipose, bone marrow and umbilical cord tissues was very low, with CXCR4, CCR7 and ACKR3 expression being restricted to visceral adipose and bone marrow derived MSCs. Inflammatory chemokines were secreted at very high levels by MSCs isolated from all of the aforementioned tissues, which induced migration of target immune cells towards all MSCs tested *in vitro* and *in vivo*, importantly however, the tissue origin of MSCs dictated the quantities of immune cells attracted. This study highlighted

that the tissue origin of MSCs could affect MSC *in vivo* migratory capacity and their ability to chemoattract surrounding immune cells, thereby potentially influencing 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.

Signature:

Printed name:

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List of abbreviations

#		
	7TM	Seven-transmembrane
A		
	ACKR	Atypical chemokine receptor
	Ad MSC	Adipose derived mesenchymal stromal cell
B		
	B2M	Beta-2 microglobulin
	BM	Bone marrow
	BM MSC	Bone marrow derived mesenchymal stromal cell
C		
	C5a	Complement component 5a
	CD	Cluster differentiation
	CFH	Complement factor H
	CFU-Fs	Colony –forming unit fibroblasts
	CIA	Collagen induced arthritis
	CLP	Common lymphoid progenitor
	CMP	Common myeloid progenitor
	CT	Cycle threshold
	CTLA-4	cytotoxic T-lymphocyte-associated protein 4
	CTLs	Cytotoxic T lymphocytes
D		
	DAMPS	Damage associated molecular pattern
	ddH_2o	Double distilled H20
	DMT1	Diabetes mellitus Type 1
F		
	FO	Follicular
G		
	GDMSCs	Granulocytic derived myeloid derived suppressor cells
	GPCRs	G-Protein coupled receptors
	GVHD	Graft versus host disease
H		
	HGF	Hepatocyte growth factor
	HKG	House keeping genes
	HLA	Human leukocyte antigen
	HSC	Hematopoietic stem cell
	HSCT	Hematopoietic stem cell transplant
I		
	IDO	indoleamine 2, 3- dioxygenase
	IFN	Interferon
	Ig	Immunoglobulin
	IL	Interlukin
	IL-1ß	Interlukin - 1 beta
	ILC	Innate like lymphocytes
	IPMSC	Induced pluripotent stem cell derived

		mesenchymal stromal cell
	IPSC	Induced pluripotent stem cell
	Is MSC	Islet derived mesenchymal stromal cell
	ISCT	International Society of Cellular Therapy
	IV	Intravenous
K		
	KIR	Killer immunoglobulin-like receptors
L		
	LN	Lymph node
	LPS	Lipopolysaccharide
M		
	MHC	Major histocompatibility complex
	MS	Multiple sclerosis
	MSC	Mesenchymal stromal cell
	MZ	Marginal zone
P		
	PAMPS	Pathogen associated molecular patterns
	PGE2	Prostoglandin E2
	PP	Payers patches
	PPC	Positive PCR control
	PRRs	Pattern recognition receptors
R		
	RA	Rheumatoid arthritis
	RBC	Red blood cell
	RBClysB	Red blood cell lysis buffer
	RT	Room temperature
	RT^2 Array	RT^2 Profiler™ PCR Array
	RTC	Reverse transcription control
S		
	SNBTS	Scottish National Blood Transfusion Service
	SOT	Solid organ transplant
T		
	T1DM	Type 1 diabetes mellitus
	TCR	T cell Receptor
	Th	T helper Cell
	TLDA	TaqMan Low Density Array
	TLRs	Toll like receptors
	TNF-α	Tumour necrosis factor- alpha
	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

Chapter 1 Introduction

1.1 Overview

As this study focussed on human MSCs, their migration and interaction with immune cells under homeostatic and inflammatory conditions, the introduction of this thesis will provide a broad overview of the immune system and MSCs and will include; i) reviewing the roles of various immune cells in orchestrating the promotion of resolution after an inflammatory insult, ii) considering the migration of immune cells during homeostasis and after an inflammatory response by exploring the chemokine family, and lastly, iii) an in depth examination of MSCs, their interaction with immune cells and how MSCs are beneficial in different clinical settings.

1.2 The immune system

We are exposed to potentially deadly threats every day in life, including a plethora of microorganisms – bacteria, viruses, fungi, parasites- and general injury which often result in inflammation. Our first line of defence against pathogens is the skin which acts as a physical barrier to deter entry. However, there are breaks in this protective barrier for digestive, reproductive and respiratory openings where chemical barriers known as mucous membranes serve as protection. Should these physical barriers be compromised, the immune system acts as our internal protection, primed and ready to respond to neutralise any breach.

The immune system is a network of molecules, cells, tissues and organs that work together to protect the host from foreign pathogens. Should a pathogen enter the tissues or circulation, it will encounter the innate immune system. The innate immune response is always immediately available to combat a wide range of pathogens within the first few hours of infection. Cells of the innate immune system are continuously circulating the body, via the blood and lymphatic systems, whilst also sampling from their environment within tissues. Should a

pathogen be encountered, the cells are equipped with rapid defences to eliminate danger whilst also recruiting additional effector cells to the infected site via secretion of inflammatory mediators. Antigen presenting cells such as dendritic cells (DCs) are among the cells recruited to the infected site. They are responsible for migrating to the lymph nodes to present antigen(s) (Ag(s)) to the adaptive immune system. If DCs have phagocytosed infectious material they will be in an activated state and will possess specific surface molecules (CD80/86) that can interact with, and influence, the activation state of the adaptive immune system. If DCs are non-activated, they will lack these molecules and induce tolerance of the adaptive immune system to the self-Ags which they bear. Adaptive immunity (also known as the acquired immunity) takes days to develop and provides long lasting protection through the production of memory cells (1). It relies on lymphocytes known as T and B cells which have Ag-specific receptors generated through a series of somatic mutations, resulting in a stronger, faster and more efficient response, should an individual be infected by that same pathogen again. Taken together, the innate and adaptive immune responses act in concert to provide protection against pathogens, whilst limiting damage to the body. The immune system is central to our survival and health and will be discussed in more detail in forthcoming sections.

1.2.1 **Innate immune system in inflammation**

As mentioned, the innate immune system is the first line of defence, responding in minutes to hours due to germline encoded receptors that recognise common features of many pathogens. Initial stages of infection and/or inflammation are characterised by five cardinal signs: calor (increased heat), rubor (redness), tumor (swelling), dolor (pain) and function laesa (loss of function). Calor and rubor are caused by vasodilation which increases blood flow and thus encourages the influx of leukocytes to the affected area which in turn leads to tumor and dolor. The combination of all of the above can lead to function laesa. This classifiction system of inflammation encapsulates a very complex interplay of molecules known as the humoral immune system and various cell types of the innate immune system, which all work together to clear infection and promote resolution (2).

1.2.1.1 **The complement system**

The complement system is a complex network of 30+ proteins that aid or "complement" the killing of bacteria and clearing of damaged cells. The complement proteins are synthesized in the liver and circulate as inactive precursors in the blood. Depending on the stimulus, one of three complement pathways can be activated; the classical pathway (activated via immune complexes), the alternative pathway (activated via pathogen surfaces and lipopolysaccharide (LPS)) or the lectin pathway (activated via bacterial surfaces) (3). Activation of these pathways causes protease cleavage of specific proteins and results in amplification processes causing further protein cleavage. All the pathways converge at the formation of C3. C3 is cleaved to form the products C3a, C3b, C5a and the membrane attack complex C5b-9. The formation of these proteins results in a wide range of cellular activity, including chemotaxis, mast cell degranulation and macrophage activation, ultimately leading to the initiation and perpetuation of the inflammatory response. To prevent aberrant complement activation and unwarranted perpetuation of the inflammatory response and potential harm to host, several complement inhibitory proteins exist in the circulation which degrade specific components of the complement systems and inhibits the cellular activity promoted by the activation of complement (4).

1.2.1.2 **Cells of the innate immune system**

The innate immune leukocytes are products of multipotent hematopoietic stem cells (HSCT) which mature and expand within the bone marrow (BM) and migrate to the periphery to perform effector functions upon relevant external signals. Innate cells derive from a common myeloid progenitor (CMP) which gives rise to neutrophils, monocytes, macrophages, DCs, eosinophils, mast cells (MC) and basophils (5).

1.2.1.2.1 Neutrophils

Neutrophils are short lived phagocytic granulocytes. Accounting for 40-70% of white blood cells in mammals, they are the most abundant white blood cells and are essential in innate immunity. Their importance in host defence is demonstrated through individuals with congenital neutrophil deficiencies who suffer from severe infections that are often fatal (6). Primarily, neutrophils are found in the bloodstream, however upon challenge, they are typically the first cell to be recruited into tissues following signals such as chemokine (CXC motif) ligand 8 (CXCL8), CXCL2, complement component 5a (C5a), and vascular endothelial growth factor (VEGF) – to name a few (7). At the site of insult, they have a plethora of mechanisms to clear infection such as phagocytosis, the release of bactericidal molecules and neutrophil extracellular traps (8). Additionally, neutrophils release a number of inflammatory cytokines and chemokines which amplify local inflammatory responses and promote further leukocyte infiltration. Therefore, neutrophils are equipped with the necessary effector functions vital in pathogen clearance, whilst also promoting the immune response. Conversely, there is increasing evidence that neutrophils are involved in the resolution of inflammation, wound healing and angiogenesis (9, 10).These opposing functions of neutrophils in inflammation and resolution have led to the notion that two distinct populations of neutrophils might exist and are recruited independently of one another. In mice, pro-inflammatory neutrophils are recruited by the pro-inflammatory chemokine CXCL2, whereas pro-angiogenic neutrophils - shown to be essential for the formation of blood vessels -are recruited via vascular endothelial growth factor A (VEGFA) and secrete matrix metallopeptidase 9 (MMP9) which facilitates tissue remodelling (11).

Whether neutrophils exist as two distinct populations – anti-inflammatory or pro-inflammatory – or if they are one multifunctional cell in different environments, responding to extracellular signals is currently debated in the literature and is reviewed elsewhere (7). From these studies, what is clear is that neutrophils are phagocytic granulocytes, capable of rapidly killing and clearing infection whilst also promoting angiogenesis and resolution of inflammation.

1.2.1.2.2 Eosinophils

Eosinophils are multifactorial granulocytes involved in host defence against parasitic helminth infections and the pathology of allergic diseases (12). They develop in the bone marrow and exist in to the peripheral blood where they briefly circulate before exiting into peripheral tissues including the thymus, ovary, lower gastrointestinal tract spleen and uterus (12, 13). Numerous stimuli recruit eosinophils to the inflammatory foci where engagement of immunoglobulin (Ig), complement and cytokine receptors results in eosinophil activation and degranulation of cytoplasmic granules filled with a plethora of chemical mediators. The contents of these granules include various cytokines (Interlukin-2 (IL-2), IL-4, IL-5, IL-10, IL-12, IL-13, IL-16 and IL-18) and chemokines (CCL11 and CCL5) which when released, result in activation and exaggeration of the surrounding immune response via the upregulation of the vascular adhesion system and an increase in vascular permeability, allowing for entry of more immune cells, leading to the perpetuation of the inflammatory response (12).

Despite being most popularly known for their involvement in allergy and end-stage parasitic infections, eosinophils also play roles in homeostasis where they are involved in post-natal mammary gland development (14) . Other roles include Ag presentation to T cells (15), promoting T cell proliferation (16) and resolution of inflammation (17).

1.2.1.2.3 Monocytes

Monocytes are circulating white blood cells that typically represent 4% (mice) and 10% (humans) of nucleated cells in the blood. They are derived from a precursor population that resides within the bone marrow which, in mice, requires the chemokine receptor CCR2 for their exit into the bloodstream (18). In the circulation -for both humans and mice- there are different populations of monocytes which are termed classical, intermediate and non-classical monocytes. When comparing human to mice, these monocyte populations may not fully overlap (19), however their roles in immune defence appear to be similar. Non-classical monocytes are involved in promoting wound healing and angiogenesis, whereas classical monocytes accumulate at sites of inflammation

in response to chemokines (namely CCL2 and CCL7) and microbial factors (20). It is unknown whether intermediate monocytes play a biologically meaningful role or whether they represent intermediates in a continuous differentiation system from classical to non-classical monocytes. Nonetheless, they have been found in increased numbers in certain pathologies such as rheumatoid arthritis (21) and patients with asthma (22), suggesting that they play a role in shaping the immune response in these pathologies. Classical, intermediate and non-classical monocytes are the precursors of monocyte –derived macrophages and DCs which differentiate when they reach peripheral tissues under specific signals (23).

1.2.1.2.4 Macrophages

Macrophage populations include 1) bone marrow derived circulating monocytes recruited to sites of tissue injury, 2) infiltrating macrophages which have differentiated from in situ monocytes and 3) yolk-sac derived tissue resident macrophages present within the spleen, lung, skin, brain, liver, pancreas and kidney (24). Macrophages can be differentially termed, depending on their tissue of residence, e.g. Kupffer cells in the liver, osteoclasts in bone and microglia in the brain. As they are hugely phagocytic, they clear cell debris, apoptotic cells and erythrocytes whilst also possessing the ability to release effector molecules that attract other immune cells upon tissue damage or infection (24). Macrophages are also equipped with a whole plethora of pattern recognition receptors (PRRs) which are vital receptors in innate immunity recognising pathogen-associated molecular patterns (PAMPS) or damage associated molecular patterns (DAMPS).The expression of these germ-line encoded receptors allows them to be primary responders to damage or pathogens (25). In responding to their surrounding environment, infiltrating macrophages can differentiate into a spectrum of cells with distinct phenotypes, popularly – but perhaps not correctly - referred to as M1 or M2 (26). M1 (or classically activated macrophages) are thought to be induced by microbial stimuli and/or interferons (IFN) and are regarded as pro-inflammatory. Conversely, M2 (or alternatively activated macrophages) are induced via IL-4 and IL-13 and are involved in resolution of inflammation (27). This terminology suggests that only these two phenotypes of macrophages exist, whereas it is likely that they represent two

extremes of a continuum of diverse functional states which respond to extracellular cues and therefore the M1/M2 paradigm is limited (26). Due to their ability to attract and instruct other immune cells, they play a major role in commencing clearance of pathogens but also contribute to pathogenesis of inflammatory and degenerative diseases (28, 29).

1.2.1.2.5 Natural Killer cells

Natural Killer (NK) cells, originally referred to as large granular lymphocytes are now categorised as innate like lymphocytes (ILC). They are known to differentiate and mature in the bone marrow, lymph nodes, spleen, tonsils and thymus and differ from B and T cells due to their lack of somatic rearranged immunoglobulin and T cell receptor (TCR) genes (respectively), thus termed innate- like lymphocytes (30). In humans, they can be identified and divided into subsets via expression levels of CD56. $CD56^{dim}$ NK cells constitute 90% of the total NK cell population and have high cytotoxic activity, whereas the remaining 10% of NK cells are $CD56^{BRIGHT}$ and are mostly involved in the production of cytokines (31). Due to their ability to be highly cytotoxic, they respond relatively rapidly (~3 days post infection) to viral infections and are pivotal in suppressing tumour formation. NK-cell cytotoxicity is a complex process, tightly regulated by a balance between inhibitory and activating signals. Killer immunoglobulin-like receptors (KIR) are composed of activating and inhibitory receptors that specifically recognise human leukocyte antigens (HLA) A, B and C and upon binding HLA, KIRs can detect virally infected cells, transformed cells and host cells. The majority of KIRs are inhibitory receptors meaning that the binding of self HLA results in inhibition of NK-cell activation and promotion of self-tolerance (32). Conversely, tumour cells and virally infected cells classically downregulate HLA molecules in an attempt to avoid recognition by cytotoxic T lymphocytes (CTLs), however this leaves them a target to NK cells, where activation receptors are no longer suppressed, resulting in strong stimulatory signals, tipping the balance towards NK cell activation (33). Once fully activated, NK cell cytotoxicity is applied via two pathways. One pathway induces apoptosis in the target cell via NK cell secretion of perforin (disrupts cell membranes) and granzymes. Alternatively, caspase-dependent apoptosis can also be induced in target cells which express death receptors (e.g. Fas/CD95)

(34). Thus, the NK cell is vital in early innate responses to protect against viral infections, formation of tumours and promotion of self-tolerance.

1.2.1.2.6 Dendritic cells

Like macrophages and neutrophils, DCs are considered professional phagocytes. However, in contrast to macrophages and neutrophils, DCs present self and non-self-Ags in the form of peptides on MHC class I (endogenous) and II (exogenous) and present it to CD8 and CD4 T cells, respectively, thus activating the adaptive immune response. Importantly, DCs can also cross present exogenous antigens on MHC I, allowing DCs to activate naïve CD8 T cells and permitting them to recognise and kill transformed or infected cells (35). It is this ability to prime and activate the adaptive immune system that has resulted in the DC being termed a professional Ag presenting cell (36). DCs are a heterogeneous population of cells derived from hematopoietic bone marrow progenitor cells and remain in an immature form until activated. Upon activation via PRRs and CD40L, the DC alters its Ag processing and presenting behaviours, ultimately resulting in the upregulation of co-stimulatory molecules CD80/86, and the chemokine CC receptor 7 (CCR7) (37, 38). Mouse models have illustrated that CCR7 upregulation is indispensable for the mobilisation of the dendritic cell through the lymphatics to the lymph node (39). Once they have arrived, they make their way to the T cell zone and depending on whether they express co-stimulatory molecules or not, will dictate whether the T cell is activated or tolerance is induced, respectively (40). Collectively, DCs are professional Ag presenting cells, branching the innate and adaptive immune system, promoting activation or inducing tolerance, paving the foundations for formation of immunological memory which is created by the somatic rearrangements of the immunoglobulin and T cell receptor genes of B and T cells in the adaptive immune system.

1.2.2 **Adaptive immune system**

Unlike the innate immune system where germline encoded receptors recognise molecular patterns of common pathogens, the adaptive immune system relies on clonal expansion of Ag specific effector cells, specifically selected for via complex processes of receptor gene rearrangements. The adaptive immune

system is only present in vertebrates and cartilaginous fish and takes roughly 5-7 days to respond to Ags whilst also forming immunological memory towards said Ags. This allows subsequent encounters with the same pathogen to be cleared more efficiently and it is this process of immunological memory that is the basis for vaccination (41). The system is termed adaptive, or acquired, due to its ability to create a plethora of Ag specific receptors which are expressed on lymphocytes. This process is known as somatic hypermutaion and results in an irreversible change in the DNA of each cells progeny. It occurs via the re-arrangement of V(D)J genes, and cells which are most specific for the Ag are positively selected for and subsequently clonally expand (42). T and B lymphocytes are the cells of the adaptive immune system capable of VDJ gene rearrangements and provide us with specific life-long immunity.

1.2.2.1 **B cells**

B-cells - termed so due to their discovery in the Bursa of Fabricius in birds – are Ag presenting cells capable of secreting cytokines and antibodies (43). They develop in the bone marrow from a common lymphoid progenitor (CLP) and undergo a complex selection process which ensures that they bear a unique B cell receptor which is highly specific but also remains non-reactive to self. Immature B cells that have not yet seen their Ag, leave the BM and circulate through the blood and lymph. B cells visit and reside within secondary lymphoid organs such as the spleen, lymph nodes (LNs), tonsils and Peyer's patches. Within the follicles of the lymph node, B cells can acquire Ag via a population of subcapsular macrophages which sample particulate Ag from the lymph (44). B cells then make their way towards the T cell zone to encounter their cognate T cell. Only when a cognate interaction occurs does the B cell clonally expand resulting in short-lived plasma cells, long lived plasma cells or memory cells (45).

Plasma cells are responsible for secreting large amounts of antibodies which are recognised as the basis for humoral immunity. Although there are 5 different kinds of antibodies involved in different aspects of immunity (IgM, IgG, IgA, and IgE & IgD), one plasma cell will produce copious amounts of one type of antibody and unlike their precursor, they cannot switch class once differentiated. These

antibodies will circulate in the blood and lymph and bind Ags causing neutralisation, complement activation or tagging for phagocytosis by other immune cells that express Fc receptors (46). Fc receptor binding to antibodies triggers cytotoxic killing or phagocytosis of the material. Several different Fc receptors exist on different types of immune cells. Each Fc receptor binds a specific type of immunoglobulin and exerts a specific function for example IgG binding to FcϒRIIA which is present on macrophages, neutrophils, eosinophils and will result in phagocytosis and eosinophil degranulation. Equally, binding of IgA or IgM to Fcα/μR present on B cells and macrophages will result in endocytosis and induction of microbe killing (47).

1.2.2.2 **T cells**

Like B cells, T cells also develop from a CLP in the bone marrow. From here, they migrate to the thymus where they undergo further maturation through a series of complex selection processes which are distinguished by the expression of different cell surface molecules. For successful development into a CD4, CD8 or T regulatory (Treg) cell, T cells must migrate through different areas of the thymus under the guidance of chemokines and their receptors (Section 1.3) and interact with thymic stromal cells presenting self-peptides on the surface of major histocompatibility molecules (MHC). This process ensures that T cells successfully develop their T cell receptor alpha, and beta chains and only thymocytes which have weak affinity for self-peptide presented by MHC will survive (48, 49). The majority of thymocytes die during this process, the ones that survive develop into i) T helper cells (CD4) - involved in shaping, perpetuating and assisting the immune response ii) cytotoxic T cells (CD8 +ve) - involved in direct lysis and killing of cancer and virally infected cells and iii) Tregs - involved in maintaining immune tolerance to self and the prevention of autoimmune diseases (50).

Naïve T cells (not yet seen their cognate Ag) circulate in the blood and lymph and reside within secondary lymphoid organs. In lymphoid organs, activated DCs present peptides to naïve T cells, if the TCR is specific for said peptide an immune synapse forms resulting in a downstream signalling cascade ultimately ending in the activation of the naive T cell. During this process CD4 T helper

cells can develop into an array of different helper cells depending on cues given from the surrounding environment as to what kind of immune response is required. These subsets, termed Th1, Th2, Th9, Th17, T follicular helper cells (Tfh) and induced T regulatory cells (iTregs), are important in the activation of the innate immune response, B cells, cytotoxic T cells and suppression of the immune response (iTregs) (50). Conversely, the activation of CD8 T cells is less ambiguous and results in a very efficient killing machine via the secretion of perforin, granzymes and interferons. Once activated, these cells clonally expand and the majority are short-lived, with only a small proportion of them become life-long memory cells. These memory cells- with the exception of T effector memory cells (Tem) - circulate between the secondary lymphoid organs sampling the lymph for their peptide in order to respond rapidly upon secondary activation (51).

1.3 Chemokines and chemokine receptors

In order for immune cells to elicit the aforementioned functions, it is essential that they migrate from where they develop, to where they reside and ultimately to sites of infection and inflammation. Additionally, immune cells must constantly move under homeostatic conditions, sampling their surrounding environment. Chemokines and their receptors are pivotal in immune cell trafficking during homeostasis and inflammation.

1.3.1 Chemokines

Chemokines are members of a large, ancient family of chemotactic cytokines consisting of approximately 50 highly conserved members. They range from 7-15 kDa in size and stimulate recruitment of leukocytes and some non-hematopoietic cells. Chemokines are secondary pro-inflammatory mediators as their secretion is activated via primary pro-inflammatory mediators such as tumour necrosis factor-alpha (TNF-α), IL-1ß and IFN-γ (interferon-gamma) (52). By binding to G-Protein coupled receptors (GPCRs) (Section 1.3.2) on the surface of immune cells, chemokines are involved in several processes such as immune development and homeostasis, activation of primary and secondary host defence mechanisms and also the initiation of wound healing (53, 54). Depending on their structure, chemokines are split into different families, dictated by the positioning of the

first two of four invariant cysteine residues and intervening amino acid residues, denoted by the letter 'X' and thus termed CC, CXC, XC or CX3C chemokines (Figure 1-1) (55).

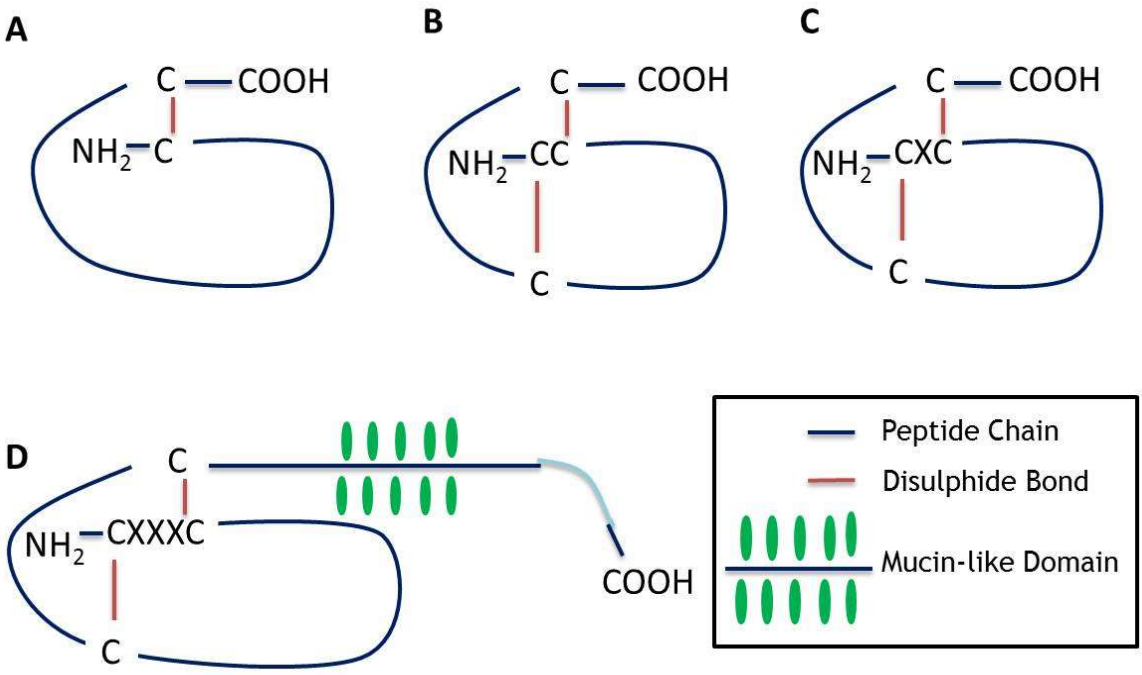


Figure 1-1 Highly conserved structures of the four chemokine families

The chemokine proteins are separated into specific families based on the arrangement of invariant cysteine residues stabilised by disulphide bonds near the amino terminus (NH2). XCL1 and XCL2 have one cysteine residue near the amino terminus (**A**), CC chemokines have 2 cysteine residues (**B**), whereas CXC chemokines have 2 cysteine residues separated by a non-conserved amino acid residue "x" (**C**). The only known member of the CX3C chemokine family (Fraktalkine) has 3 non-conserved amino acid residues separating the two amino terminus cysteine motifs (**D**). CX3CL1 and CXCL16 both have a mucin stalk domain which consists of a membrane-spanning hydrophobic α-helix and a short cytoplasmic tail. This mucin stalk allows these chemokines to be bound on the surface of cells, although soluble forms of fraktalkine exist.

1.3.1.1 **CC-Chemokines**

CC chemokines -also known as the ß- chemokines- have 27 members and are numbered in the order they were discovered from CCL1 – CCL28 (56). They can be broadly categorised into homeostatic or inflammatory chemokines with some exceptions. The inflammatory chemokines are generally lower CC numbers with CCL1 being implicated in lung mucosal inflammatory responses (57), CCL3 in a model of colitis (58) and CCL8 in allergic responses (58). Despite these chemokines being implicated in these diseases, it is difficult to associate one inflammatory chemokine with a specific pathology due to the infidelity of the chemokine system. Put simply, one inflammatory CC chemokine will bind several

different CC chemokine receptors resulting in a massive amount of apparent redundancy in the system (Figure 1-2Figure 1-2), however this redundancy allows for efficient fast clearing of infection. Conversely, the homeostatic CC chemokines are produced at much lower quantities and are constitutively expressed by certain cell types in specific locations and often only bind one receptor. CCL19 and CCL21 bind to CCR7 which is expressed on DCs and they are involved in lymphoid organ development, trafficking of DCs and T cells to the lymph nodes and maintenance of lymphoid tissue microarchitecture (59). Similarly, CCL25 binds to CCR9 and is constitutively expressed in the thymus and the small intestine. CCL25, along with CCL28 (binds CCR10) are involved in immune cell gut homing during homeostasis (60). As mentioned, some chemokines serve as both inflammatory and homeostatic chemokines. CCL2 binds CCR2 and is a potent monocyte and memory T cell chemoattractant during inflammation (61). This chemokine and chemokine receptor pair are implicated in several inflammatory diseases such as atherosclerosis (62) and neurodegenerative disorders (63), however, they are also involved in the homeostatic egress of LY6C^{hi} monocytes from the bone marrow in mice and therefore play a dual role in inflammation and homeostasis (64).

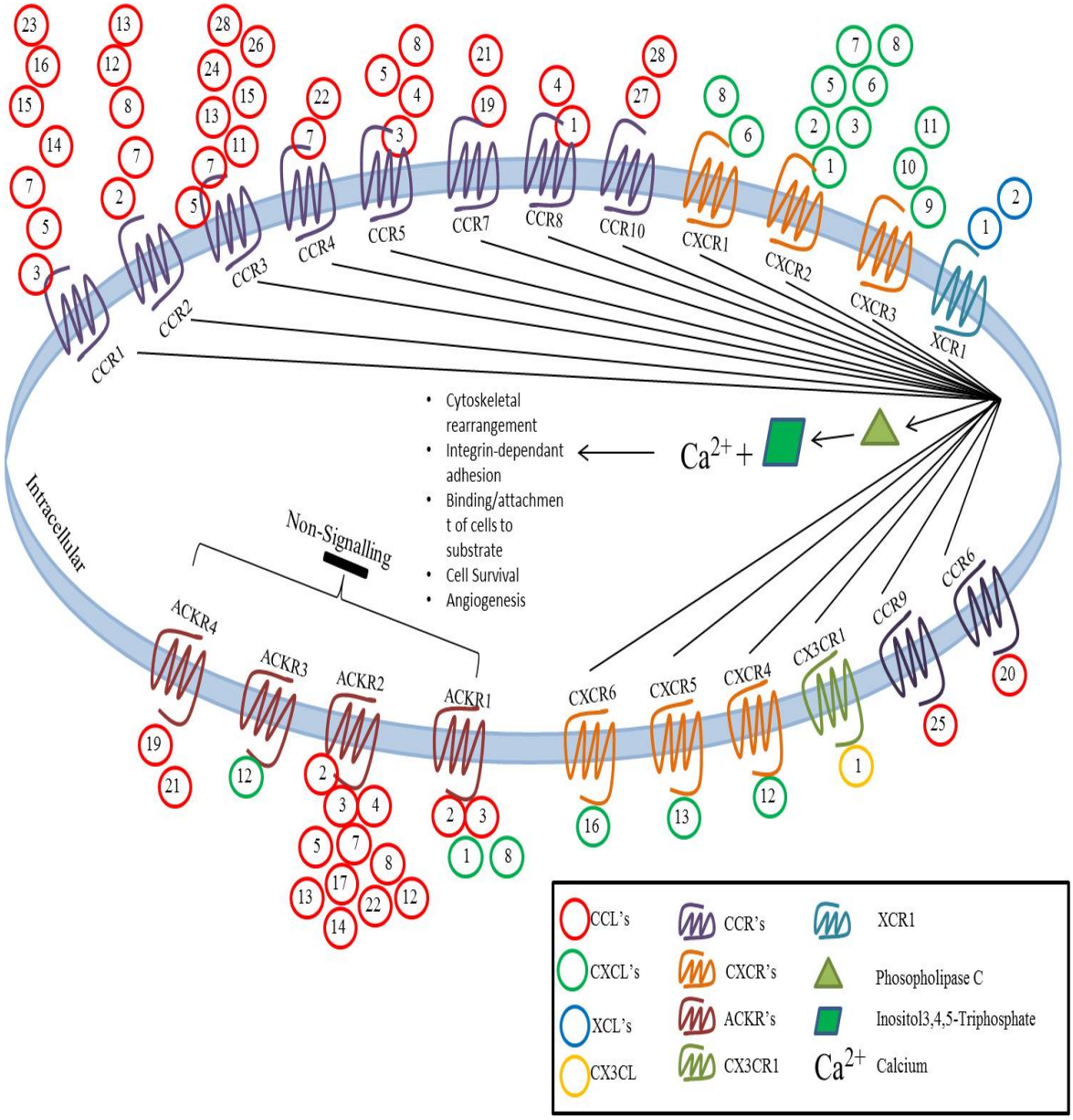


Figure 1-2 Chemokine and chemokine receptor family binding patterns – human.

Chemokine receptors bind several different chemokines and chemokines bind several different receptors. CCRs (purple receptors) will only ever bind CCLs (red circles), CXCRs (orange receptors) will only ever bind CXCLs (green circles). Apart from the atypical chemokine receptors (red receptors), ligand binding, induces an intracellular signalling cascade causing a calcium flux which ultimately results in cytoskeletal rearrangements, cell survival, angiogenesis, integrin-dependent adhesion, among others. The atypical chemokine receptors (red receptors) lack the signalling motif DRYLAIV and therefore are unable to signal like conventional chemokine receptors. Despite an 'unconventional' signalling pattern, ACKRs are able to internalise their ligand and in some cases (ACKR2, ACKR3 and ACKR4) degrade it before recycling the receptor back to the surface of the cell.

1.3.1.2 **CXC Chemokines**

CXC chemokines are so called due to an amino acid in between the first two cysteine residues. Also known as the α-chemokines, there have been 17 described in mammals that can be divided into two different sub-classes dependent on the presence or absence of an amino acid motif – Glu-Leu-Arg. These groups consist of ELR-positive (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8) or ELR-negative chemokines (CXCL4, CXCL9, CXCL10, CXCL11 and CXCL17). As well as leukocyte chemoattraction, ELR-positive chemokines are involved in angiogenic processes, whereas ELR-negative chemokines are angiostatic (65). Like the CC chemokines, they can also be segregated into homeostatic or inflammatory chemokines. The most primordial chemokine, CXCL12, is a homeostatic chemokine that binds CXCR4. Particularly well described for its pivotal involvement during development and retention of hematopoietic stem cells within the bone marrow, it is essential throughout all stages of life, from embryogenesis to adulthood. Being a homeostatic chemokine, CXCL12 is constitutively expressed by the BM stromal compartment, however during inflammation CXCL12 can be secreted by most stromal cells, resulting in chemotaxis of lymphocytes towards the affected area, promotion of vascular formation (angiogenesis) and maintenance of tissue stem cells (66, 67). Unlike CXCL12, CXCL8 - formerly known as IL-8 - is considered an inflammatory chemokine which binds to CXCR1 and 2 and is involved in the chemoattraction of neutrophils and other granulocytes towards sites of inflammation, promoting phagocytosis. CXCL8 is also a strong promoter of angiogenesis, fibrosis and tumorigenesis. Unlike other chemokines, CXCL8 can be stored as a fully formed protein in endothelial cell vesicles called Weibel-Palade bodies, allowing for rapid release (68, 69).Together, CXC chemokines are essential for development and initiation of inflammatory processes whilst also possessing the ability to promote resolution.

1.3.1.3 **CX3C and XC – Chemokines**

Fractalkine, otherwise known as CX3CL1 or neurotactin (in mice), is the only chemokine in the CX3C chemokine receptor family. Structurally, it is quite distinct from the other chemokines as it possesses three amino acid residues and a mucin-like stalk domain. Although it exists in a soluble form, the mucin-like

stalk allows it to be embedded and presented on the surface of cells. It is the only member of its family and binds one receptor – CX3CR1 (70). Similar to fractalkine, XCL1 and XCL2 only bind one receptor (XCR) and exist in a small family of their own. Their function is not fully appreciated or understood (71).

1.3.2 **Chemokine receptors**

As mentioned, chemokines signal through chemokine receptors which are members of the seven-transmembrane (7TM), G-protein-coupled receptor (GPRC) superfamily. There are roughly 20 signalling receptors which upon ligand binding at the N-terminal (Figure 1-3) induce downstream signalling causing a calcium flux. This ultimately leads to cellular responses such as cytoskeletal rearrangements, chemotaxis, cell survival and angiogenesis (52, 72).

Similar to the chemokines which they bind, chemokine receptors are numbered in the chronological order in which they were discovered. Despite each receptor subtype binding several different ligands, they only ever bind ligands from one chemokine subfamily and therefore are named based on the chemokines they bind (CC, CXC, CX3C, XC) followed by "R" for receptor (73). There are 10 known CC receptors, 8 known CXC receptors and the CX3C and XCR receptor families consist of one known member each. Leukocytes express different chemokine receptor profiles depending on their maturation and activation status. Chemokine receptors can define a specific leukocyte population, for example the expression level of CCR2 is often used to distinguish mouse monocyte populations (74). Additionally, the expression of CCR7 is arguably what defines a mature dendritic cell and its upregulation is indicative of a DC's activation state (75). The CC receptor family bind several ligands as does 3 out of 6 of the CXC receptor family. Fractalkine receptor (CX3CR1) binds one ligand CX3CL1 and XCR binds XCL1 and XCL2. The expression of particular chemokine receptors will dictate leukocyte migration to specific sites and govern entry of cells into specific tissues, for example, CCR7 +ve cells will migrate towards the lymph node, CCR10 +ve cells will migrate towards the skin, CCR9 +ve cells will migrate to the intestine and the expression of CXCR4 can result in migration towards the BM, lung and spleen (76-79).

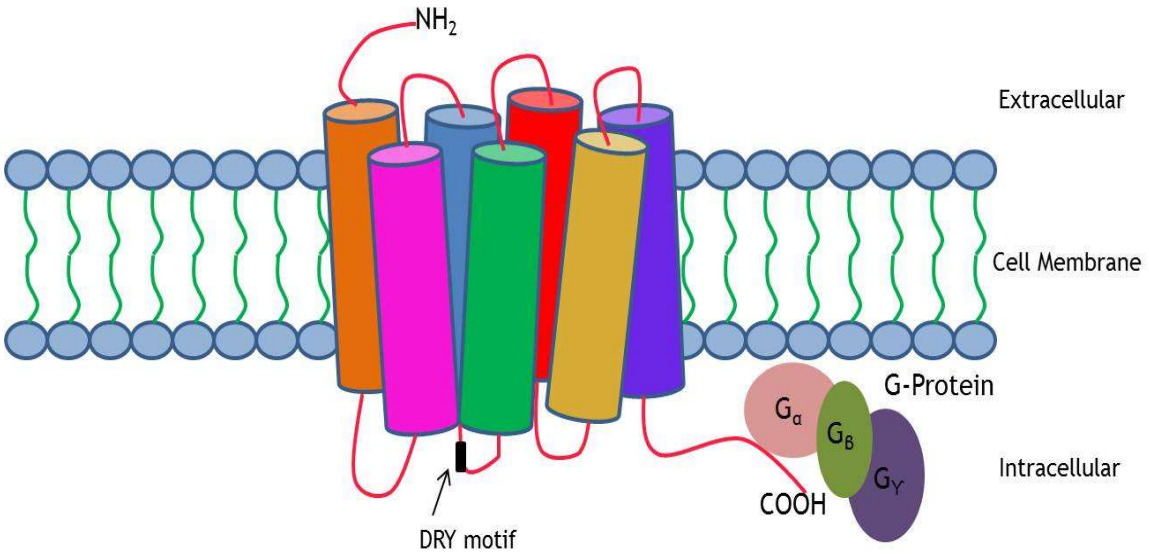


Figure 1-3 Typical Structure of a Chemokine Receptor

Each chemokine receptor has 7-transmembrane regions (7TM) connected by extramembranous loops. The ability of a chemokine receptor to signal relies on the presence of the DRYLAIV motif in the second intracellular loop (not present in atypical chemokine receptors). The ligand binding N terminus is extracellular, whereas the C-terminus, coupled to G-proteins is intracellular. Upon ligand binding, the G-proteins change conformation ultimately resulting in a physiological response.

1.3.2.1 **Atypical Chemokine Receptors**

Atypical chemokine receptors (ACKRs) are structurally homologous to GPRCs, however they lack the DRYLAIV motif and therefore are unable to couple to G-proteins and fail to signal in a “classical” manner, thus termed atypical and segregated into their own family. Unlike conventional chemokine receptor ligand binding, biding of ACKRs to their ligands does not result in chemotaxis, however, they are able to internalise their ligands. There are 4 members of the ACKR family termed ACKR1-4, some of which are involved in well-defined processes, whilst the function of others is currently unclear. Perhaps the best understood atypical chemokine receptor is ACKR2 (formerly known as D6), known for its ability to internalise and degrade inflammatory chemokines, thus considered a scavenging receptor with pivotal roles in the regulation of inflammation (80, 81). Like ACKR2, ACKR3 and ACKR4 are also known for their ability to scavenge their ligands. However, unlike ACKR2, both ACKR3 and ACKR4 bind significantly fewer ligands with only two known ligands for ACKR3 (CXCL11 and CXCL12) and three for ACKR4 (CCL19, 21, 25). The high expression of ACKR4 on lymphatic endothelial cells has led to the belief that it is involved in finely tuning chemokine gradients in the lymph node by binding CCL19 and CCL21 and therefore controlling the availability of these ligands for CCR7 expressing cells

such as DCs (82). ACKR3 is involved in germ cell migration during embryogenesis through the formation of tissue chemokine gradients (CXCL12) and is expressed at high levels in tumour microenvironments, promoting angiogenesis (83, 84). Thus, atypical chemokine receptors finely tune the immune response by scavenging chemokines whilst also playing important roles in development and the regulation of cell migration.

1.3.2.2 **Chemokines and Their Receptors in Migration of Leukocytes**

Neither the innate, nor the adaptive immune responses would occur without immune cells migrating from the blood into the tissue. This processes is termed diapedesis (Figure 1-4) and relies on immobilised chemokines presented on the surface of the endothelium by glycosylamminoglycans (GAGs). The migration of a leukocyte into a tissue is a tightly regulated process involving several different steps which depend on the leukocyte expressing a combination of adhesion molecules and chemokine receptors. This combination of molecules serves as tissue specific “address codes” and governs leukocyte entry into specific tissues during homeostasis and inflammation (85). Put simply, leukocytes come in contact with the endothelium and begin rolling which is mediated by selectins on the endothelial surface and glycosylated sugar ligands on the leukocyte membrane. If the leukocyte expresses the appropriate chemokine receptor - which bind specific chemokines presented by GAGs on the endothelial surface – the leukocyte begins to tether. Hereafter, G-protein coupled signalling results in conformational changes within the leukocyte which results in integrin clustering and activation. Activated integrins bind to adhesion molecules on the endothelium, resulting in firm adhesion of the leukocyte to the endothelium. Thereafter leukocytes undergo diapedesis through endothelial tight junctions into specific tissues (86). Once within tissues, it is relatively unclear how chemokines coordinate chemotaxis of leukocytes. Perhaps the best understood example of how chemokines dictate the movement of leukocytes within tissues is the role of CCR7 and its ligands CCL19 and 21 and CXCR5 and its ligand CXCL13 in the movement and retention of cells at specific sites within the LN (87, 88). Moreover, the evidence of chemokine gradients and their necessity *in vivo* is sparse, however, the first documented *in vivo* existence of a chemokine gradient has been described for CCL21 in the LN (89).

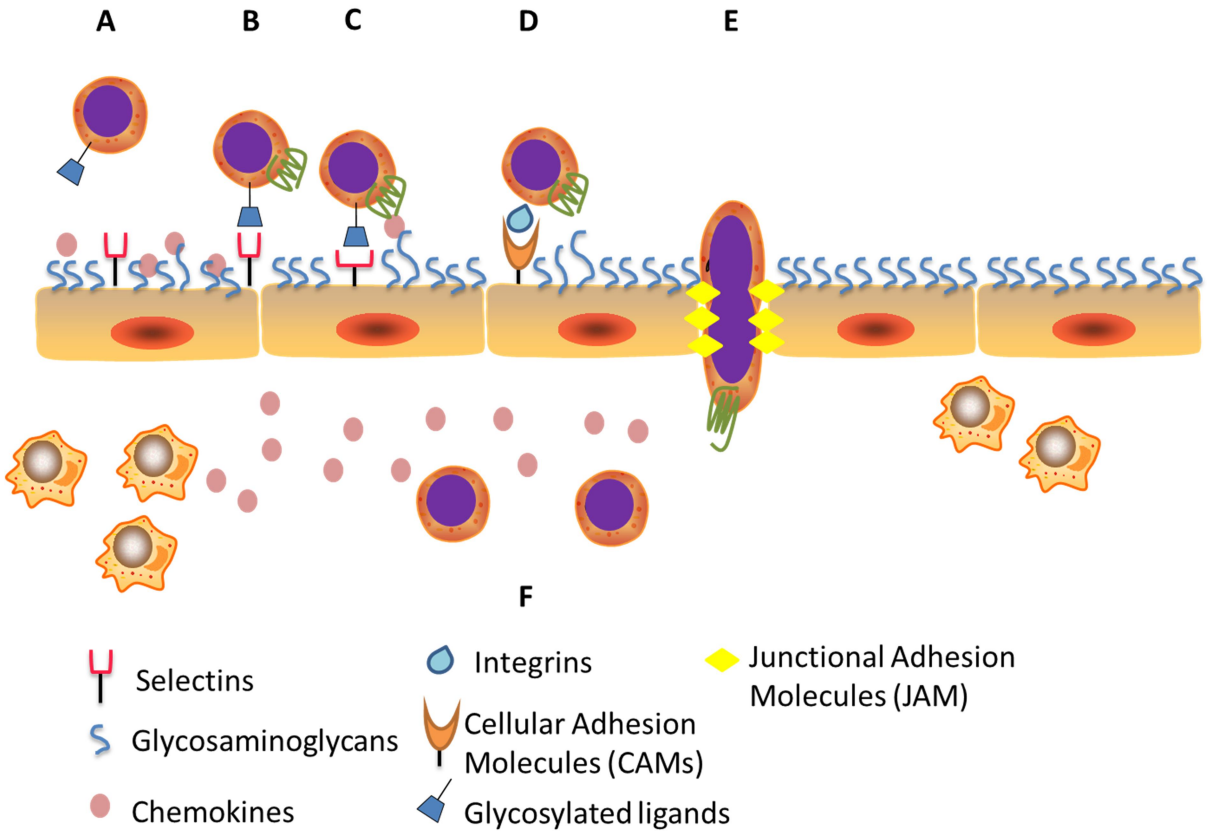


Figure 1-4 Recruitment of leukocytes into tissues – rolling, tethering, tight binding and diapedesis

The process of leukocyte migration into tissues is a serious of adhesive steps that can be categorised into rolling (**B**), tethering (**C**), tight binding (**D**), and transendothelial migration (**E**). Factors released by immune cells and endothelium during insult lead to endothelial cell upregulation of adhesion molecules, known as selectins (**A**). Glycosylated ligands on the immune cell bind to endothelial expressed selectins, resulting in a cell rolling across the endothelial surface (**B**). Chemokines in the surrounding environment, presented on the endothelial surface via GAGs will bind to immune cells with the appropriate chemokine receptor. This chemokine/chemokine receptor binding results in integrin clustering and a conformational change in integrin molecules on the leukocyte, leading to tight binding and immobilisation of the leukocyte (**C, D**). The cytoskeleton of the leukocyte rearranges and the leukocyte passes through the gaps in the endothelial cells, aided by junctional adhesion molecules (**E**). Once within the tissue, chemokines direct cellular movement towards the desired location, perhaps assisted via chemokine gradients (**F**).

1.4 The Stromal Compartment

Stromal cells are responsible for the secretion of several mediators, as well as Ag presentation, that initiate and shape the hematopoietic immune response. The stromal compartment is the connective tissue of organs and consists of a complex mixture of phenotypically similar, and somewhat functionally distinct, cells such as MSCs, fibroblasts and pericytes.

1.4.1 **Mesenchymal Stromal Cells**

MSCs are multipotent precursor cells that give rise to cells from the mesoderm lineage. First described by Alexander Friedenstein in the 1970's as spindle shaped, clonogenic cells in monolayer cultures that can be isolated from the bone marrow, he termed them as colony-forming unit fibroblasts (90, 91). Friedenstein later described them as cells that can serve as feeder cells in the bone marrow for hematopoietic stem cells (HSCs) and this lead to a series of studies which resulted in Arnold Caplan terming them mesenchymal stem cells due to their origin and supposed ability to self-renew (92). In more recent years, it has become apparent that MSCs can be isolated from most tissues in the body, however, their self-renewal capabilities *in vivo* are debated (93). Thus, due to a lack of true stem cell activity, the International Society of Cellular Therapy (ISCT) suggested a nomenclature alteration from mesenchymal stem cells to mesenchymal stromal cells - the two terms are used interchangeably within the literature, however, they describe the same cell type and are denoted the acronym MSC (singular) or MSCs (plural)(94).

1.4.2 **Definition of a Mesenchymal Stromal Cell**

For a cell to be defined as a MSC, it must reach specific criteria stated by the ISCT. These criteria include; adherence to plastic when maintained in standard culture conditions, capacity to differentiate into adipocytes, chondrocytes and osteocytes along with the expression of the surface markers CD73, CD90, CD105 whilst lacking hematopoietic markers such as CD45, CD34, CD14, CD19 and HLA-DR (95). MSCs also express several other markers including variable levels of CD29, CD44, CD166, CD146 and CD271. Some of these markers might be useful in the isolation of a particular type of MSC for example CD271 in BM MSC isolation (96). The variability of these surface markers on MSCs could be due to culture conditions, passage number, tissue or species origin.

1.4.3 **Tissue origin of MSCs impacts their phenotype and function**

As mentioned previously, MSCs can be isolated from a large variety of tissues around the body including, dental pulp, Wharton's jelly, skin, placenta, umbilical cord (UC), periodontal ligament, adipose (Ad), pancreas and from the islets within the pancreas (Is) - to name but a few. BM MSCs are considered the

gold standard MSC to which other MSCs are compared. More recently however, it has become apparent that other tissue sources may be more suitable for clinical use due to them being more easily accessible and isolated in higher quantities from tissues that would often be regarded as medical waste, thus being relatively low cost to acquire, with no burden on the donor (97). Subsequently, studies comparing MSC source, phenotype and function have led to the belief that MSCs from different sources are phenotypically similar (similar morphology and surface CD expression levels) but differ in their proliferation capacity, their ability to differentiate into certain lineages, their immunomodulatory capacity and their cytokine secretion profiles (98-103). Despite numerous studies on the comparison of MSCs isolated from different tissue sources, not all avenues have been fully explored. Studies comparing their immunomodulatory capacity have focussed mainly on their ability to suppress T cells (104). Additionally, studies comparing MSC migratory potential are minimal. Moreover, in depth studies on MSC migration and immunomodulation have largely focussed on BM MSCs. Understanding and comparing the migratory and immunomodulatory capacity of MSCs isolated from various tissue sources will be important for the progression of clinical studies with MSCs.

1.4.4 **MSCs and the immune system**

When MSCs were discovered to be weakly immunogenic - due to their lack of HLA Ags and co-stimulatory molecules (e.g. CD80/86) - whilst also possessing the ability to be immunosuppressive, immunomodulatory and regenerative in both humans and animal models, they became particularly interesting to immunologists (104, 105). Their location in the stromal compartment within epithelial niches in close proximity to vessel walls, results in MSCs being one of the first epithelial responders in mechanical and microbial breach. Once breached, they are in the perfect position to recruit immune cells, whilst also promoting resolution via blood vessel formation (106). It is therefore, perhaps, of no surprise that MSCs can interact with, immunosuppress and immunomodulate the humoral, the innate and the adaptive immune systems. Their ability to interact with the immune system and promote resolution is enhanced during inflammation, a process known as MSC licensing, i.e. in order for MSCs to function as anti-inflammatory and pro-resolving cells they must be surrounded by an inflammatory environment, which is popularly mimicked *in*

vitro by stimulating MSCs with cytokines such as IFN-γ, TNF-α and/or IL-1ß. Licensing MSCs in this way highlights how MSCs might perform in a clinical setting, as they are predominantly used as anti-inflammatory and immunomodulatory mediators in inflammatory environments (107).

1.4.5 **MSCs and the humoral immune system**

The humoral immune system is composed of macromolecules, secreted antibodies, the complement system and antimicrobial peptides. MSCs have been reported to have an effect on, respond to and interact with several components of humoral immunity. Perhaps the most comprehensively explored avenue of humoral immunity and MSCs is the complement system (Section 1.2.1.1).

Studies suggest that MSCs express complement receptors C3aR and C5aR, allowing chemotaxis of MSCs towards the complement components C3 and C5, which are abundantly expressed in inflamed tissue. The binding of C3 and C5 to their receptors on the surface of MSCs ultimately results in MSCs having increased resistance to oxidative stress – a favourable attribute in an inflamed environment, leading to prolonged survival of MSCs (108). Moreover, MSCs secrete complement factor H (CFH) which is a complement inhibitor and exerts its function via inhibiting the formation of C5 and C3 convertases, whilst also accelerating their degradation (109). Taken together, MSCs are able to interact with the complement system to migrate towards sites of inflammation. Once within the inflamed site, MSCs are able to survive longer by binding complement factors whilst also secreting factors that inhibit complement formation and degrade it.

Secreted antibodies from B cells are heavily involved in humoral immunity as described in (Section 1.2.2.1). The antibodies IgM, IgA and IgG secreted by B cells are reduced in the presence of BM MSCs, the mechanisms behind this phenomenon are largely unknown, however it is suggested to be T cell mediated (110, 111).

1.4.6 **MSCs and the innate immune system**

As mentioned in Section 1.2.1, the innate immune system plays a crucial and essential role in initiating and resolving inflammation. Additionally, the innate immune system has been implicated in transplant rejection and autoimmune disease (112). Thus the observations documenting the immunomodulatory and anti-inflammatory effect of MSCs on various components of the innate immune system are crucial for understanding MSCs potential in the clinic.

1.4.6.1 **MSCs and monocytes and macrophages**

As mentioned previously, monocytes and macrophages are vital in the early stages of inflammation where they help clear debris whilst also perpetuating the inflammatory-immune response. The monocyte/macrophage system is a complex mixture of cells consisting of bone marrow derived circulating monocytes recruited to sites of tissue injury, infiltrating macrophages which have differentiated from in situ monocytes and yolk-sac derived resident macrophages. Monocytes and macrophages are involved in the persistence and perpetuation of several inflammatory and autoimmune diseases such as atherosclerosis and Crohn's disease (113-115). Several studies have demonstrated that MSCs have an immunomodulatory effect on monocytes and macrophages and therefore could prove an advantageous treatment option for monocyte/macrophage driven inflammatory diseases (116, 117). Although the precise molecular mechanisms remain unclear, it has been suggested that MSCs can promote the differentiation of the anti-inflammatory "M2" macrophages from monocytes via MSC indolamine 2,3-dioxygenase (IDO) activity (118). This leads to IL-10 production from macrophages, which in turn has an inhibitory effect on surrounding proliferating T cells (119). Additionally MSC exposure to the anti-inflammatory M2 macrophage prolongs MSC survival when compared to pro-inflammatory M1 exposure (120). Further, in a rat model of spinal cord injury BM MSC infusion was linked with elevated levels of M2 cytokines IL-4 and IL-13. The elevated levels of cytokines matched the observation of increased M2 cell numbers and decreased M1 cell numbers. This was associated with less scarring, preserved axons and increased myelin sparing (121). Collectively, MSCs promote the development of M2 macrophages from monocytes, which in turn

provide MSCs with survival signals. These M2 macrophages secrete anti-inflammatory cytokines which act on surrounding immune cells such as T cells to prevent their proliferation.

1.4.6.2 **MSCs and neutrophils**

Neutrophils are involved in early immune responses, releasing reactive oxygen species along with other inflammatory mediators to clear infection. Along with monocytes and macrophages, they too have an important role in recruiting plentiful immune cells to the site of inflammation. Literature surrounding the interaction of MSCs and neutrophils is sparse, however, it has been reported that MSCs modulate neutrophil function in several ways that lessens their inflammatory capacity. Through the secretion of IL-6, MSCs have been shown to downregulate the respiratory burst of neutrophils, along with a delay in spontaneous apoptosis in resting and activated neutrophils (122). This downregulation of the respiratory burst is thought to promote the longevity of the neutrophil and could be important in the reservation of immature neutrophils in specific sites such as the BM (123).

1.4.6.3 **MSCs and dendritic cells**

As professional Ag presenting cells, presenting Ag to T cells, B cells (124) and NK (125) cells, depending on their activation status, DCs are capable of activating the adaptive immune system or inducing tolerance. Activated DCs express co-stimulatory molecules such as CD80/86 as well as a marked upregulation of MHC II which allows them to activate T cells. Conversely, a peptide loaded DC which is still immature, i.e. low levels of CD80/86 and MHC II will not provide a T cell with sufficient activation signals resulting in T cell anergy or apoptosis. MSCs have the ability to interfere with the maturation and activation state of DCs. GMCSF and IL-4 stimulated monocytes were inhibited from maturing into DCs via MSC secreted IL-6 and PGE2 (126, 127). In addition to this, DC's ability to endocytose, upregulate co-stimulatory molecules and secrete IL-12 were impaired when co-cultured with MSCs (128). This repressed activation state of the DC had a knock on effect on the DCs ability to activate T cells.

1.4.6.4 **MSCs and NK cells**

NK cells are well known for their ability to kill other cells without needing any prior stimulation. This results in them being relatively unregulated killing machines, perhaps accounting for their well-documented involvement in autoimmune diseases (129). As previously discussed (Section 1.2.1.2.5) NK cells kill those cells expressing low levels of MHC I, leaving the MSC as a target for NK cells. Administration of IFN-γ (MSC licensing) to MHC:NK co-cultures upregulates MHC I on MSCs and protects them from NK cell cytotoxicity (130). Additionally co-culture of MSCs and NK cells downregulates the expression of activation receptors on NK cells (131). Moreover via the secretion of TGF-β1 and PGE_2, MSCs are able to repress proliferation and IFN-γ production of IL-2 or IL-15 activated NK cells (132). Together, the microenvironment surrounding the NK cells and MSCs may determine which cell has the upper hand. During inflammation, MSCs are able to regulate the activation state of NK cells whilst in the absence of IFN-γ, NK cells could be capable of cytotoxic killing of MSCs.

1.4.7 **MSCs and the adaptive immune system**

1.4.7.1 **MSCs and T cells**

Upon TCR engagement T Cells are activated to perform a variety of effector functions including cytokine production to shape the surrounding immune response and cytotoxic killing of virally infected and tumour cells. They are the primary mediators of many inflammatory and autoimmune diseases, therefore, the observation that MSCs can inhibit T cell proliferation resulted in sequential studies dissecting the immunomodulation of MSCs on T cell effector pathways (133). MSCs ability to supress T cell proliferation occurs directly or indirectly. Direct suppression of proliferation occurs through cellular contact or soluble mediators, whereas indirect suppression of T cells occurs via MSCs effects on other immune cells such as DCs. BM MSCs are able to inhibit the proliferation of T cells via the engagement of programmed cell death protein 1 (PD-1) *in vitro* (134). Additionally, MSC production of soluble factors such as indoleamine 2 3-dioxygenase (IDO) (secretion leads to T cell anergy) (135), prostaglandin E_2 (PGE_2) (suppresses TCR signalling) (136), Hepatocyte Growth Factor (HGF) and IL-

10 have been reported to inhibit T cell proliferation and promote Tregs (137). The anti-proliferative effect MSCs have on T cells is associated with the arrest of T cells in a state of quiescence, which can be reversed upon administration of IL-2. Additionally, due to the MSC mediated downregulation of co-stimulatory molecules on DCs, MSCs are able to promote T cell anergy indirectly via other immune cells. MSCs are also able to skew the type of T helper cell response along with downregulating CD8 T cell cytotoxicity (138). Co-culturing human MSCs with Th1 cells resulted in reduced IFN-γ secretion whereas IL-4 production from Th2 cells was increased. Moreover, the number of Tregs was increased (139). Similar observations were noted in a mouse model of inflammatory bowel disease, where the administration of xenogenic, allogenic or autologous Ad MSCs ameliorated disease (140). This was associated with a downregulation of IFN-γ alongside an upregulation of FOXP3 Tregs. Thus, MSCs can modulate the intensity of the immune response by inhibiting Ag specific T cell proliferation whilst also promoting Tregs.

1.4.7.2 **MSCs and B Cells**

B cells have been implemented in several autoimmune diseases such as Multiple Sclerosis (MS), systemic lupus erythematosus and rheumatoid arthritis (141, 142). Their ability to form memory plasma cells and produce antibodies specific for self-Ag for a lifetime is what renders them key players in autoimmune diseases. The immunomodulatory effect of MSCs on B cells are contradictory, this could be due to a number of factors such as MSC isolation technique, passage number of MSCs and different experimental procedures. When co-cultured with human BM MSCs and a B cell stimulus, purified B cells were arrested in the G0/G1 phase of the cell cycle and unable to proliferate. Additionally, soluble factors secreted from MSCs impaired IgM, IgA and IgG production. Moreover, B cell's ability to migrate towards CXCL12, CXCL13 and CCL19/21 was diminished via the downregulation of the receptors CXCR4, CXCR5 and CCR7 on the B cell (110). Interestingly, the ability of MSCs to immunomodulate B cells is thought to be dependent upon the presence of T cells (143).

1.4.8 **MSCs migratory capacity**

Like hematopoietic cells, in order to carry out efficient effector functions, MSCs must migrate to sites of tissue injury. MSCs have been shown to possess the ability to home to sites of injury from the periphery. Human and rat MSCs have demonstrated the capability to migrate into sites of brain injury after cerebral ischemia (144, 145). Moreover, in a model of allograft rejection, MSCs delivered IV found their way towards sites of rejection (146). Others have observed MSC capability to migrate towards the lung in response to injury when injected IV (147). However, due to a large body of literature describing MSCs getting trapped in the lungs of mice when delivered systemically (148), it is hard to determine if the observation of MSCs in the lung is a result of specific homing to the injured lung or whether it is entrapment within the lung. The low engraftment rate of MSCs within target tissues could suggest that poor pulmonary passage is also occurring in humans (149, 150).

The precise mechanisms that MSCs use to migrate from the periphery into tissues are unknown but it is likely that they use a combination of adhesion molecules and chemokine receptors to achieve this. In support of this, there is a large body of literature describing chemokine receptor expression on human MSCs, however the findings are somewhat contradictory.

Expression levels of the homeostatic chemokine receptor CXCR4 is perhaps the most widely disputed within the literature. Some groups found no expression (151), some found very little (152) whilst others observed functional CXCR4 on MSCs (153). MSCs have also been reported to express other functional chemokine receptors such as CCR1, CCR7, CCR9, CXCR5 and CXCR6 (151, 154). What is important to note is that all of these studies have only assessed BM MSC chemokine receptor expression. Interestingly, other studies have suggested that Ad MSCs express fewer adhesion molecules than BM MSCs and this allows for more rapid egress from the lung into sites of damage (155). This could suggest that MSCs isolated from different sources possess differential migratory capacity and therefore a particular tissue for MSC isolation might be favourable to enhance migration to specific anatomical locations.

1.4.9 **MSCs clinical use**

As a result of the aforementioned observations of (BM) MSCs interactions with immune cells and migratory potential, MSCs are intensively studied as a cellular therapy in a wide range of inflammatory and autoimmune disorders. In the first instance, MSCs received attention due to their differentiation capabilities, thus interest grew in their potential application within the clinic for tissue degrading diseases such as rheumatoid arthritis and MS (156, 157). Secondly, their ability to immunosuppress and immunomodulate several components of the immune system, led to the concept of MSCs being used to suppress graft rejection, graft versus host disease (GVHD) and autoimmune disorders (158). Moreover, MSCs can be easily isolated from a variety of human tissue with little effort. To add to this, they can be easily and quickly expanded for mass production of a good manufacturing practice (GMP)-grade cell. Perhaps most importantly, MSCs have poor immunogenicity *in vitro*, in pre-clinical and in human studies (105, 159). This is due to their low expression of HLA molecules resulting in the possibility of MSCs being obtained from allogeneic donors. It is for these reasons that MSCs have gained much interest in numerous pre-clinical and clinical trials.

Animal models of human disease are a tool in pre-clinical trials and have been indispensable in understanding the effect of MSCs within different disease models. Mouse models have been used to demonstrate the immunosuppressive capacity of MSCs *in vivo*. More specifically, mouse models have been used to understand the effects of MSCs in chronic obstructive pulmonary disease, Type 1 Diabetes, experimental autoimmune arthritis, graft versus host disease and others.

1.4.9.1 **MSCs in Graft Versus Host Disease and Solid Organ Transplantation**

Graft versus host disease occurs after an allogeneic hematopoietic stem cell transplant (HSCT) which is a treatment for hematopoietic malignancies and immune deficiencies. It arises as a result of transplanted donor immune cells mounting a response to recipient tissues and/or organs, occurring in around 50% of transplants (160). Mouse models have allowed insight into the pathophysiology of the disease, allowing for better understanding of GVHD and improved success rate of HSCT. Mouse models of GVHD are created by the depletion of endogenous

hematopoietic cells in recipient mice via radiation or chemotherapy and then reconstituting the immune system with allogeneic bone marrow cells. The severity of GVHD in these animals is determined via the degree of MHC mismatch between donor and recipient, with the readout of disease severity being determined via weight loss, survival and histological examination of organs (161).

Due to the properties of MSCs, they have therapeutic potential for the treatment and prevention of GVHD. A mouse model demonstrated that BM MSCS reduced the severity of GVHD, whilst also prolonging the survival of the HSCT recipients. These observations were coupled with the diminished infiltration of T cells into target organs and inhibition of the co-stimulatory molecules CD80/86 on DCs (162).

In humans MSCs have been used in several clinical trials for patients with steroid-refractory GVHD (163). MSCs have shown to significantly lower the rate of acute GVHD and MSC infusions seem to be tolerated well in all patients (164-166).

Due to the success and safety of pre-clinical and clinical trials with MSCs in GVHD, MSCs are being investigated for use in solid organ transplantation (SOT). Interestingly, a mouse model of kidney allograft transplantation highlighted the importance of timing of MSC delivery. They demonstrated that a pre-transplant infusion of MSCs resulted in the localisation of MSCs within lymphoid organs and this promoted the expansion of Tregs. Conversely, an infusion of MSCs post-transplant was associated with premature graft dysfunction coupled with neutrophils and complement deposition (167). This study suggests that infusing MSCs post-transplant does not allow the MSCs to be licensed (in an inflammatory environment) for long enough to exert their anti-inflammatory properties, an issue which has been associated with the failure of several pre-clinical and clinical trials (168).

In humans, the use of MSCs in patients undergoing a renal transplant resulted in a lower case of acute graft rejection, reduced risk of opportunistic infection along with improved renal function at one year (169).

1.4.9.2 **MSCs in Diabetes Type1**

Diabetes mellitus type 1 is an autoimmune disease that results from the destruction of the insulin producing pancreatic beta cells. Macrophages, dendritic cells, B cells and T cells have been shown to be involved in the pathogenesis of T1DM (170). In 2014, the number of people in the UK living with T1DM was 350,000, costing the NHS £1 billion per year, with this figure estimated to double by 2035 if current treatment options remain unchanged (171).

There are several treatment options for individuals with T1DM including the most common method of daily insulin injections, along with other methods such as an insulin pump, an artificial pancreas and more invasive options such as whole pancreas transplantation and islet transplantation (172-174). The aforementioned options aim to restore blood glucose levels by replacing insulin production in some way. Insulin therapy was the first therapy discovered for T1DM however patients quality of life is still affected by frequent episodes of hyper and hypoglycaemia which can lead to undesirable side effects such as weakness, shortness of breath, fainting and in more severe cases coma and sometimes death. The only way to restore blood glucose levels and combat progression of diabetic complications is to replace ß-cells. Whole pancreas and islet transplantation are two treatment options that can achieve this outcome, however, pancreas transplants are associated with early mortality and lifelong immunosuppressive drug use. Conversely, islet transplantation is a significantly less invasive surgery and presents as a much smaller immunogenic tissue (174). Moreover, investigation into protecting the islets from recipient immune system attack is underway. Current strategies include physically encapsulating the islets within a semi-permeable membrane consisting of polymer (175), along with infusing MSCs to act as an anti-inflammatory and regenerative cell to improve graft survival (176).

A number of animal models exist to study the immunological pathogenesis and long term consequences of ß-cell loss. The Non-Obese diabetic (NOD) mouse model is used as a model for spontaneous autoimmune disease which shares many similarities with T1DM. These mice have a mutation in exon 2 of the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) gene, which plays a vital

role in the suppression of T cells. Without a functional CTLA-4 gene, the ß-cells are susceptible to autoimmune attack from T cells (177). Additionally, the administration of streptozotoan (STZ) which damages ß-Cells results in the accumulation of immune cells and immune mediated destruction of ß-cells. The STZ administration model is used to assess progressive loss of ß-cells. Both of these models have been used to study the effect of MSC administration in T1D, and have shown that MSC administration can protect against disease (MSCs administered before onset), reverse disease (MSCs administered after onset) and can revert hyperglycaemic animals to normal blood glucose levels. These observations have been paired with a reduction of inflammatory CD4 T cells, a shift in cytokine production towards a Th2 mediated response and an induction of T regulatory cells (178). Due to the success of MSC infusion in T1D disease models, studies have focussed on understanding if the co-infusion of MSCs with islets is protective for the islets. Results show that MSCs can significantly improve islet survival (179), insulin secretion (180) and delay allograft rejection (181).

1.5 **Thesis aims**

Previous sections highlight how MSCs are of great interest for use as a cellular therapy to act as anti-inflammatory mediators in several diseases. As SNBTS provide an islet transplant service, our particular interest lies in the co-infusion of MSCs with pancreatic islets in order to improve graft function and longevity. However our interest is not limited to this and extends to understanding the potential roles of MSCs within any clinical setting. What is important to note from the previous sections is that the majority of our understanding of MSCs phenotype, function and performance within clinical trials comes from BM derived MSCs, with a relative lack of understanding of other MSC populations. Therefore we sought to understand if MSCs isolated from tissues that would be regarded as medical waste – islet (Is), visceral adipose surrounding the pancreas (Va), adipose (Ad), and umbilical cord MSCs (UC) – are comparable to BM derived MSCs. More importantly, this study aimed to elucidate if one population of MSC could be more beneficial than others in specific clinical settings.

To understand this, we thought it would be appropriate to understand the chemokine receptor expression of all MSC types. This would help us gain insight

into whether one MSC would be more suited to migrating to specific target tissues if infused systemically. Equally, if MSCs were infused locally, low expression of chemokine receptors might be more desirable to ensure that MSCs do not migrate away from the graft site.

In addition, it was important to address how MSCs isolated from various tissues might interact with their surrounding environment once infused into a patient. With several reports highlighting BM MSCs anti-inflammatory, immunomodulatory and pro-regenerative capacity in various clinical settings, understanding how MSCs isolated from various sources interact with surrounding immune cells is pivotal in elucidating their potential immunomodulatory and pro-regenerative capacity *in vivo*. In an attempt to examine this, we aimed to assay the chemokine secretion profiles of MSCs isolated from various sources and how this impacted their interaction with immune cells. The layout of this study and overall aims of each chapter are outlined below:

1. The aim of Chapter 3 was to fully phenotype Is, Va, Ad, BM and UC 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 all chemokines and their receptors by MSCs at rest and under inflammatory stimulation.

3. The aim of Chapter 5 was to determine if the expression patterns of chemokines and their receptors would persist at a protein level at rest and under inflammatory stimulation. Moreover, through assessing the immune cell attraction profile of MSCs at rest and under inflammatory stimulation, the work in this chapter aimed to address whether the chemokines secreted by MSCs were functional.

4. Chapter 6 - having observed immune cell attraction in an in *vitro* system, the work carried out in this chapter aimed to understand if this immune cell attraction profile persisted *in vivo*.

Chapter 2

Materials and Methods

Chapter 2 Materials and Methods

2.1 General solutions and consumables

Solutions used in these studies, their composition and their concentration are detailed in Table 2-1. All Laboratory chemicals and plasticware were obtained from a variety of manufacturers and are detailed throughout this chapter.

Table 2-1 List of solutions and their compositions

Solution	Composition	Concentration
1xs Dulbecco's Phosphate-Buffered Saline	Potassium Chloride (KCL) Potassium Phosphate Monobasic (KH_2Po_4) Sodium Chloride (NaCl) Sodium Phosphate Dibasic (Na_2HPO_4)	2.7mM 1.76mM 137mM 10mM
Fluorescence-activated cell sorting (FACS) buffer	DPBS FCS Sodium-Azide (NaN_3) EDTA	1% 0.02% 5mM 0.01%
Freezing down solution	AB Pooled Plasma Dimethylsulphoxide (DMSO)	90% (v/v) 10% (v/v)

2.2 Cell culture methods

2.2.1.1 Primary cell maintenance

All cells used during this study were primary cells, unless stated otherwise. Cells were handled in a sterile environment (Laminar flow hood with HEPA filtration) with sterile equipment at all times. Surfaces and equipment were sprayed with 70% industrial methylated spirits prior to use. All centrifuge steps for cell culture were carried out at 200g for 7 minutes - unless stated otherwise, using a Biofuge primo centrifuge (Thermo Scientific). All cell cultures were incubated at 37°C / 5%CO_2 / 95% humidity. Mesenchymal stromal cells (MSCs) were seeded at a density of 3000 cells/cm^2, unless stated otherwise.

Islet (Is), visceral adipose (Va), adipose (Ad), bone marrow (BM) and umbilical cord (UC) MSCs were maintained in 0.26mL/cm^2 (per tissue culture flask)

Dulbecco's minimal essential medium (DMEM), high glucose, GlutaMAX™ Supplement (Life Technologies) plus 10% AB pooled plasma (provided by Scottish National Blood Transfusion Service), 4mM penicillin and streptomycin (Invitrogen) – this will be referred to as "MSC medium" for the remainder of this thesis.

The cells were examined daily for growth using a phase-contrast microscope (Ziess) and maintained in culture until they reached 70-80% confluence. At this time, cells were washed twice with DPBS (sigma) and detached following a 10min incubation at 37°C with 10mL of 1 x TryplE (Life Technologies), cells were then placed into a 15mL Flacon and spun down at 200g for 7 mins. Supernatant was discarded and the cell pellet was resususpended in the appropriate amount of MSC medium, counted as outlined in Section 2.2.3 and re-seeded at 3000 cells/cm^2. When cells were detached from flasks and re-seeded into new flasks as described above, this was termed as cell passaging and each time it was performed counted as one passage. Cells of lower passage were used when possible (Passage 1-3). This detachment method was used consistently throughout all experimental procedures.

2.2.2 **Isolation of cells**

Isolation of MSCs from various tissues was carried out by staff at the Scottish National Blood Transfusion Services (SNBTS). Single cell MSC cultures were transported from Edinburgh to Glasgow at Passage 1 (P1) for experimental purposes. Details on how MSCs were isolated are provided below.

2.2.2.1 **Isolation of adipose and visceral adipose mesenchymal stromal cells**

Human Adipose tissue isolated from liposuction and visceral adipose tissue (isolated from the adipose tissue surrounding the pancreas) were received from SNBTS and the protocols approved by Glasgow National Health Service Trust-East Ethics committee. Adipose tissue was manually disociated with scissors until the tissue was liquidised. The sample was then spun down at 300g for 10 mins. Supernatant was aspirated and the remaining adipose tissue was digested with 1mg/ml of collagenase (Sigma Aldrich) + 2% Human Serum Ovalbumin (HSA) (Sigma Aldrich) in PBS for 1 hour. The sample was then spun down at 300g for 20

mins. The supernatant was aspirated and the cell pellet was cultured in MSC culture medium. After 2 days, non-adherent cells were discarded, MSC medium was replaced and adherent cells were left to grow. From then on, cells were checked daily for growth and medium was changed every 2-3 days. When cells were 80% confluent they were passaged.

2.2.2.2 **Isolation of islet mesenchymal stromal cells**

Waste islet fractions were obtained from SNBTS Islet isolation lab - where the preparation of islets isolated from deceased donor's pancreas occurs before islet transplantation.
High purity islets (HPI) or low purity preparations were centrifuged and plated out in T125, T75 or T25 flasks at approximately 10 islets/cm^2. All materials were treated as an explant. These materials were cultured at 37^oC in 5% CO_2 in MSC culture medium. At day 7 explant outgrowth was assessed and adherent MSC were observed migrating from the explanted materials. The medium was carefully changed and then further changed every 3-4 days.
Once the cells had reached 80% confluency the adherent MSCs were passaged as described in Section 2.2.1.1. To remove any larger islets and cell debris the material was passed through a 100μM cell strainer. The cells were counted using a haemocytometer and classified as passage 1. These MSC were either cryopreserved at 1x10^6 cells per vial in 10% DMSO or plated out at 3000 cells/cm^2.

2.2.2.3 **Isolation of umbilical cord mesenchymal stromal cells**

The cord was dissected by stripping the epithelial tissue to expose the vessels and Wharton's Jelly (WJ). Firstly, using a sterile bullclip the cord was clamped onto a dissection board. The cord was kept wet by the addition of PBS. The cord was cut by making a shallow circumferential incision into epithelium close to the clamp with a scalpel. Using dissection forceps, the epithelium was separated from the incision site around entire circumference of cord to expose underlying Wharton's Jelly gelatinous tissue. Using forceps the loose epithelium was slowly pulled away, along the entire length of the cord, from the vessels and the Wharton's Jelly (WJ) (Figure 2-1). This was performed for the length of the cord and the epithelium discarded. The vessels were separated by cutting down the

cord into the 3 vessels (2 arteries and 1 vein) with the associated WJ using forceps the WJ was carefully removed from the vessel walls and collected into a 50mL conical tube containing PBS. Once all WJ tissue was collected it was cut into small 1-2mm fragments using a scalpel. This tissue was then used as explant material into tissue culture flasks. Approximately 1mL of MSC culture medium per cm^2 of tissue was added to a T75 flask. Cells were assessed for outgrowth from explanted material at day 5 and fed with fresh media on day 7. Flasks were assessed for outgrowth every few days and when cells were 80% confluent they were passaged as described in Section 2.2.1.1. Prior to re-seeding MSC into new tissue culture flasks, they were passed through a 100 micron filter, to remove large clumps, before re-plating.

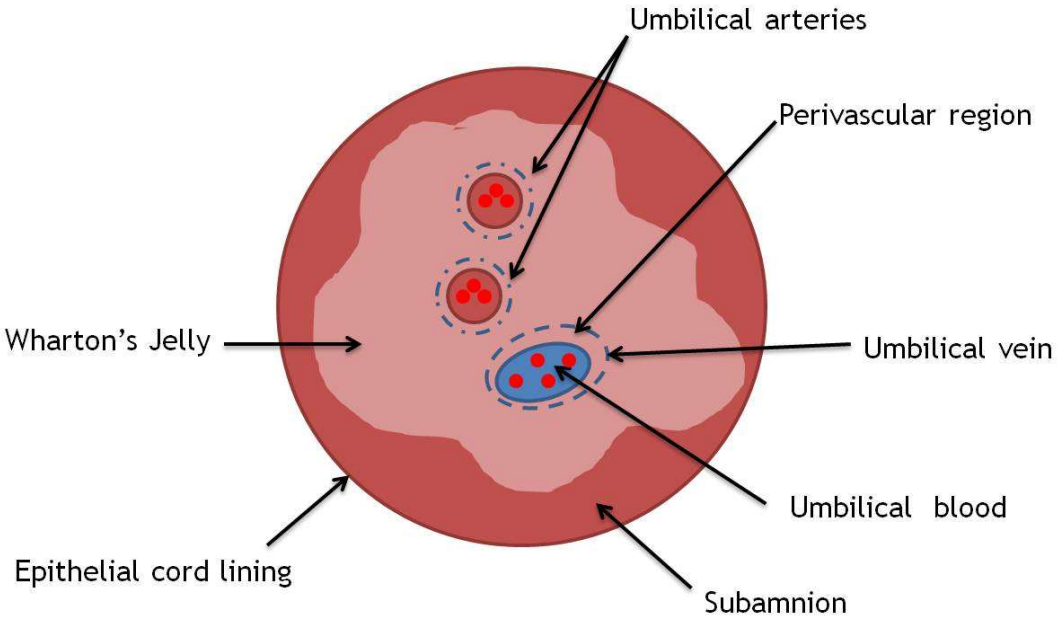


Figure 2-1 Schematic of the different compartments of the umbilical cord
The diagram depicts the anatomy of the umbilical cord showing the 2 umbilical arteries and umbilical vein which are surrounded by an associated perivascular region. A gelatinous substance known as the Wharton's Jelly - where UC MSCs in this study were isolated from – fills the centre of the cord, which is held together by the epithelial cord lining.

2.2.2.4 **Isolation of bone marrow mesenchymal stromal cells**

The bone marrow (BM) was received from patients undergoing hip revision surgery. The BM sample was weighed and dissected using a scalpel into 0.5-1g pieces. The BM fragments were digested with Collagenase II (Sigma) at a final concentration of 0.25mg/mL at 37°C in a 50mL centrifuge tube for 30-60mins.

Following digestion, the cells were centrifuged at 200g for 15mins. The supernatant was carefully discarded and the cell pellet retained. The pellet was re-suspended in Hanks Balanced Salt Solution (HBSS) (Thermo Fisher), passed through a 100μM cell strainer (Falcon) and cells were counted using a haemocytometer. Cells were then plated out in T75 flasks at 3000 cells/cm^2 in MSC culture medium. Flasks were incubated at 37^oC, 5% CO_2. Cells were assessed for outgrowth daily and media changed every 3-4 days. Once at 80% confluence, the adherent MSC were passaged as described above and labelled P1. Cells were either cryopreserved or re-passaged by plating at a density of 3000cells/cm^2.

2.2.3 **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 PBS. 50μl of diluted Trypan blue was mixed with 50μl of cell suspension and incubated for 2-3 mins at room temperature (RT) before loading 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 give the number of cells per 1 mL of the cell suspension.

2.2.4 **Freezing**

Primary cells were frozen at early passage from all cell sources. Cells were washed in PBS, detached with TryplE as discussed in Section 2.2.1.1, spun down and resuspended at a density of approximately 1x10^6 cells/mL in freezing down solution (see Table 2-1). 1 mL aliquots were transferred to 2mL cryo-vials (Thermo-scientific) and stored in a freezing vessel (Nalgene), containing isopropanol. This was then placed at -80^oC (cooling 1℃ per minute) and transferred to liquid nitrogen tanks within two days.

2.2.5 **Thawing of primary cells from frozen**

Cells were recovered from liquid nitrogen and rapidly thawed in a 37^oC water bath. Once defrosted, the cells were transferred from a cryo-vial to a 15mL falcon tube. Warmed culture medium was slowly added drop by drop until cells were suspended in 7mL of MSC medium. The cells were then spun down at 200g

for 7 mins and the supernatant was discarded. The remaining pellet was then resuspended in the appropriate volume of MSC culture medium and transferred to tissue culture flask(s).

2.2.6 **Stimulation of cells with inflammatory cytokines**

When MSCs reached 80% confluency, culture medium was pipetted off and cells were washed twice with PBS. MSC culture medium was replaced and supplemented with 10ng/mL of Interferon-γ, Tumor Necrosis Factor-α and Interlukin-1ß (Peprotech) in MSC culture medium. After 24 hours, the inflammatory culture medium was drawn off (and used for later experimentation where appropriate) and MSCs were ready for experimental procedures.

2.2.7 **Differentiation Assays**

MSCs were differentiated into adipocytes, chondrocytes and osteoclasts using the R&D Systems Stem Cell Kits. All Reagents and materials are listed in the manufacturer's handbook (page 2). Constituents of the differentiation supplements are listed below in Table 2-2.

Table 2-2 Differentiation supplements and their constituents

Differentiation Supplement	Constituents
Adipogenic Supplement	0.5mL of a 100X concentrated solution containing hydrocortisone, isobutylmethylxanthine and indomethacin in 95% ethanol.
Osteogenic Supplement	2.5mL of a 20X concentrated solution containing dexamathasone, ascorbate-phosphate and ß-glycerolphosphate.
Chondrogenic Supplement	0.5mL of a 100X concentrated solution containing dexamethasone, ascorbatephosphate, proline. Pyruvate and recombinant TGF-ß3

2.2.7.1 **Adipogenesis and osteogenesis**

For adipogenic and osteogenic differentiation, 13mm sterile coverslips (VWR) were inserted into the bottom of a 24 well plate. MSCs from different sources were plated at $2.1x10^4$ (adipogenesis) or $4.2X10^3$ (osteogenesis) on top of the coverslips within the 24 well plate. Cells were cultured in 0.5mL/well 90% α-

MEM, 10% (v/v) FBS 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 (Table 2-2) 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 (Table 2-2) added to α-MEM culture medium. From then on, in both cases, fresh medium including supplement was replaced every 3-4 days for 14-21 days. Cells were then fixed and stained as stated in Section 2.4.1.1.

2.2.7.2 **Chondrogenesis**

For the differentiation of MSCs into chondrocytes, $2.5x10^5$ MSCs were transferred into a 15mL conical tube in chondrogenic culture medium (99% D-mem/F12, 1% ITS supplement and 1% 100X Penicillin-streptomycin-Glutamine) and spun down at 200g for 5 mins at RT. Supernatant was poured off and MSCs were resuspended in chondrogenic culture medium and spun again at 200g for 5 mins. MSCs were left in the chondrogenic culture medium as a pellet and placed into the incubator with the 15mL conical tube lids slightly loosened for gas exchange. Chrondrogenic 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 (Section 2.4.2) and further staining (Section 2.4.1.1)

2.3 **Molecular Biology**

2.3.1 **RNA**

In an attempt to reduce degradation of RNA from environmental RNases, various measures were taken. All plastic and glassware was supplied RNase free or autoclaved. Additionally, sterile filtered tips were used, water was RNase/DNase free and bench tops and pipettes were sprayed with RNAzap before commencing work.

2.3.1.1 **Isolation of RNA from primary cells using silica-membrane technology**

RNA was isolated from cells using the RNeasy mini Kit protocol (QIAGEN). Cells were pelleted and lysed according to manufacturer's instructions using the appropriate amount of RLT buffer:

$\geq 1x10^6$ cells 350µl RLT

$\geq 5x10^6$ cells 600 µl RLT

A micro kit was used for anything below $1x10^6$ cells. The lysed cells were then homogenized using a QIAshredder spin column (QIAGEN). RNA was then extracted according to the Qiagen mini kit instructions. Unless specified otherwise, genomic DNA was digested as described in the protocol. RNA was eluted from the RNeasy spin column by adding 30µl of nuclease free water ((QIAGEN). RNA was quantified by using a nanodrop (Nanodrop 1000 Thermo Scientific) and stored at -80ºC in an eppendorf until needed.

2.3.2 **cDNA**

2.3.2.1 **cDNA synthesis - RT^2 PCR™ Profiling Arrays**

When preparing cDNA to be used on the RT^2 PCR™ profiling arrays (RT^2 Array), cDNA was synthesised using the RT^2 First Strand Kit (QIAGEN) (Kit used for all PCR reactions in results Chapter 3). Instructions in the RT^2 Array Handbook were followed for the synthesis of cDNA and in all cases, cDNA was synthesised to a final concentration of 400ng/µl (as advised by RT^2 Array Handbook). The appropriate amount of RNA, RNase-free water and 2µl of Buffer GE were mixed and incubated at 42ºC for 5 minutes and immediately placed on ice for at least 1 minute to eliminate genomic DNA. The reverse transcription mix was prepared according to RT^2 Array Handbook and incubated at 42ºC for exactly 15 minutes, followed by 95ºC for 5 minutes. 91µl of RNase-free water was added to each reaction and placed at -20 ºC until required for gene expression studies.

2.3.2.2 **cDNA synthesis for routine qRTPCR**

High capacity RNA to cDNA kit (Life Technologies) was used to synthesise cDNA for standard QRTPCR. In this case, for each experimental group, a minus reverse transcription control was included:

–RT control: 20x enzyme mix was substituted with nuclease free water.

cDNA was synthesised to a final concentration of 168ng/µl. 10µl of 2X Buffer Mix and 1.0µl RT enzyme mix and the appropriate amount of RNA and RNase-free water to bring the volume to 20µl, were mixed thoroughly and incubated at:

37^{o}C for 60 minutes

95^{o}C for 5 minutes

4^{o}C 'forever'

2.3.3 **Quantitative real-time PCR – QRT-PCR**

2.3.3.1 **RT^{2} Profiler™ PCR Arrays**

RT^{2} Arrays were used to assess the expression of chemokines and their receptors by MSCs and the results are presented in Chapter 3. RT^{2} Arrays Human Chemokines & receptors (Format E 384 well [4x96] HT option) were prepared and amplification carried out using a 7900HT (ABI) sequence detection system in accordance with manufacturer's guidelines. The RT^{2} Array consists of 84 genes in quadruplicate, including 5 house-keeping genes, genomic DNA contamination controls and internal controls which monitor PCR and reverse transcription efficiency.

Plate set-up was performed as recommended in the RT^{2} Array user's manual. In short, 102µl of cDNA synthesis reaction was added to 650µl of RT^{2} SYBR Green Mastermix and 548 µl of RNase-free water. Volumes were scaled up appropriately depending on sample size. 10µl of the PCR mastermix was added to each well of the RT^{2} Array. Once loaded, the plate was sealed using an optical adhesive film. The plate was centrifuged at 1000g for 1 min at RT to remove

bubbles and run on an Applied Biosystems 7900HT cycler. Cycling conditions were as follows: 1 cycle for 10 mins at 90°C followed by 40 cycles of 15s at 95°C/ 1min at 60°C.

2.3.3.2 **Standard qRT PCR**

cDNA was prepared using the high capacity RNA to cDNA kit (Section 2.3.2.2). Prior to use, it was diluted 1 in 5 and then added to a mastermix consisting of PerfeCTa SYBR Green FastMix with ROX reference dye (VWR-international) custom made primers (IDT) and nuclease free water. Details of volumes per well are listed below:

5µl SYBR Green

3.85µl nuclease free H_2O

0.15µl pre-mixed primer pair (Section 2.3.3.3)

The desired final volume was calculated and the above volumes adjusted accordingly to provide enough master mix for the reaction. Using a multi-channel pipette, 9µl of the master mix was dispensed into each well of the 384 well plate. Using a repeat dispenser pipette, 1µl of sample cDNA or –RT cDNA was dispensed into designated wells. For no-template control wells, cDNA was substituted with nuclease free water. The plate was then sealed using an optical adhesive film and then spun at 500g at 4°C to remove any air bubbles and run on an Applied Biosystem 7900HT thermal cycler. Cycling conditions were as described below:

(95°C for 10 minutes) X1 cycle

(95°C for 3 seconds then 60°C for 30 seconds) X40 cycles

To confirm the specificity of the QPCR primers a dissociation curve was generated using the following cycling conditions at the end of the QPCR run:

95°C 15 seconds

60°C 1 minute

2.3.3.3 **Primer Design**

To perform quantitative real-time PCR (QRTPCR), a pair of forward and reverse primers were designed to amplify each target gene. The design of these primers adhered to a set of strict criteria to ensure accurate amplification of a specific product.

All primers were designed using Primer3 Input software version 4.0.0 (http://primer3.ut.ee/).

Criteria for QPCR primers:

Primer size:	18 to 24 base pairs (bp) (20bp optimal)
Melting temperature (Tm):	59.5°C to 61°C (60°C optimal)
GC content:	40% to 65% (50% optimal)
Max self-complementarity:	3 (<2 optimal)
Max 3' complementarity:	1
Amplicon Size:	<150bp

All primers were synthesised by Integrated DNA Technologies (IDT). The specificity of the primer sequences was first assessed using the free online bioinformatics resource, Basic Local Alignment Search Tool (BLAST) (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer specificity was then physically tested by running a PCR reaction with cDNA containing the transcript of interest. Specificity was confirmed if the primers amplified a single product of the appropriate size. Primers used throughout this study (Results Chapter 6) are detailed in Table 2-3. Where possible, to avoid amplification of any potential genomic DNA contamination, primers were designed so that they spanned exons. Exon spanning primers are marked with an asterisk.

Table 2-3 List of left and right primer sequences

Gene Name	Left Primer	Right Primer
IDO*	GCAAGAACGGGACACTTTGC	TGCCTTTCCAGCCAGACAAA
CFH*	GGAAGGGAGAATGGGTTGCT	GATGTCCACAGGGCCTTTTCT
CD274	GCCTCCACTCAATGCCTCAA	CTGTCCCGTTCCAACACTGA
HGF*	TTGCCTGAAAGATATCCCGACAA	CGGGTGTGAGGGTCAAGAG
TGF-beta*	AAGTGGACATCAACGGGTTCA	GGGTGGCCATGAGAAGCA
IL-10*	TGCTGGAGGACTTTAAGGGTTAC	CGCCTTGATGTCTGGGTCTT
MMP9*	GCCCCAGCGAGAGACTCTA	ATTGGCCTTGGAAGATGAATGGA
GMCSF	ACTTCCTGTGCAACCCAGATT	CCAGCAGTCAAAGGGGATGA
TSG-6*	AGCACGGTCTGGCAAATACA	GCAGCACAGACATGAAATCCAAT
B2M	GCTCGCGCTACTCTCTCTTT	TGAATCTTTGGAGTACGCTGGAT
RPLP0*	GGAAGGCTGTGGTGCTGAT	CGGATATGAGGCAGCAGTTTCT

2.3.3.4 **Standardisation of RT^2 Profiler™ PCR Arrays**

As all of the samples could not fit on one plate, inter-plate variability had to be controlled for. For this, one sample (induced pluripotent stem (IPSC) cells, differentiated into MSCs (IPSMSC)) was split over three different plates. cDNA synthesis, plate set up and plate reading were carried out on different days to replicate the typical experimental time course. The IPSMSC samples Ct values from all of the genes on each plate were compared to assess their correlation. The Pearson correlation coefficient was used to assess the relationships between each plate. The Pearson correlation runs from -1 (the two variables are in perfect opposites), through 0 (no correlation of the values at all) to 1 (indicates the two values are perfectly correlated).

2.3.3.5 **Analysis of results (RT^2 Profiler™ PCR Arrays)**

Data analysis was performed using QIAGEN's GeneGlobe data analysis centre (http://www.qiagen.com/gb/shop/genes-and-pathways/data-analysis-center-overview-page/). The reference gene *Beta-2 Microglobulin (B2M)* was chosen for normalisation. Any gene with a CT value of >35 was determined as undetectable as advised by QIAGEN's GeneGlobe data analysis centre. For each sample, the Ct values generated from duplicate wells were averaged and data were presented as $2^{(-\Delta CT)}$ and calculated as below.

ΔCT = Ct (sequence of interest) – Ct (reference sequence- B2M).

Each bar represents an n of 3 and is plotted as mean ± SEM. The appropriate statistical analysis was used and is stated in the figure legends.

2.3.3.6 **Analysis of results – standard QRT-PRC**

Data analysis was performed in a similar manner to the RT^2 Profiler™ PCR Arrays. In this case, the reference gene used to calculate $2^{(-\Delta CT)}$ was Ribosomal Protein Lateral Stalk P0 (RPLP0). For each sample, the Ct values generated from triplicate wells were averaged. Each bar represents an n of 3 and is plotted as mean ± SEM. The appropriate statistical analysis was used and is stated throughout the figure legends.

2.4 Protein Analysis

2.4.1 Immunohistochemistry

2.4.1.1 **Immunohistochemistry for adipocytes, osteocytes and MSC ACKR4 expression**

MSCs that had undergone adipogenic and osteogenic differentiation were grown and treated as described in Section 2.2.7.1. MSCs being tested for ACKR4 expression were grown on a 2-well Nunc™ Lab-Tek™ chamber slide system in 1mL of MSC culture medium. When cells reached 80% confluency, MSC cluture medium was aspirated and MSCs were washed thoroughly with warmed PBS. The walls of the chamber slide were removed, leaving a flat microscope slide. A wax pen (Sigma) was used to create a hydrophobic barrier around MSCs for further staining. The following staining procedure was adopted for adipogenic, osteogenic and ACKR4 immunohistochemistry:

Cells were washed twice with 1mL of PBS and cells were then fixed with 0.5mL of 4% paraformaldehyde in PBS for 20 mins at RT. Cells were washed three times with 0.5mL of 1% bovine serum albumin (BSA) (Thermo Fisher Scientific) in PBS for roughly 5 mins each wash. The cells were permeabilized and blocked with 0.5mL 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 300µl/well of the appropriate primary antibody (Table 2-4). Cells were left at 4^{o}C overnight. Cells were washed three times with 0.5mL of 1% BSA in PBS for

5 mins each wash. Cells were then incubated with a diluted appropriate secondary antibody (Table 2-4) for 60 mins, in the dark, at RT. Cells were washed three times with 0.5mL of 1% BSA in PBS for 5 mins each wash. PBS was aspirated from the wells and replaced with 0.5mL distilled water. Coverslips were carefully removed with forceps and placed cell side down onto a drop of mounting medium (Vectashield + DAPI (Vector Labratories Inc) on a superfrost glass slide (Thermo Scientific). All slides were imaged with a Zeiss epifluorescent microscope using the appropriate fluorescent channels and magnifications. Images were prepared using Zen software.

2.4.1.2 **Immunohistochemistry for chondrocytes**

The pellet of cells was washed twice with 1 mL of PBS, then fixed with 0.3mL of 4% paraformaldehyde in PBS for 20 mins at RT. The pellet was then washed twice with 1mL PBS for 5 mins. The pellet was carefully removed and placed into a cryomold. Cryosectioning was carried out as detailed in Section 2.4.2. Using a liquid barrier pen, a hydrophobic barrier was drawn around each section and cells were then blocked and permeabilised as described before. After blocking, sections were incubated with the Goat Anti-human Aggrecan (R&D) working solution overnight at $4^{o}C$ in a container with adequate moisture. 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 2-4 Antibodies used in immunohistochemistry). Sections were washed three times with PBS containing 1% BSA for 5 mins. Sections were then washed once with distilled water. Excess water was removed and a drop of Vectashield + DAPI was added to each section before placing a coverslip on top of sections, being careful to avoid air bubbles. All slides were imaged using a Zeiss epifluorescent microscope using the appropriate fluorescent channels and specified magnifications. Images were prepared using Zen software.

Table 2-4 Antibodies used in immunohistochemistry

Experiment	Primary	Secondary	Company
Differentiation			
Adipogenesis	10µg/mL Goat Anti-mouse Fatty Acid Binding Protein 4 (FABP4)	Northern Lights 557-conjugated Anti-goat. **Dilution- 1:200**	Primary Ab: R&D Systems Secondary Ab: R&D Systems
Osteogenesis	10µg/mL Mouse Anti-human Osteocalcin	Northern Lights 557-conjugated donkey anti-mouse **Dilution- 1:200**	Primary Ab: R&D Systems Secondary Ab: R&D Systems
Chondrogenesis	10µg/mL Goat Anti-human Aggrecan	Northern Lights 557-conjugated Donkey Anti-goat **Dilution- 1:200**	Primary Ab: R&D Systems Secondary Ab: R&D Systems
Receptor Staining			
ACKR4	10µg/mL Mouse Anti-human CCX-CKR	Northern Lights 557-conjugated donkey anti-mouse **Dilution- 1:200**	Primary Ab: BioLegend Secondary Ab: R&D Systems

2.4.2 **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^{o}C freezer until sectioning. Frozen moulds were placed into the cryostat (Bright Instruments) at -25^{o}C and sectioned 8µm thick onto superfrost glass slides.

2.4.3 **Flow cytometry**

2.4.3.1 **Flow cytometry staining**

For every experiment, the same number of cells were added (~1x10^6 cells) to polystyrene tubes (BD Falcon) and washed with FACS buffer (Table 2-1). The Cells were then incubated at 4^{o}C with 5µl FcR block, to reduce non-specific binding via Fc receptors. The cells were then washed twice with FACS buffer and incubated for 20 minutes with the appropriate antibody. The cells were washed

with FACS buffer again and data were acquired using the MACSQuant flow cytometer, LSR II (BD Sciences) or the FACSAria III (BD Sciences) and analysed using MACSQuantify version 2.6 or FlowJo version 10 software. All antibodies used for Flow Cytometry are listed in Table 2-5.

2.4.3.2 **Mesenchymal stromal cell phenotyping**

At Passage 3, MSCs were grown until they were 80% confluent, detached from the flasks as discussed previously and prepared for flow cytometry (as detailed in Section 2.4.3.1) using the "MSC phenotyping" panel of antibodies listed in Table 2-5 and run on the MACSQuant.

2.4.3.3 **Mesenchymal stromal cell phenotyping in inflammation**

Once MSC had reached 80% confluence at passage 2, MSCs -derived from one donor- were detached and re-seeded into two T75 flasks (Passage 3). When MSCs reached 80% confluency, culture medium was removed and cells were washed with warmed PBS. For each MSC donor, one T75 flask was stimulated with 10ng/mL of TNF-α, IL-1ß and IFN-ϒ in 20 mL of MSC medium for 24 hours, whilst the second T75 flask from each MSC donor remained in 'homeostatic conditions' through the addition of 20 mL of MSC medium alone for 24 hours. After 24 hours, MSC conditioned medium was removed and MSCs were detached and prepared for flow cytometry (as stated in Section 2.4.3.1) using the "MSC phenotyping in inflammation" panel of antibodies listed in Table 2-5. Samples were run on the MACSQuant.

2.4.3.4 **Mesenchymal stromal cell surface chemokine receptor expression**

MSCs were grown and treated as detailed in Section 2.4.3.3. MSCs were prepared for flow cytometry as described in Section 2.4.3.1 and MSCs were stained with each antibody listed in the "Chemokine receptor expression" section of Table 2-5. Samples were run on the MACSQuant.

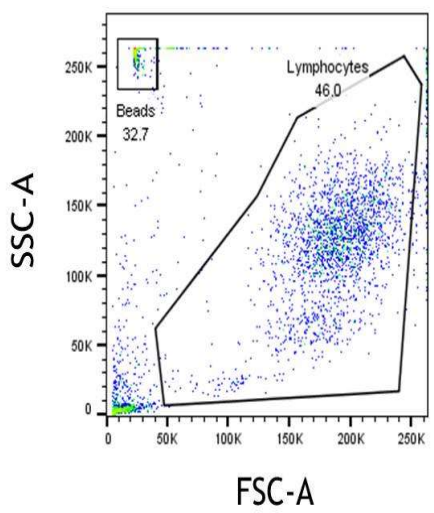
Table 2-5 List of antibodies used for flow cytometry

	Antigen	Flurochrome	Clone	Dilution	Company
Phenotyping MSCs	CD105	FITC	266	1:200	BD Biosciences
	CD90	Pe Vio770	DG3	1:200	Miltenyi
	CD73	PE	AD2	1:200	Miltenti
	CD14	APC	TÜK4	1:200	Miltenti
	CD45	VioBlue	REA747	1:200	Miltenti
	Fixable Viability Dye	eFluor 780		1:200	eBioscience
MSC phenotyping in Inflammation	HLA-DR	FITC	Tu39	1:200	BD Biosciences
	HLA ABC	PeVio770	REA230	1:200	Miltenyi
	CD146	VioBlue	541-10B2	1:200	Miltenyi
	CD166	PE	3A6	1:200	BD Biosciences
	CD271	APC	ME20.4-1H4	1:200	Miltenyi
	CD73	Percp710		1:200	
	Fixable Viability Dye	eFluor 780		1:200	eBioscience
Chemokine Receptor Expression	CCR10	PE	6588-5	1:200	Biolegend
	CCR7	PE	G043H7	1:200	Biolegend
	CXCR4	PE	12G5	1:200	Biolegend
	CXCR6	PE	K041ES	1:200	Biolegend
	ACKR3	APC	358426	1:200	R&D Systems
	DRAQ7	APC CY7		1:200	Biostatus
Transwell Panel	CD16	PercPCy 5.5	3G8	1:200	Biolegend
	CD56	APC Cy 7	HCD56	1:200	Biolegend
	HLA-DR	AF700	LN3	1:200	Biolegend
	CD1c	Biotin	AD5-8E7	1:200	Miltenyi
		SA 605		1:200	Biolegend
	Siglec 8	PE	7C9	1:200	Miltenyi
	CD4	FITC	VIT4	1:200	Miltenyi
	CD 8	FITC	BW135/80	1:200	Miltenyi
	CD14	VioBlue	TÜK4	1:200	Miltenyi
	CD19	Pe Vio770	LT19	1:200	Miltenyi
	CD66b	APC	REA306	1:200	Miltenyi
	Fixable Viability Dye	eFluor 506		1:200	eBioscience
Adherent Panel (Transwell)	CD16	PercPCy 5.5	3G8	1:200	Biolegend
	CD14	VioBlue	TÜK4	1:200	Miltenyi
	CD4	PeVio770	REA623	1:200	Miltenyi
	CD8	FITC	BW135/80	1:200	Miltenyi
	CD45	PE	5B1	1:200	Miltenyi

	DRAQ7	APC Cy7		1:200	Biostatus
NSG Mouse Model (Mouse Antibodies)	CD11c	APC	NF18	1:200	Biolegend
	F480	PeCy7	BM8	1:200	eBioscience
	Siglec F	PE	E50-2440	1:200	BD Biosciences
	CD11b	APC Cy7	M1/70	1:200	eBioscience
	Ly6c	AF700	HK1.4	1:200	Biolegend
	Ly6g	Pacific Blue	1A8	1:200	Biolegend
	CD105 (HUMAN)	FITC	266	1:200	BD Biosciences
	CD45	PercP	A20	1:200	Biolegend
	Fixable Viability Dye	eFluor 506		1:200	eBioscience
OT-1 Mouse Model (Mouse Antibodies)	CD4	APC	GK1.5	1:200	eBioscience
	CD8 alpha	PE	53-6.7	1:200	Biolegend
	B220	APC Cy7	RA3-6B2	1:200	Biolegend
	CD45	PercP	A20	1:200	eBioscience
	CD105	FITC	266	1:200	BD Biosciences
	NK1.1	BV421	PK136	1:200	eBioscience

2.4.3.5 Cell counting using CountBright™ Absolute Counting Beads for flow cytometry

When samples were acquired using the LSR II or the FACSAria III, CountBright™ absolute counting beads were used to obtain accurate cell numbers in each sample. CountBright™ absolute counting beads are a calibrated suspension of microshperes which are fluorescent in every channel (Excitation: UV to 635nm, Emission: 385-800nm). Beads were thoroughly vortexed and 50µl were added to each sample immediately before acquisition. How to calculate the number of cells in a sample is detailed in Figure 2-2 Identification of CountBright BeadsTM on the flow cytometer and subsequent cell counting analysis



Calculation of Cell concentration:

$$\frac{A}{B} \times \frac{C}{D} = \text{Concentration of sample as cells/µl}$$

A= No. of cell events
B= No. of bead events
C= assigned bead count of the lot (beads/50µl)
D= volume of sample (µL)

Figure 2-2 Identification of CountBright BeadsTM on the flow cytometer and subsequent cell counting analysis
CountBright beads are roughly 5-50 times brighter than the anticipated intensities of typically stained cells and fluoresce in every channel. For each experiment, the channels used to identify beads varied in order to isolate a clean, total bead population. Using the FSC and SSC parameters as an example, beads were easily identifiable (top left gate). Beads were gated to generate the number of bead events. This value, along with the assigned bead number of the lot ($4.9x10^4$/50µl), number of cell events and the volume of each sample was used to calculate the total number of cells in each sample.

2.4.3.6 **Flow cytometry analysis**

Irrespective of the flow cytometry software used for analysis, a similar approach was taken to identify live cells. When acquiring samples, FSC and SSC voltages were set to a suitable value to ensure all of the appropriate events were included for analysis. When analysing flow cytometry data, cells of interest were gated on using FSC and SSC, doublets were excluded using SSC-A and SSC-H or FSC-A and FSC-H, and live cells were selected based on lack of fluorescence of dead cell markers. This gating strategy was used prior to any further analysis for all flow cytometry experiments (Figure 2-3).

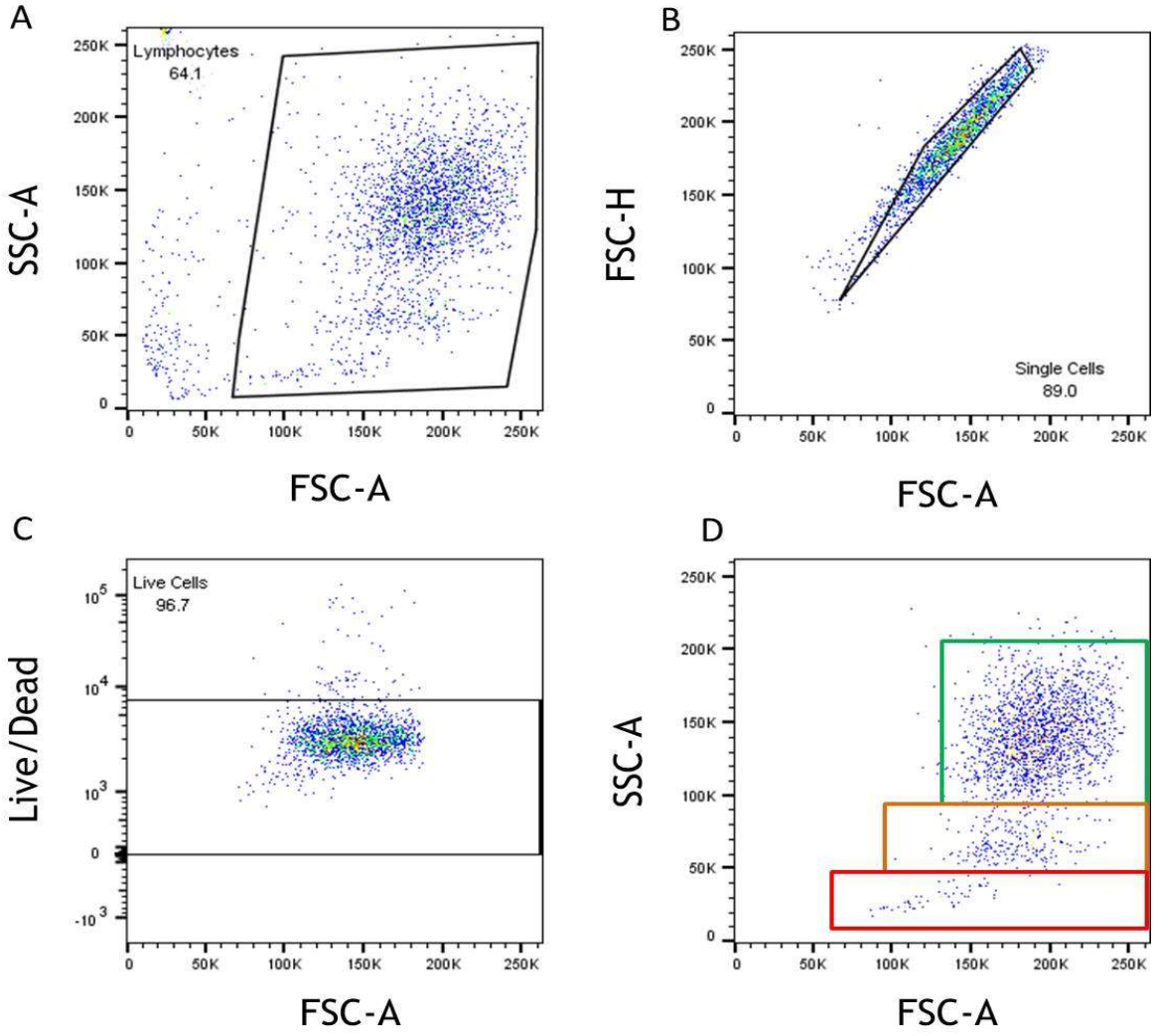


Figure 2-3 Initial steps in flow cytometry gating strategies
Initial steps in flow cytometry gating strategies were consistent for each sample of every experiment in order to identify live cells. First steps included gating on cells of correct size and granularity (A), followed by doublet discrimination, to ensure cells were not clumped together (B). Cells negative for live/dead marker were indicative of intact, live cells (C) and were gated on for further analysis. In this example, granulocytes (green box), monocytes (orange box) and leukocytes (red box) are all live (D) and suitable for further analysis.

2.4.3.7 **Fluorescent minus one controls**

Fluorescence minus one (FMO) controls were used to accurately assess and gate on positive staining (Figure 2-4). In every antibody panel, an FMO was made for each fluorophore. The missing fluorophore in each FMO sample would be used to assess any spill-over of fluorescence from other channels, whilst also serving as a negative control, allowing clear visualisation of positive staining.

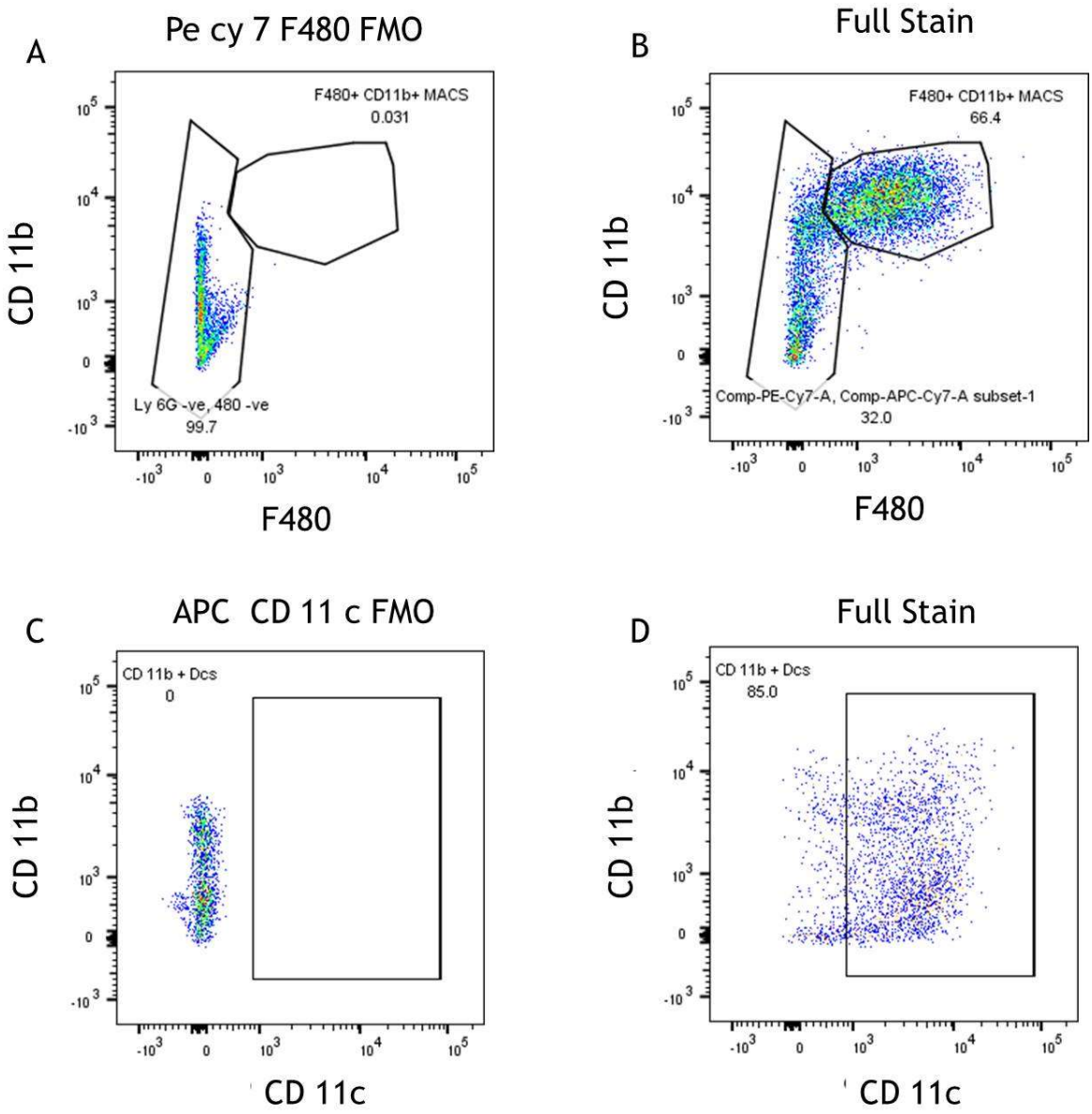


Figure 2-4 Using FMOs to accurately analyse flow cytometry data
Fluorescence minus one controls are designed to identify spectral overlap into the channel of interest. The control is performed by adding all of the fluorochromes in your panel with the exception of one. When the data are displayed, the channel of interest – Pecy7 (A) and APC (C) will show you all spill-over from other channels. This allows one to draw a gate around true positive staining for Pecy7 (B) and APC (D) in fully stained samples.

2.4.4 **Luminex**

Conditioned media from the samples used for transcript work were collected for luminex analysis. The human personalised premixed magnetic multi-analyte kits were 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 2 fold with calibrator diluent (75µl in 75µl). 10 µl of the pre-coated microparticle cocktail was added to each well of

the 96 well microplates, followed by either 50µl sample or 50µl standard, sealed and placed on an orbital shaker (0.12mm orbit at 800 ± 50rpm) for 2 hours at RT. 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 (0.12mm orbit at 800 ± 50rpm). The plates were washed as before and 50 µl/well of streptavidin-phycoerythrin was added and incubated for 30 mins at RT. Microparticles were resuspended in 100 µl/well of wash buffer and immediately read on the Bio-Rad analyser.

2.4.4.1 **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. Background protein levels from serum in medium were recorded and served as background controls (Chapter 5). Each bar represents an n of 3 and is plotted as mean ± SEM. Statistical tests included a One Way ANOVA in conjunction with a Tukey's compare all comparisons test when comparing across MSCs from different sources. A Students T test was used when comparing stimulated vs unstimulated within one tissue source. Significance was marked where appropriate and detailed throughout the text in Chapter 5.

2.5 In Vitro Transwell Migration Assays

MSCs were seeded at 3000cells/cm^2 and grown as a monolayer on the bottom of 5µm-pore transwell plates (Fisher Scientific). When MSCs were 80% confluent the appropriate wells were stimulated with 10ng/mL of the cytokines detailed in Section 2.2.6 or left in MSC medium alone (600µl). Medium with inflammatory cytokines or medium alone was also added to control wells (no MSCs). After 24 hours, all wells were washed twice with PBS, then 600µl of fresh MSC medium was added to all wells and left for a further 24 hours. 5µm-pore inserts were placed into the wells on top of the MSCs, ensuring there was no air bubbles

underneath them. Inserts were left to incubate with MSCs alone for 10 mins before the addition of leukocytes. Leukocytes were isolated as detailed in Section 2.5.1. $5.5x10^5$ leukocytes in 100µl of MSC medium was placed on top of the insert. The transwell plate was put in the incubator at 37°C for 3 hours. After 3 hours, the inserts were carefully removed and discarded. The supernatant in the bottom wells was placed into appropriately labelled 15mL Falcon tubes. The wells were then thoroughly washed with PBS and placed into the same respective Falcon tubes. Adherent cells, attached to the bottom of the transwell were detached from the bottom of the wells with 1x TryplE as previously described and placed into different 15 mL falcon tubes. All cells were spun at 300g for 5 mins and transferred to FACS tubes for staining (Section 2.4.3.1). Cells that were collected in the supernatant were labelled with the "transwell" panel detailed in Table 2-5 and run on the LSR II, and adherent cells were labelled with the "adherent" panel and run on the MACSQuant. 50µl of countbright beads (Section 2.4.3.5) were added to each sample immediately before flow cytometric analysis in order to gain accurate cell numbers.

2.5.1 **Isolation of white blood cells from whole blood**

Buffy coats were obtained from SNBTS under sample governance no. 6839/15, and used as a source of blood cells. To isolate leukocytes, 5-10mLs of buffy coat was added to a 50mL Falcon tube and spun at 300g for 20 mins. The top layer of plasma was pipetted off and discarded. To lyse the red blood cells (RBCs) 5mL of Red Blood Cell lysis buffer (RBClysB) (Miltenyi Biotech) was diluted with 45mL of double distilled H_20 (ddH_20). 45mLs of diluted RBClysB was added to the remaining layers of blood, vortexed and left at RT for 12 minutes, then spun at 300g for 10 minutes. The supernatant was carefully poured off and any residue pipetted off. RBC lysis was repeated by adding 5mL RBClysB and incubated for 5 minutes, followed by a 5 minute spin at 300g. The supernatant was discarded as before. White blood cells (WBCs) were washed with 10mL PBS and spun at 300g for 5 minutes (X2). The supernatant was discarded and WBCs were resuspended in 10mLs of PBS for counting. Typically, 5mL of whole blood yielded $1.5\text{-}2.5x10^8$ WBCs.

2.6 *In Vivo* procedures – murine air pouch model

2.6.1 Animal welfare

All animals were housed within the Biological Central Research Facility and the Beatson Institute at the University of Glasgow. Animals were maintained in specific pathogen-free conditions within filter-top cages and given access to food and water *ad libitum*. All experiments received ethical approval and were performed under the auspices of a UK Home Office License.

2.6.2 Mice

2.6.2.1 NOD *Scid* gamma (NSG) mice

Genotype of mouse: NOD.Og-Prkdc^{scid} Il2rg$^{tm1Wjl/}$SzJ gamma (NSG).

This mouse has defects in both the innate and adaptive compartments of the immune system. Defects in antigen presentation, NK cell function and macrophage cytokine production, C5 complement and wound healing, coupled and IL2 receptor gamma chain deficiency which disables cytokine signalling resulting in a lack of mature T cells and B cells.

Breeding pairs were obtained from Charles River Europe and the colony was maintained in house. 14-16 week old female mice were used for the air pouch model. Before experimental procedures were carried out, mice were given 7 days to adjust and settle after being moved from the Beatson Institute to the Biological Research Facility. After experimental procedures, mice were sacrificed using a recognised Schedule 1 technique. All procedures on animals were carried out by Dr Kenny Pallas and Paul Burgoyne.

2.6.2.2 OT-1 Mice

Genoytpe of Mouse: OT-1.

The T cell receptor of these mice have been genetically modified to only recognise the ovalbumin peptide in the context of MHC-1 (CD8 T cell restricted). 14 week old male C56BL/6 mice were bred and maintained in house. After

experimental procedures, mice were culled using a recognised scheduel 1 technique.

2.6.2.3 **Induction and maintenance of the air pouch model**

One day prior to experimental procedures, the dorsal skins of mice were shaved in preparation for the generation of the air pouches. All procedures for NSG mice were performed in a laminar flow hood. All mice were put under general anaesthesia before and during the procedure. To ensure the air injected into the air pouch was sterile, the syringes were pre-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 1mL sterile air was injected 2 days later, followed by an injection of $5.5x10^5$ pre-stimulated Islet MSCs (treated animals) in 1 mL of sterile PBS or sterile PBS alone (control animals) one day later. Cells or PBS controls were left in the air pouch for 24 hours before mice were sacrificed. Detailed timeline in Chapter 6 Figure 6-1.

2.6.2.4 **Dissection and draining of the air pouch**

Immediately after sacrifice, 1.5mL of PBS was injected into the hollow air pouches of the mice. In an attempt to obtain an optimal number of immune cells from the air pouch, mice were gently shaken to allow the PBS to mix throughout the air pouch.

To separate the overlying soft tissues from the upper air pouch membrane (Figure 2-5), a small incision was made into the dorsal skin overlying the air pouch to reveal the apex of the upper membrane. The membrane was punctured with a needle and the pouch content was lavaged, transferred to an eppendorf tube and placed on ice (the contents of the lavage are referred to as “air pouch samples” throughout Chapter 6). Using blunt dissection with curved scissors, the upper membrane was separated from the overlying skin. The air pouch collapsed and the upper membrane was clipped off using scissors (the contents of this membrane are referred to as the “upper membrane” throughout Chapter 6). A thin sample of the lower membrane was also clipped off, avoiding blood vessels and the spinal cord (the contents of this membrane are referred to as the “lower

membrane" throughout Chapter 6). The upper and lower membranes were placed into 1mL of Hank's balanced salt solution (Sigma-Aldrich) and put on ice.

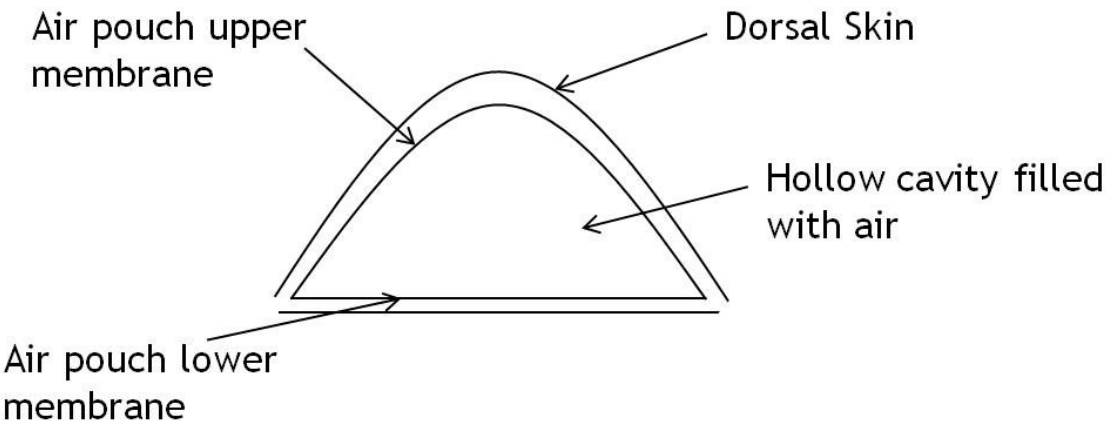


Figure 2-5 Simplistic diagram of a cross-section of the air pouch
Sterile air was injected subcutaneously into the intracapsular area of the mouse creating a hollow pocket of air surrounded by a membrane. The membrane directly underlying the dorsal skin but overlying the hollow pocket of air was termed the upper membrane and the membrane underlying the hollow pocket of air and overlying the spinal cord was termed the lower membrane.

2.6.2.5 **Digestion of the upper and lower membranes**

To digest the upper and lower membranes of the air pouch, 35µl of liberase (Sigma) (stock conc = 2.5mg/ml) was added to each membrane sample and incubated at 37^{o}C for 1 hour on a thermo-shaker incubator (800 rpm) (Thermo Shaker Incubator MS-100). Once membranes had fully digested into a single cell suspension, cells were passed through a 40µm sterile cell strainer (Corning) to remove any remaining debris. Cells were washed twice with 1mL of PBS and counted. The remaining portion of the cells was prepared for flow cytometry as detailed in Section 2.6.2.6 and 2.6.2.7.

2.6.2.6 **Staining for flow cytometry – NSG mice**

Lavaged contents from the NSG air pouches were washed 2X with PBS. Cells were counted and an aliquot of 2x10^{4} cells was put in an eppendorf tube for cyto-spin analysis (Section 2.6.2.8). Flow cytometry staining is detailed in Section 2.4.3.1, using the "NSG innate" antibody panel in Table 2-5 and samples were run on the Aria III.

2.6.2.7 **Staining for flow cytometry – OT-1 mice**

Lavaged contents from the OT-1 air pouch were washed twice with PBS. Cells were counted and an aliquot of ~2x10^4 cells was placed in an eppendorf tube for cyto-spin analysis (Section 2.6.2.8). For flow cytometry analysis, the remaining samples were split over two polystyrene tubes in order to stain with two antibody panels- "OT-1 innate" and "OT-1 adaptive" panels (Table 2-5). The staining process is detailed in Section 2.4.3.1 and samples were run on the Aria III.

2.6.2.8 **Cytospins**

~20x10^4 cells were prepared in 100µl of PBS. Superfrost slides, filter paper, funnels and holders were prepared and assembled as detailed in Figure 2-6 Preparation of slides for cytospins.. Each 100µl sample was loaded into an individual funnel which was anchored to an appropriately labelled slide and loaded into Shandon cytospin 2 and spun at 300rpm for 3 mins. Samples were left to dry overnight in preparation for histological staining.

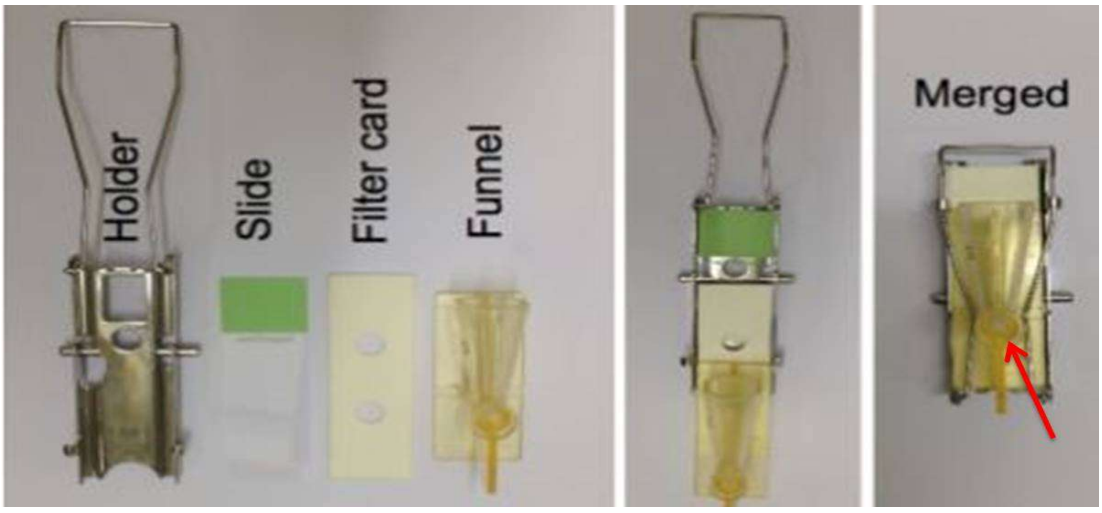


Figure 2-6 Preparation of slides for cytospins.
Appropriately labelled super frost slides were loaded into the holder with filter card and a funnel on top. The holder was clipped together to hold everything in position and 20x10^4 cells in 100µl of PBS were loaded into the top of the funnel (red arrow). Picture modified from: http://bio-protocol.org/e1303.

2.6.2.9 **Histology**

Slides that were prepared from cyto-spins were histologically assessed following Giemsa staining. Giemsa (Sigma) was diluted 1 in 10 with deionised water and poured into a glass staining jar. Slides were carefully placed into the jar

containing diluted Giemsa for 30 seconds. After 30 seconds, the staining jar and slides were transferred to a sink where tap water was added to the jar until the purple Giemsa ran colourless. Slides were left overnight to dry in a fume hood. Once dried, coverslips were placed on top of the slides using one drop of DPX mounting media (Sigma), making sure to avoid air bubbles. Slides were left to dry overnight and images were captured using a Zeiss epifluorescent microscope – bright field.

Chapter 3

Phenotyping MSCs

Chapter 3 Phenotyping MSCs

3.1 Introduction and aims

Mesenchymal Stromal Cells (MSCs) are broadly categorised as a cell type which is capable of differentiating into a variety of cells and immunosuppressing/immunomodulating the immune system. More recently however, it is becoming apparent that their phenotype and biological activity can differ depending on where MSCs are isolated from. Differences in surface marker expression (CD49d, CD54, CD34 and CD106) and proliferative capacity have been observed in bone marrow (BM) versus adipose (Ad) MSCs (182). Comparatively, variation in adipogenic differentiation potential has been linked to tissue specificity of MSCs, with umbilical cord (UC) MSCs showing little or no adipogenic differentiation compared to Ad and BM isolated MSCs (183). Moreover, MSC tissue source also has an effect on their immunomodulatory and immunosuppressive capacity, for example, Ad MSCs have been shown to have greater immunosuppressive capabilities than BM and UC derived MSCs (184) where their immunosuppressive capacity was measured by their ability to inhibit the activation and proliferation of T cells. Conversely, a separate study demonstrated that BM MSCs have greater immunosuppressive activity with regards to the activation of T cells than Ad MSCs (185).

Discrepancies like the aforementioned are not uncommon within the MSC literature and are often due to a lack of standardised approaches in this field, resulting in differences in; isolation procedure, variations in culture medium and different passage number of MSCs (186-189). As a consequence of these variations between studies, it is becoming increasingly more challenging to compare results across the literature and therefore the International Society of Cellular Therapy (ISCT) has suggested minimal criteria to define a MSC. These criteria include adherence to plastic whilst maintained in standard culture conditions and surface expression of CD105, CD73, CD90 whilst lacking expression of CD45, CD14, CD34 or CD19 and HLA-DR. Finally, they must be able to differentiate into two out of three of adipocytes, chondrocytes and osteocytes (95).

Part of this study aimed to objectively compare cells isolated from different tissues using identical isolation methods and assays. Therefore, this chapter will focus on the phenotype of Islet (Is), Visceral adipose (attached to the pancreas at donation) (Va), (liposuction) adipose (Ad), BM and UC MSC morphology, surface molecule phenotype and differentiation potential using standardised assays. Additionally, as MSCs are generally used in an inflammatory setting, their morphology and surface phenotype was assessed after 24 hours of inflammatory stimulation – 10ng/mL of IFN-γ, TNF-α and IL-1ß. Thus, this chapter aims to assess the cells isolated from Is, Va, Ad, BM and UC tissues for standard MSC functions and to understand the effects of tissue origin and inflammation on their phenotype.

The study was begun by comparing Is, Va and Ad MSCs. BM and UC MSCs became available and were added to the data sets during the study. These latter two cell types are thus added to comparative data sets where available. 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

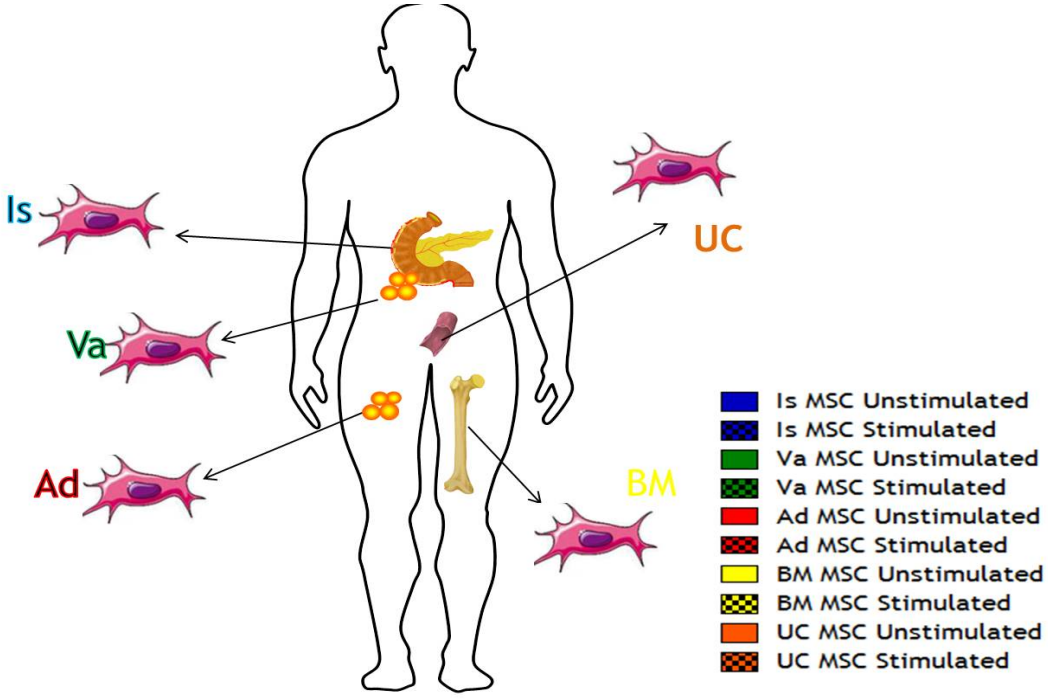


Figure 3-1 Schematic highlighting the colour scheme used in all figures throughout this study
The above diagram illustrates the colour scheme throughout this thesis, linking a specific tissue source of MSC to a particular colour. When data are graphed, Is MSCs under homeostatic conditions will be graphed as a blue bar, and when they have been under inflammatory conditions, they will be graphed as a blue bar with black checks. This pattern is used for all tissue sources of MSCs with Va being graphed in green bars, Ad MSCs in red, BM MSCs in yellow and UC MSCs in orange.

Results

3.2 Physical morphology of MSCs

To ensure that MSCs isolated from the Is, Va, Ad, BM and UC possess and maintain the typical spindle-like morphology of MSCs through passage (P), cells were seeded at P0 and grown up to P8, and their morphology was monitored by phase contrast light microscopy. Within this study, a passage is defined as seeding MSCs at 3000 cells/cm^3, letting them grow until they reach 80% confluence, detaching them and reseeding into new flasks - this would be classified as one passage. Upon seeding, cells isolated from all sources were left to attach for 5-7 days during which time small spherical cells transformed into plastic adherent small spindle-shaped cells. At P1, small spindle-shaped cells had enlarged slightly into fibroblast-like spindle-shaped cells. From here, their morphology remained consistent through passage (Figure 3-2). Cells from all sources were similar in size and shape. This demonstrates that in routine culture conditions and standard culture medium, cells isolated from all tissue sources exhibited an MSC-like morphology which was maintained through passage. This satisfies one of the ISCT criteria of MSCs being plastic-adherent, spindle-shaped cells in standard culture conditions.

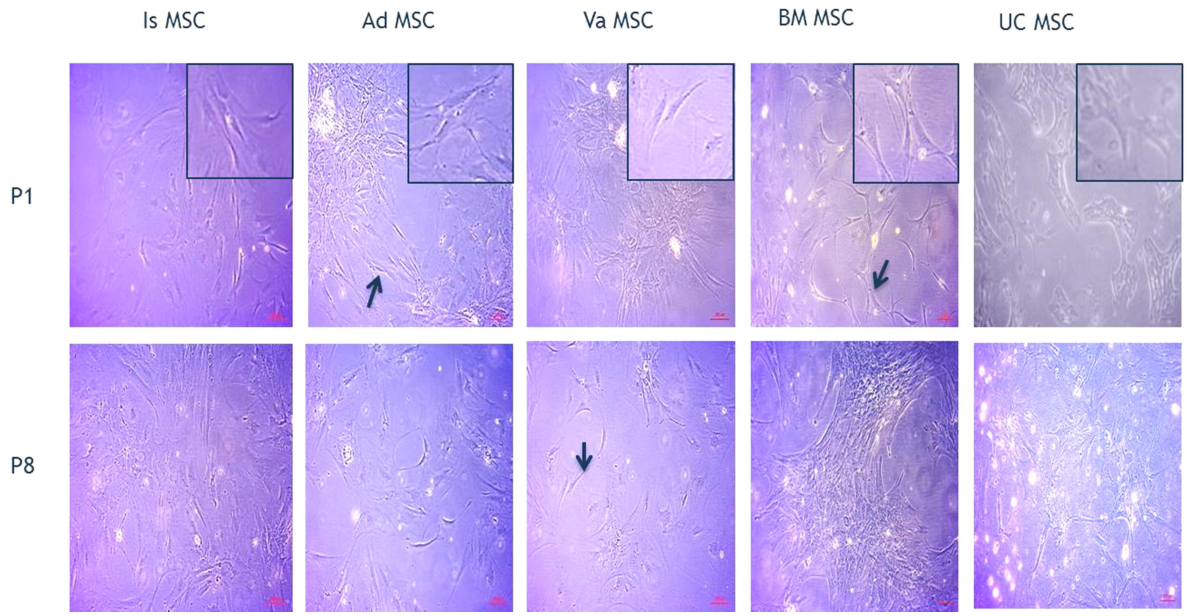


Figure 3-2 MSCs isolated from all tissue sources exhibit an MSC spindle-like morphology, which is maintained through passage.
MSCs were seeded at P0 and grown until P8. Their morphology was observed and monitored using a phase-light microscope. All MSCs were adherent to plastic and possessed the typical spindle-like morphology. Black arrow heads point to typical spindle shaped cells and thumbnail images show magnified, individual MSCs. Spidle-like morphology and adherence to plastic was not affected through prolonged passage (P1-8).

3.3 Surface Phenotype of MSCs

To establish that cells isolated from each tissue source express surface markers indicative of MSCs according to ISCT criteria, flow cytometry was used to assess their surface expression of MSC markers at P3. A cocktail of markers typically used to asses MSC phenotype was used and included; CD90, CD105, CD73, CD166, CD14 and CD45. Results show that all MSCs were negative for hematopoietic markers CD45 and CD14 (Figure 3-3 B.i, C.i, D.i, E.i & F.i) and positive for MSC markers CD90, CD105 (B.ii, C.ii, D.ii, E.ii &,F.ii), CD73 (B.iii, C.iii, D.iii, E.iii & F.iii) and CD166 (B.iii, C.iii & D.iii) (Figure 3-3 B-F).

To assess whether the MSC tissue source had an effect on MSC marker expression, the percentage of live cells positive for each marker is graphed (Figure 3-3 G-L).

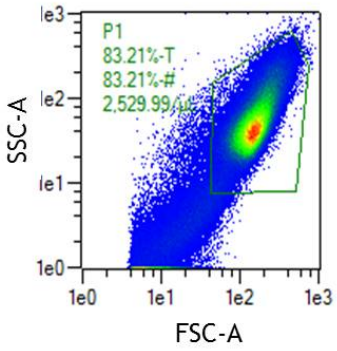
The adhesion molecule CD90 (Thy-1) was positively expressed on a similar percentage of MSCs, irrespective of MSC tissue source (G). Similarly, CD105 (Endoglin) a cell surface glycoprotein, was also expressed by the majority of MSCs, with very little variance in % positive cells when comparing across tissue

sources (H). Additionally, CD73, an enzyme that converts adenosine monophosphate to adenosine, was also expressed by a similar percentage of MSCs from all sources (I). Small variations in the % of positive cells were observed for the adhesion molecule CD166 in those MSCs tested (J) with Is MSCs having the highest % positive cells and Va MSCs having the lowest % positive cells (BM and UC MSCs not tested).

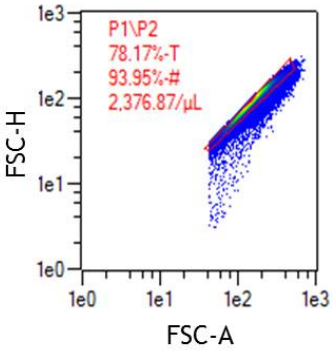
As well as being positive for an array of markers, MSCs must be negative for others such as CD45 and CD14. The hematopoietic marker CD45 was consistently absent on all MSCs with only a very small percentage of cells showing positive staining (K). Similarly, CD14, the co-receptor for TLR4, was not expressed by MSCs and this was consistent among different MSC sources (L).

These data indicate that MSCs isolated from Is, Va, Ad, BM and UC all express the relevant MSC markers meeting ISCT criteria. Importantly, the tissue source of MSC does not seem to affect the percentage of live cells expressing these markers.

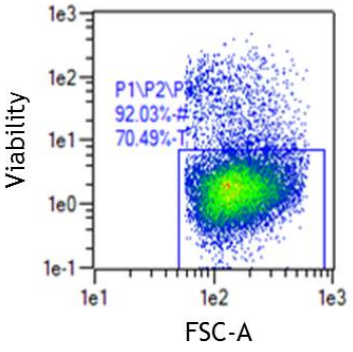
A.i



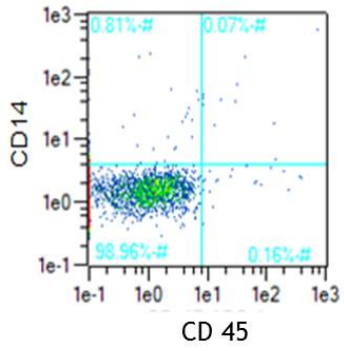
A.ii



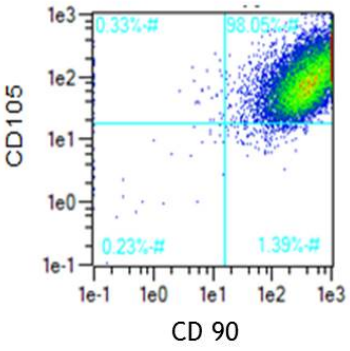
A.iii



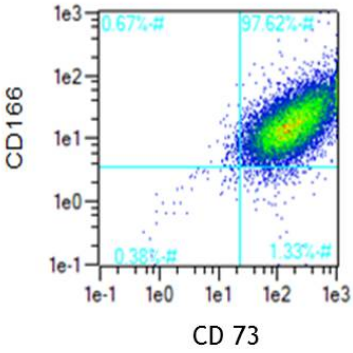
B.i



B.ii

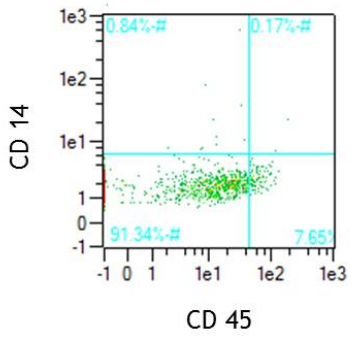


B.iii

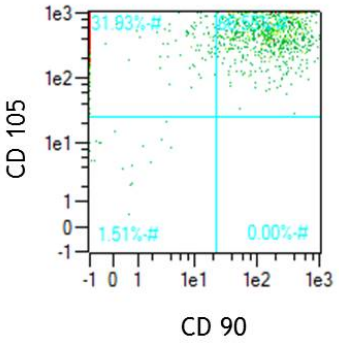


Is MSC

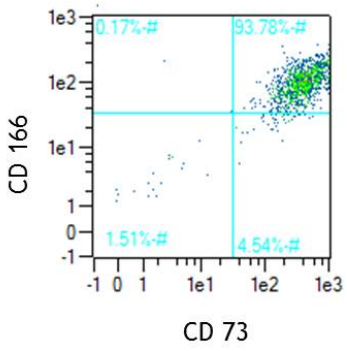
C.i



C.ii

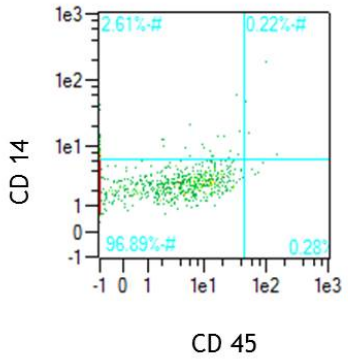


C.iii

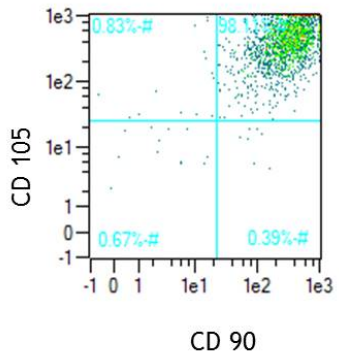


Ad MSC

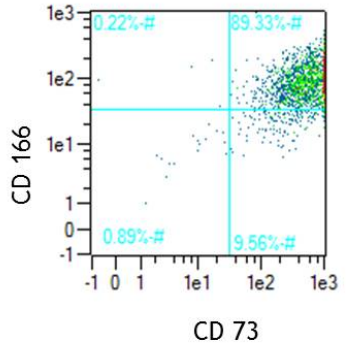
D.i



D.ii

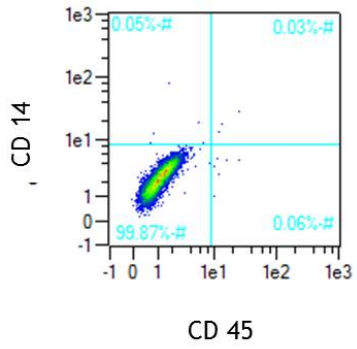


D.iii

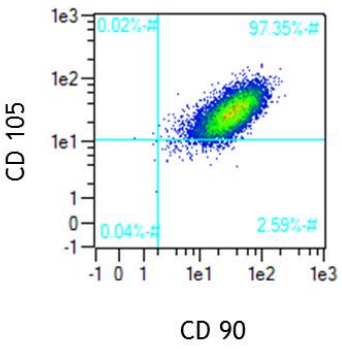


Va MSC

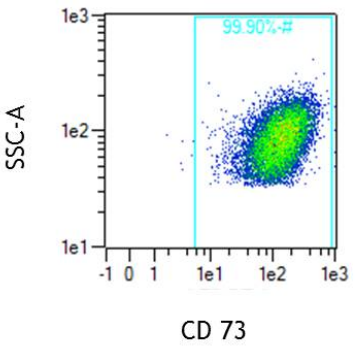
E.i



E.ii

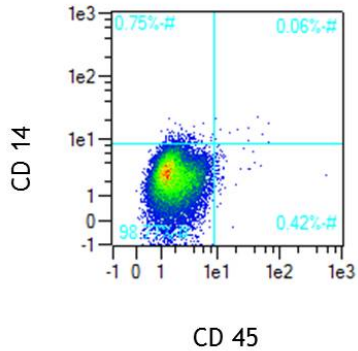


E.iii

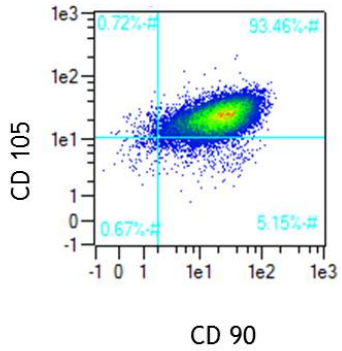


BM MSC

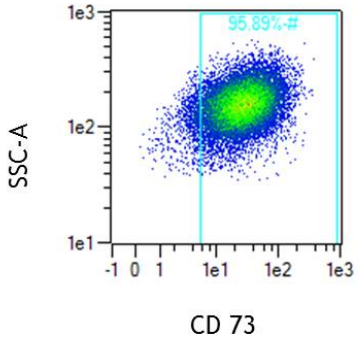
F.i



F.ii



F.iii



UC MSC

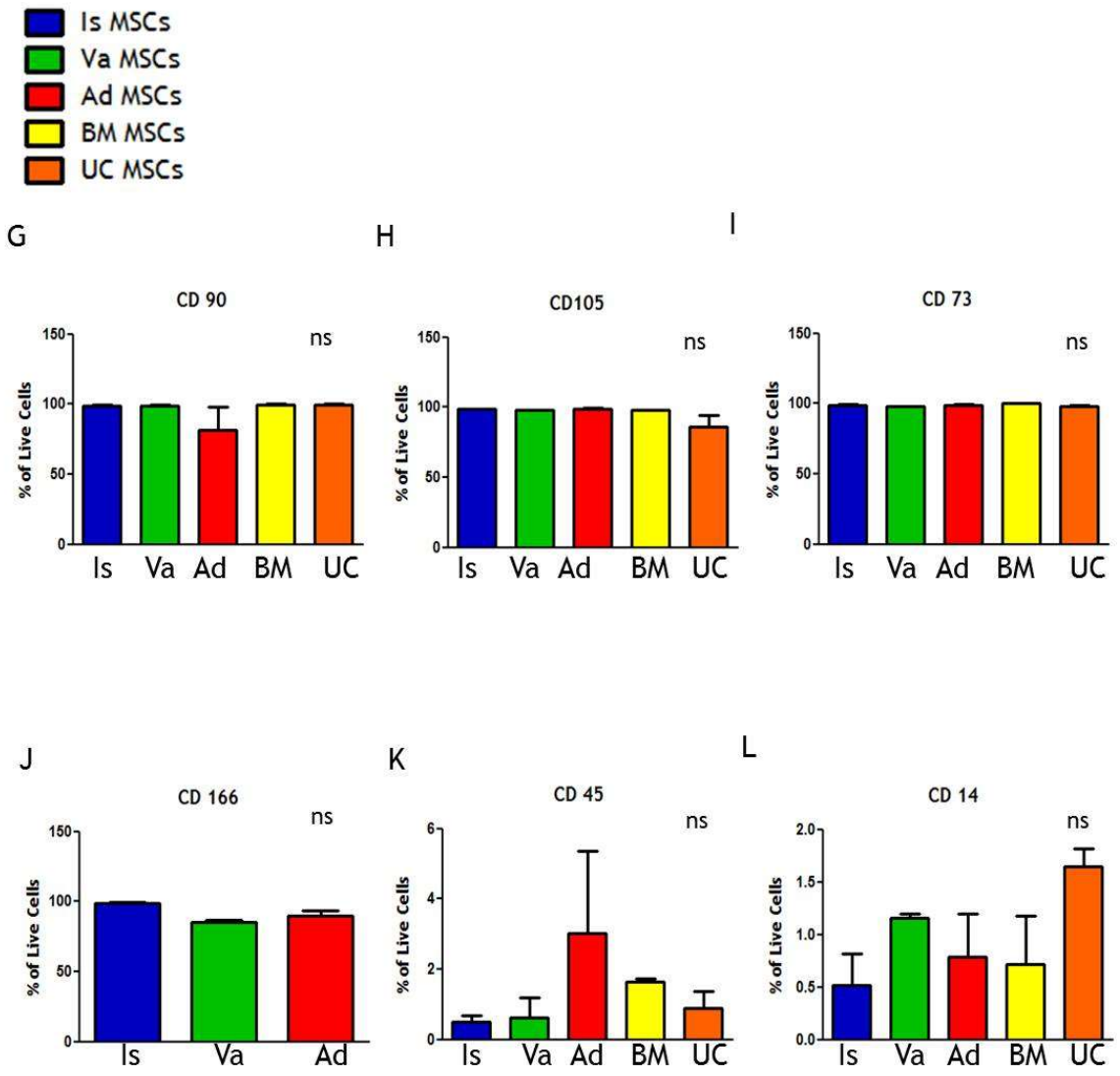


Figure 3-3 Cells isolated from all tissues express MSC Markers
MSCs isolated from Is, Va, Ad, BM and UC tissues were grown in culture until passage 3. MSCs were then stained with a cocktail of antibodies, to allow characterisation of MSCs by flow cytometry.

Gating strategy: FSC and SSC were used to gate on cells of correct size and granularity (**A.i**), doublets were excluded **(A.ii**) and live cells were selected (**A.iii**) in all samples before assessing surface CD phenotype (**B-F**). All MSCs were negative for hematopoietic markers CD45 and CD14 whilst being positive for MSC markers; CD90, CD105 and CD73 (**B-F**). Due to slightly different panel designs, Is, Va and Ad MSCs were also tested for their expression of another MSC marker - CD166- which they were all positive for (**B.iii**, **C.iii** & **D.iii**). The % of live MSCs which stained positive for each of the tested markers is graphed to compare expression between MSC tissue sources (**G-L**). Each bar represents an n of 3 and is graphed as mean ± SEM. ONE WAY ANOVA with Tukey's post-test analysis was used for statistical assessment.

3.4 **Differentiation of MSCs**

To determine the ability of MSCs isolated from Is, Va and Ad to differentiate into two out of three of adipocytes, chondrocytes or osteocytes, cells were cultured with specific differentiation factors to push them down a particular differentiation pathway. After 2-3 weeks of differentiation, MSCs were stained

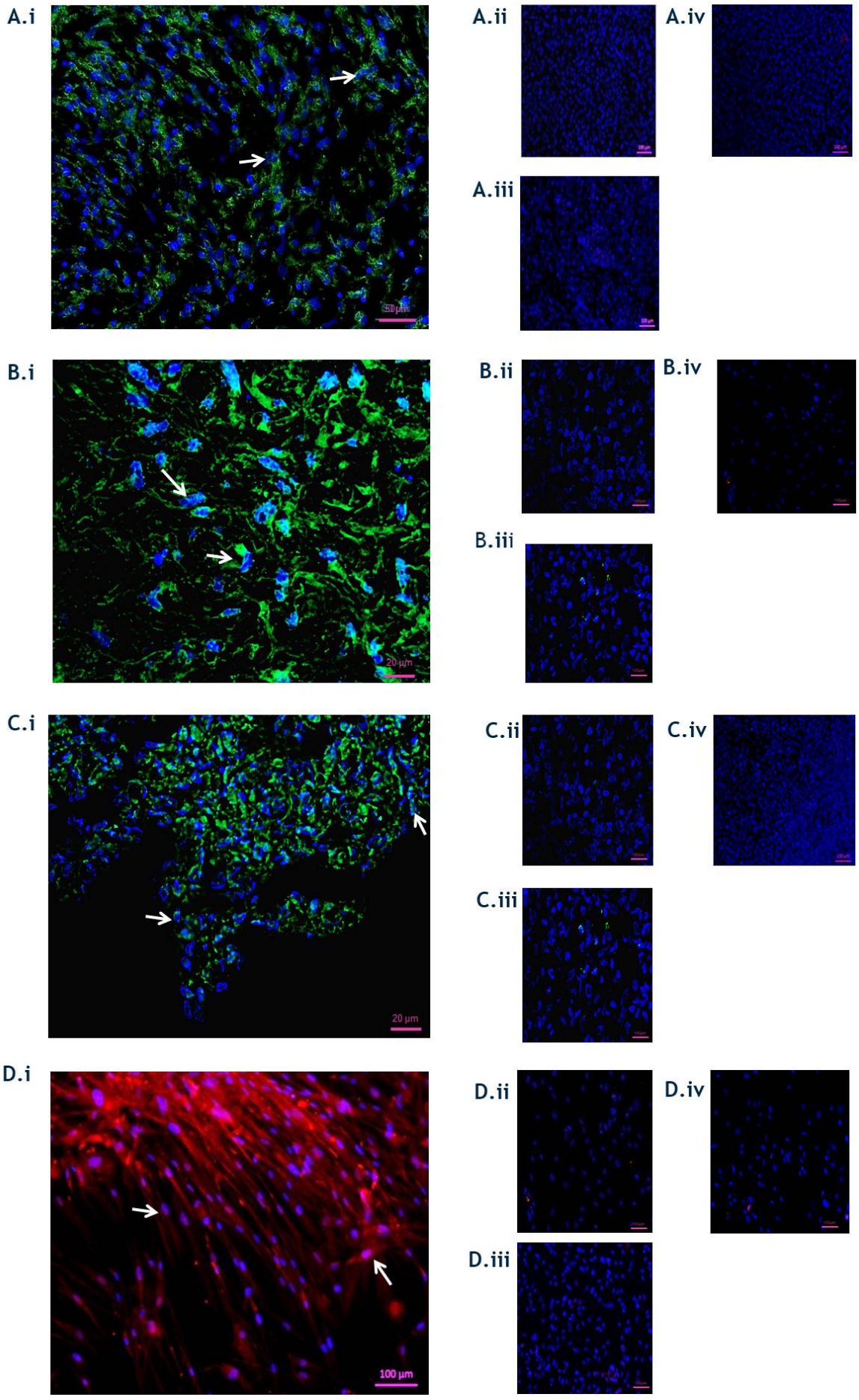
with antibodies specific for adipocytes (anti-FABP4), chondrocytes (anti-aggrecan) or osteocytes (anti-osteocalcin) and fluorescence was assessed by an epifluorescent microscope (**Figure 3-4** MSCs can successfully differentiate into adipocytes, chondrocytes and osteoblasts. Due to BM and UC MSCs not being available during the time course of this experiment, differentiation of these MSCs was not carried out.

Within 21 days the majority of Is, Va and Ad MSCs were all able to differentiate into chondrocytes (**Figure 3-4** A.i, B.i, C.i**),** where the majority of MSCs had transformed into aggrecan expressing cells. To ensure that undifferentiated MSCs were not aggrecan positive, MSCs were grown in the same conditions and culture medium, minus the differentiation cocktail, and stained with anti-aggrecan (A.**iv**, B.**iv** & C.**iv**). The lack of fluorescence in the non-differentiated controls, isotype controls (A.ii, B.ii, C.ii) and no-primary antibody controls (A.iii, B.iii, C.iii) compared to differentiated samples suggests that positive staining was specific for differentiation induced aggrecan expression and that MSCs isolated from the Is, Va and Ad were fully capable of differentiating into chondrocytes.

Is MSCs were able to differentiate into osteocytes (**Figure 3-4** D.**i**). Being the only MSC tested for their ability to differentiate into osteocytes, the potential of other MSCs to differentiate into osteocytes cannot be commented on. The majority of MSCs isolated from Islets were able to differentiate into bone, which was confirmed by the large signal from the anti-osteocalcin antibody. The specificity of this antibody was confirmed using isotype controls (D.ii), no-primary antibody (D.iii) and non-differentiated controls (D.iv).

Similarly to chondrogenic differentiation, Is, Va and Ad MSCs were able to differentiate into adipocytes within 21 days (**Figure 3-4** E.**i**, F.**i** and G.**i**). Positive staining for fatty acid binding protein 4 (FABP4), identified MSC adipogenic differentiation. Lack of signal in isotype controls (E.ii, F.ii, G.ii), no-primary antibody controls (E.iii, F.iii, G.iii) and non-differentiated controls (E.**iv**, F.**iv**, G.**iv**) confirmed specificity of the anti-FABP4 antibody, highlighting that positive staining is indicative of successful differentiation.

The ability of Is, Va and Ad MSCs to differentiate into adipocytes, chondrocytes and - in the case of Is MSCs - osteocytes within 21 days was confirmed by specific positive staining of particular antibodies. Where tested, all differentiations were successful, thus satisfying ISCT criteria of MSC differentiation capabilities. The ISCT criteria suggests that MSCs must be able to differentiate into two out of three of adipocytes; chondrocytes and osteocytes which Is, Ad and Va MSCs are capable of. Moreover, all controls carried out during the experiment confirmed that the antibodies used were specific and that positive staining was indicative of full differentiation of MSCs.


A.i
A.ii
A.iii
A.iv
B.i
B.ii
B.iii
B.iv
C.i
C.ii
C.iii
C.iv
D.i
D.ii
D.iii
D.iv
20 µm
20 µm
100 µm

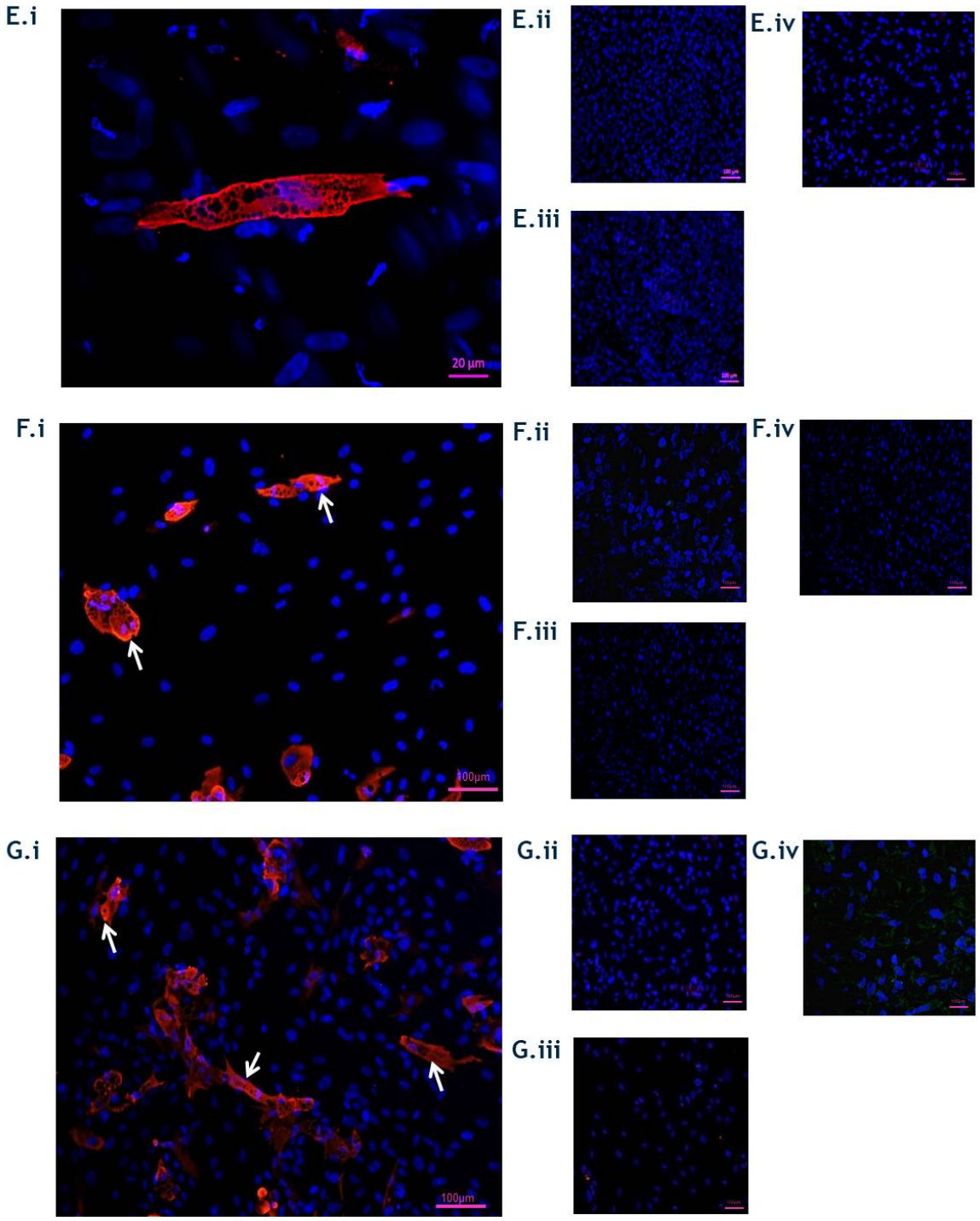


Figure 3-4 MSCs can successfully differentiate into adipocytes, chondrocytes and osteoblasts

Is, Va and Ad MSCs were differentiated into chondrocytes (A-C).

Is (**A**), Va (**B**) and Ad (**C**) MSCs (P3) were seeded at $2.5x10^5$ total, 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 cultured in medium without differentiation factors. After 21 days of culture, spherical balls were removed, snap frozen and sectioned. All samples were stained with anti-aggrecan (GREEN), with the exception of isotype controls (**A.ii, B.ii, C.ii**) and no-primary controls (**A.iii, B.iii, C.iii**). Is MSCs differentiated into chondrocytes: n=2 (A). Va MSCs differentiated into chondrocytes: n=1 (**B**). Ad MSCs differentiated into chondrocytes: n=1 (**C**).

Is MSCs were differentiated into osteocytes (D).

For osteogenesis, cells were seeded at $2.21x10^3$ per cm^2 and grown in 24 well plates. Once they had reached roughly 80% confluence, differentiation factors were added every 2-3 days for 14-21 days. Undifferentiated control samples (**D.iv**) were maintained in medium without differentiation factors. After 14-21 days of differentiation, cover slips within the 24 well plates were removed and stained with anti-osteocalcin (RED), with the exception of isotype controls (**D.ii**) and no-primary controls (**D.iii**). Is MSCs differentiated into osteocytes: n=1 (**D**).

Is, Va and Ad were differentiated into adipocytes (E-G).

For adipogenesis, Is (**E**), Va (**F**) and Ad (**G**) MSCs were seeded at $1.11x10^4$ per cm^2 and grown in 24 well plates. Once they had reached roughly 80% confluence, differentiation factors were added every 2-3 days for 14-21 days. Undifferentiated control samples (**E.iv, F.iv, G.iv**) were maintained in medium without differentiation factors. After 14-21 days of differentiation, cover slips within the 24 well plates were removed and stained with anti-FABP4 (RED), with the exception of isotype controls (**E.ii, F.ii, G.ii**) and no-primary controls (**E.iii, F.iii, G.iii**). Is MSCs differentiated into adipocytes: n=1 (**E**). Va MSCs differentiated into adipocytes: n=2 (**F**). Ad MSCs differentiated into adipocytes: n=2 (**G**).

In all cases, all samples were stained with specific fluorescent secondary antibodies. DAPI (blue) marks the cell nuclei. White arrow heads point to fully differentiated MSCs.

3.5 Phenotype of MSCs during Inflammation

In the clinic, MSCs are often infused into a pre-existing inflammatory environment (190, 191). Therefore, it is important to understand the effects of inflammation on the phenotype of MSCs isolated from various tissue sources. In order examine this, the effects of an inflammatory environment were assessed and mimicked by splitting MSCs isolated from one donor into 2 flasks, leaving one flask in "normal" conditions and stimulating the other with 10ng/ml of TNFα, IL-1ß and IFN-Ɣ for 24 hours. From there, surface phenotype of several MSC markers was assessed by flow cytometry (**Figure 3-5** and **Figure 3-6**), as was the size and granularity of MSCs (**Figure 3-7**).

Although true homeostasis *in vitro* is hard to define, MSCs which were maintained in normal culture medium are referred to as being in a homeostatic environment. Equally, when MSCs were stimulated with the inflammatory cytokine cocktail, this is referred to as an inflammatory environment - this terminology will be used throughout.

3.6 Surface Molecule Phenotype of MSCs in Homeostatic Vs Inflammatory Conditions

Representative dot plots illustrate the percentage of Is MSCs expressing the markers HLA ABC, HLA-DR, CD271, CD166, CD73 and CD146 during normal and inflammatory conditions (Figure 3-5 A-C). Positive staining was determined by

the use of Fluorescent minus one's (FMO's) and isotype controls. The percentage of Is, Va and Ad MSCs expressing these markers are graphed in **Figure 3-5 D-I**.

During "normal" conditions, 4.90% (± 1.66%) of Is MSCs expressed HLA-DR, which slightly increased to 14.06% (± 5.76%) after 24 hours of stimulation. Like Is MSCs, Ad MSCs had a slight increase in the % of cells expressing HLA-DR when stimulated, going from 4.26% (± 1.39%) to 16.90% (± 4.66%). The % of Va MSCs expressing HLA-DR went from 7.77% (± 3.46) in normal conditions to 14.81% (± 2.74%) when inflamed (**Figure 3-5 D**).

Unlike the small % of HLA-DR expressing MSCs under homeostatic and inflammatory conditions, a large % of MSCs expressed HLA-ABC under both homeostatic and inflammatory conditions, with 81.28% (± 16.59%) of Is MSCs expressing HLA-ABC in homeostatic conditions, rising to 97.92% (± 0.82%) after inflammatory stimulation. The percentage of Ad MSCs expressing HLA-ABC rose from homeostatic (90.49% ± 4.90%) to inflammatory (98.97% ± 0.27%) conditions. By contrast, a smaller increase in percentage of Va MSCs becoming HLA-ABC positive was observed from 94.77% (±1.56%) in normal conditions to 96.716% (± 2.04) under inflammatory stimulation (**Figure 3-5 E**). This upregulation of HLA-ABC was more prominently reflected in the increase of mean fluorescence intensity (MFI) (**Figure 3-6 A**), where Is MSCs went from an MFI of 25.75 (± 11.22) to 47.17 (± 8.0), Va MSCs from 31.33 (± 6.60) to 51.58 (± 9.03) and Ad MSCs from 22.63 (± 5.36) to 60.03 (± 13.15). No significant differences in the percentage of HLA-ABC expressing MSCs were observed going from homeostatic to inflammatory conditions or between MSC tissue sources.

As previously mentioned, CD73 is a widely used marker for MSCs. The percentage of MSCs expressing CD73 was barely affected by tissue source or inflammatory stimulation. A marginal increase in the percentage of CD73 expressing Is MSCs was observed after inflammatory stimulation, from 91.43% to 98.54%, whereas the percentage increase in Ad MSCs expressing CD73 was slightly lower, going from 95.94% to 96.76%. The percentage of CD73 expressing Va MSCs was the same under homeostatic (96.90%) and inflammatory conditions (96.47%) (**Figure 3-5 F**). The effects of inflammatory stimulation on CD73 was better reflected by the MFI (Figure 3-6 A), where all MSCs showed a reduction in MFI under inflammatory stimulation (Is=63.73 (± 21.81) to 57.25 (± 7.85), VA=65.55 (± 6.91)

to 56.38 (± 12.21) and Ad=64.24 (± 64.25) to 61.67 (±10.82)). No significant differences in the percentage of CD73 expressing MSCs or in CD73 MFI were observed comparing homeostatic to inflammatory conditions or between MSC tissue sources.

Similarly, as mentioned before, CD166 is also a marker of MSCs. A uniform pattern of increase in the percentage of MSCs expressing CD166 was observed during inflammation in all MSCs tested, with Is MSCs increasing from 31.13% (± 23.69) to 68.18% (± 18.41), Va MSCs from 66.55% (± 10.10%) to 78.82% (± 7.33) and Ad MSCs from 49.60% (± 10.52) to 74.13% (± 9.42) (**Figure 3-5 G**).

CD271 is also a marker of MSCs (192) and is expressed at variable levels and sometimes not at all (193). Reflecting that, the percentage of MSCs positive under 'homeostatic' conditions varied from source to source as does the effects of inflammation on the percentage of CD271 expressing MSCs. Is MSCs displayed a small % of CD271 expressing cells with 10.03% (± 8.63%) under homeostatic conditions, dropping to 7.17% (±3.72%) under inflammatory conditions. Similarly, Va MSCs showed a decrease in the % of CD271 expressing MSCs under inflammatory stimulation, going from 33.50% (±15.77%) under homeostatic conditions to 20.72% (±14.56) under inflammatory stimulation, whereas the percentage of Ad MSCs expressing CD271 increased slightly going from 42.53% (± 5.66%) to 51.08% (± 8.86%) (**Figure 3-5 H**). No significant differences in the percentage of CD271 expressing MSCs or in CD271 MFI were observed going from homeostatic to inflammatory conditions or between MSC tissue sources.

CD146 is another marker commonly used to identify MSCs, however like many of the markers used, its expression is variable on MSCs (194, 195). All MSC sources showed a small percentage of CD146 expressing MSCs, which rose after inflammatory stimulation. 2.94% (±1.39%) of Is MSCs were positive for CD146 under homeostatic conditions, whilst under inflammatory stimulation, this rose to 21.74% (± 8.53%) of Is MSCs being CD146 positive. Similarly Ad MSCs had a small percentage of CD146 expressing cells under homeostatic conditions (1.67% ± 0.51%), which increased under inflammatory conditions to 12.81% (± 1.70%). Va MSCs showed the largest proportion of CD146 expressing cells under homeostatic conditions (6.24% ± 4.9%), which rose to 27.55% (± 7.57%) under inflammatory conditions (**Figure 3-5 I**).

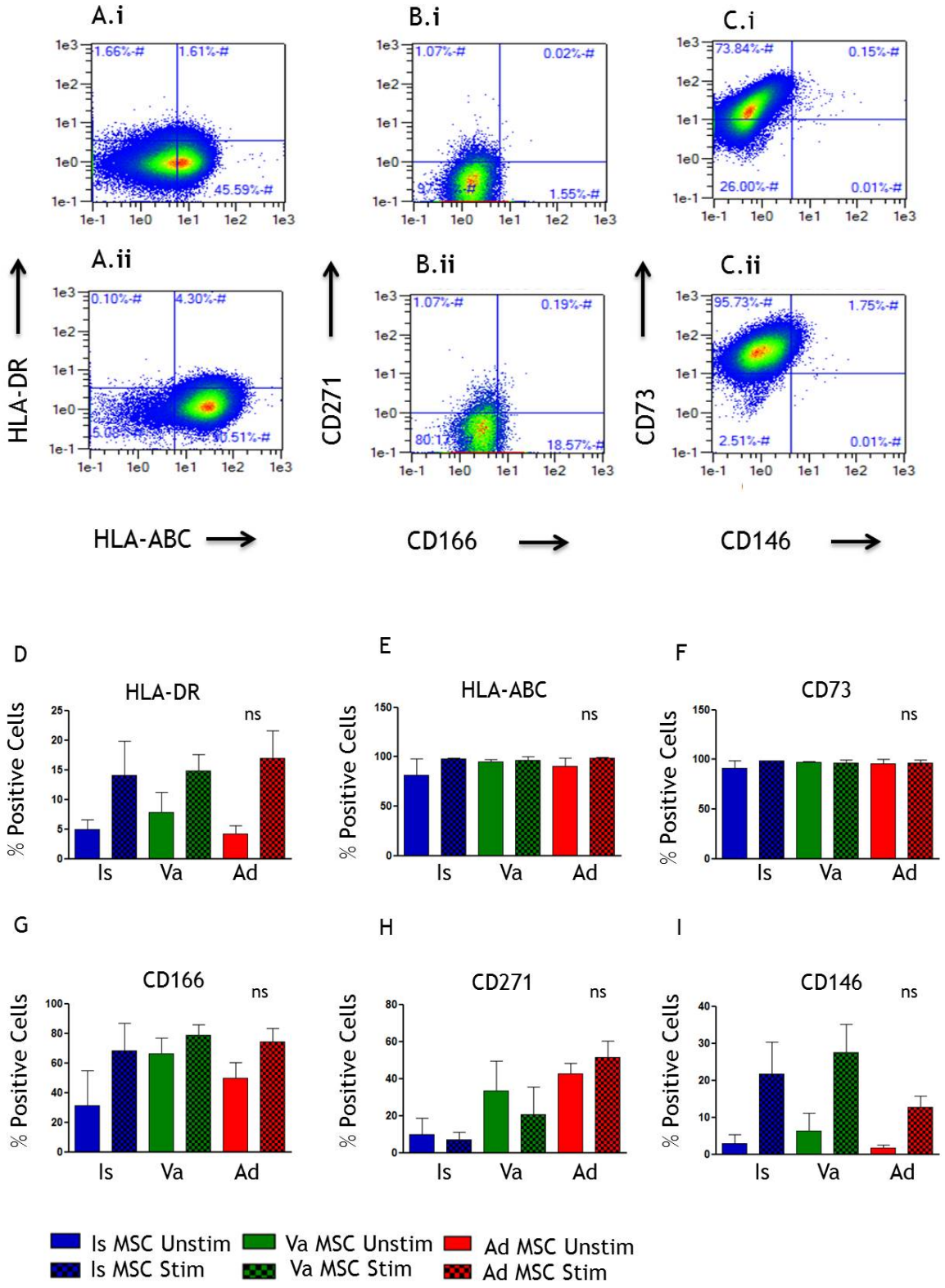


Figure 3-5 The surface molecule phenotype of MSCs from the tissue sources tested following inflammatory stimulation

MSCs isolated from the Is, Va, and Ad were seeded into 2 flasks at P3. Once MSCs had reached 80% confluence, one flask was stimulated with 10ng/mL of IFN-Ɣ, TNFα and IL-1β (inflammatory conditions) and the other flask was left in MSC culture medium (homeostatic conditions) for 24 hours. After 24 hours, cells were stained with an array of antibodies, including; HLA-ABC, HLA-DR, CD73, CD271, CD146 and CD166 and analysed by flow cytometry to examine the effects of inflammation on MSC surface marker phenotype. Representative dot plots show the expression of the aforementioned markers on unstimulated (**A.i,B.i,C.i**) vs stimulated (**A.ii, B.ii, C.ii**) Is MSCs. Positive staining was measured by the use of fluorescence minus ones and isotype controls and

gates were drawn appropriately. The data in the dot plots were graphed and presented as the percentage of MSCs positively expressing each marker under homeostatic and inflammatory conditions for Is, Va and Ad MSCs (**D-I**). Each bar represents an n of 3 and graphed as mean ± SEM. Student's T test was used to assess statistical differences between unstimulated and stimulated MSCs from one source. To assess statistical differences between MSC sources, a ONE WAY ANOVA was used in conjunction with Tukey's multiple comparison tests.

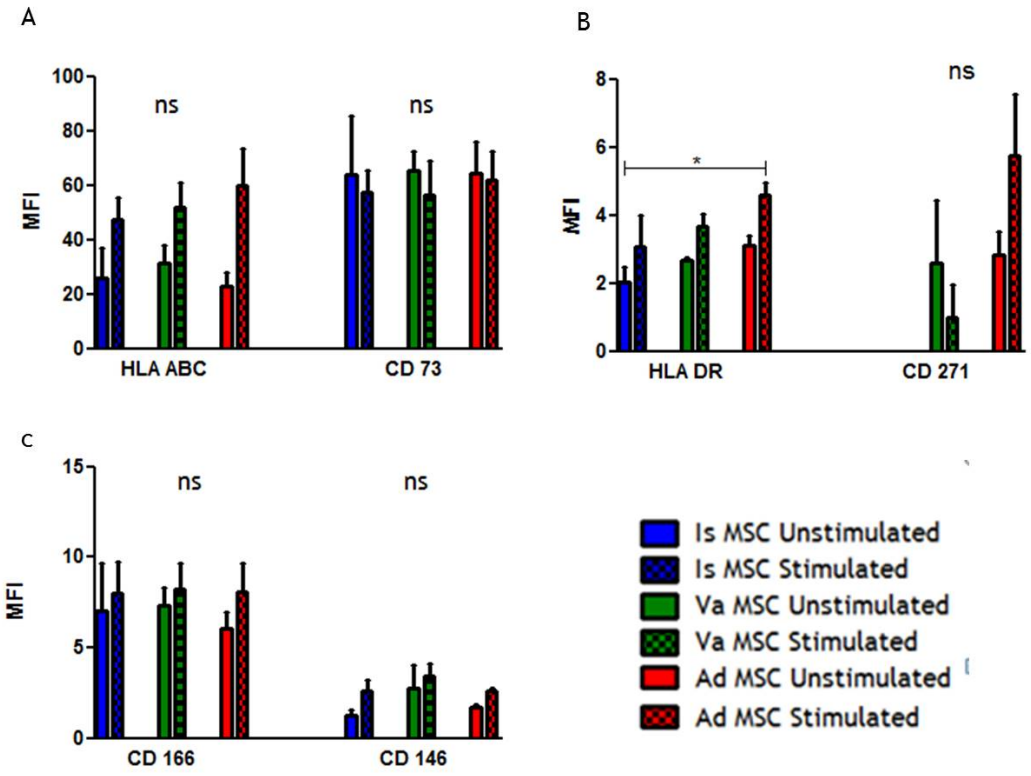


Figure 3-6 MFI of selected markers fluctuates slightly under inflammatory stimulation
Applying the same experimental set up as Figure 3-5, this is the same data, expressed as mean fluorescence intensity, allowing for more subtle changes in marker expression on MSCs to be observed. Each bar represents an n of 3 and graphed as mean ± SEM. Statistical analysis performed = ONE WAY ANOVA with Tukey's multiple comparison post-test. A Student's T test was used when comparing one tissue source of MSC- unstimulated vs stimulated.

In summary, HLA-DR, CD271, CD166 and CD146 were slightly upregulated in all MSCs when maintained under inflammatory conditions, with the exception of CD271 on Is MSCs, where expression was extremely low/not detectable under both homeostatic and inflammatory conditions. HLA-ABC was markedly upregulated in all MSCs under inflammatory stimulation and CD73 was slightly downregulated in all MSCs under inflammatory stimulation, however expression levels of CD73 remained the highest of all markers tested. Importantly, there was no significant change in MSC surface molecule phenotype after 24 hours of stimulation. Moreover, there was no significant difference in the expression of these markers between MSC tissue source under homeostatic or inflammatory conditions.

3.7 Size and granularity of MSCs in homeostatic vs inflammatory conditions

Using flow cytometry, the parameters forward scatter (FSC) and side scatter (SSC) were used to measure the size and granularity (respectively) of Is, Va, Ad, BM and UC MSCs under homeostatic and inflammatory conditions (**Figure 3-7**). The size of a cell is measured by the amount of laser light that can pass around the cell, whereas granularity is measured by the amount of light that bounces off particles within the cell. Thus the size and complexity of a cell can be measured using these parameters where a small FSC value is indicative of a small cell, and a large SSC value suggests a granular, complex cell.

When MSCs were maintained under homeostatic conditions, Is MSCs had the lowest SSC, whereas BM MSCs had the highest. A significant difference was observed between Is MSCs compared to all other sources of MSCs (Is vs Va $P<0.01$, Is vs Ad $P<0.01$, Is vs BM $P<0.001$, Is vs UC $P<0.01$) under homeostatic conditions (**Figure 3-7 A.i**). Under inflammatory stimulation, the SSC increased in all MSCs, however a significant increase was only observed in Is MSCs ($P=0.0015$). When comparing the SSC of MSCs from all sources under inflammation, it mirrors the patterns observed during homeostatic conditions, i.e. Is MSCs exhibited the smallest SSC during inflammation, and BM MSCs exhibited the largest, however, the only significant difference observed between SSC of stimulated MSCs was between Is and BM ($P<0.01$). The same data are represented as histograms to clarify the graphed observations (**Figure 3-7 A.ii-A.iii**).

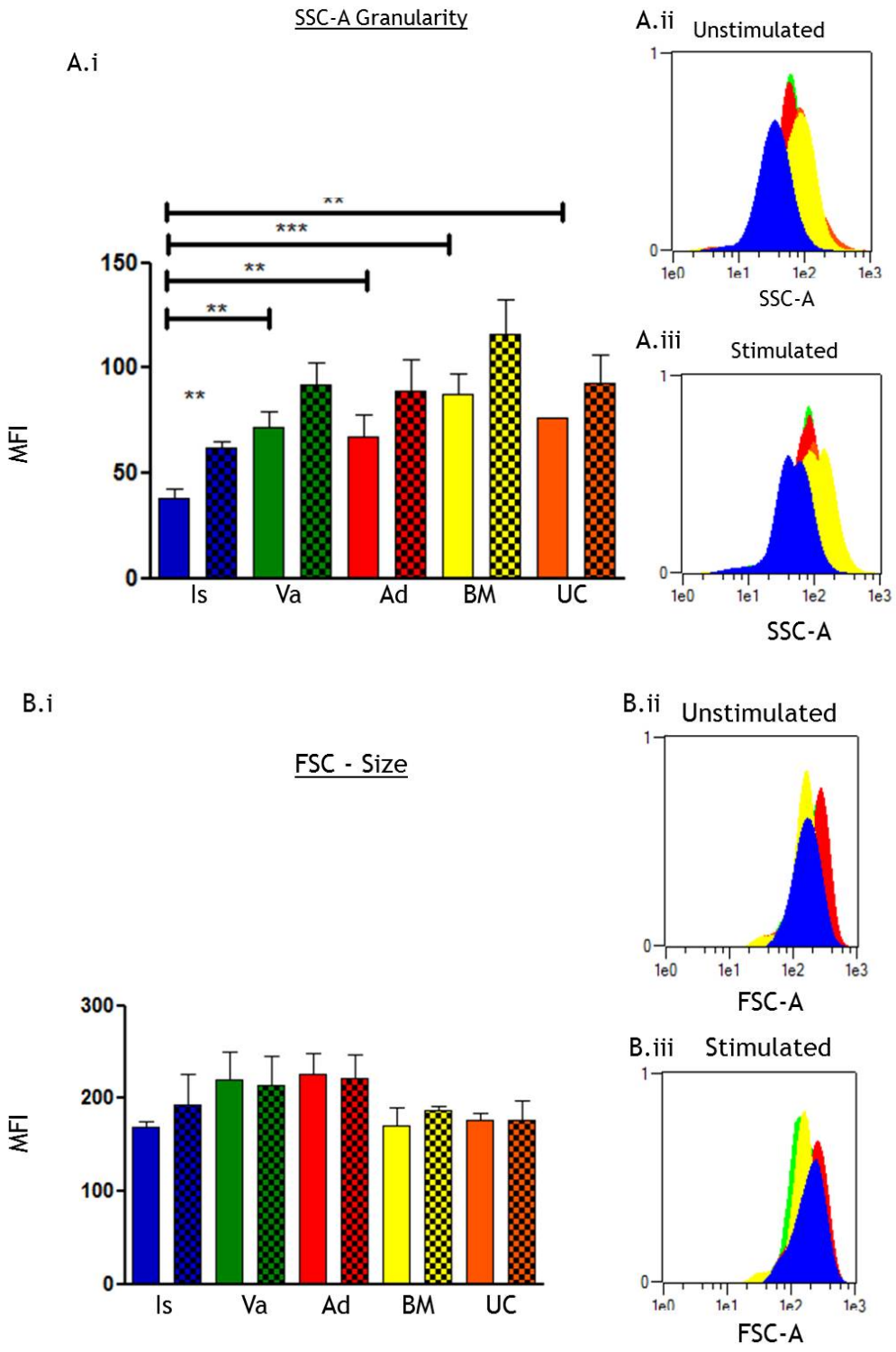


Figure 3-7 Granularity of MSCs increases under inflammatory stimulation but size remains unchanged.
Adopting the same experimental set up as Figure 3-5 and Figure 3-6 with the addition of UC and BM MSCs, flow cytometry was used to measure FSC and SSC to assess size and granularity of MSCs during homeostatic and inflammatory conditions. In all MSCs, side scatter increased after inflammatory challenge (**A.i**). Size of MSCs was unaffected by tissue source or inflammation (**B.i**). Each bar represents an n of 3, and plotted as the mean ± SEM. A Student's T test was used when comparing one tissue source of MSC- unstim vs stim. When comparing all tissue sources of MSCs a ONE WAY ANOVA was used with Tukey's multiple comparisons test.

The graphed data were represented in histograms for clarity (**A.ii, A.iii, B.ii, B.iii**).

The size of MSCs did not vary significantly between tissue sources, nor was it affected by inflammatory stimulation (**Figure 3-7 B.i**). The graphed data are represented in histograms (**Figure 3-7 B.ii-B.ii**).

These data show that tissue source of MSC does not have an effect on the size of MSCs, nor does inflammation. In contrast, tissue source appears to have some effect on the granularity of MSCs, with Is MSCs being significantly less granular than Va, Ad, UC and BM MSCs. When MSCs were maintained under an inflammatory environment, MSCs from all tissue sources became more granular, however, this increase in granularity was only significant in Is MSCs. When comparing the granularity of MSCs from all sources under inflammatory conditions, Is MSCs were the least granular and BM MSCs were the most, with this difference in granularity between Is and BM MSCs being significant.

3.8 Discussion and Conclusions

The aim of this chapter was to discover if cells isolated from the Is, Va, Ad, BM and UC meet the ISCT criteria for MSCs. In addition, this chapter also aimed to examine whether MSCs isolated from different human tissues had variations in their phenotype. Finally, as there is a large body of literature supporting the concept of MSC licensing - where MSCs need inflammatory stimulation in order to exert their anti-inflammatory and immunomodulatory capacity – the phenotype of MSCs was also assessed under inflammatory conditions (168).

From their first description by Friedinstein in 1976, MSCs are now well established as being adherent, spindle-shaped cells (196). ISCT outlines that this morphology must be met in order to be defined as a MSC (95). Is, Va, Ad, UC and BM MSCs all satisfied this criteria from early (P1) through to later passage (P8). Importantly, this morphology was uniform among all MSCs, irrespective of the tissue they were originally isolated from. Akin to this, ISCT also states that MSCs must be able to differentiate into two of three cell types out of adipocytes, chondrocytes and osteoblasts, which Is, Ad and Va MSCs were all capable of.

The third criteria that must be met in order to be classified as a MSC, is the expression of an array of markers including CD90, CD105, and CD73, whilst

lacking others such as CD14 and CD45 (95). Other markers are often used to identify MSCs such as CD166 and CD271, however the expression of these markers is not uniform and is known to fluctuate substantially for several reasons including passage number and tissue source of MSCs (194, 197).

CD90 (also known as Thy-1), is a cell surface glycoprotein involved in cell/cell matrix interactions (198), inflammation and wound healing (199). Whilst being a marker for hematopoietic stem cells (200), it is also a marker for MSCs and has a suggested function of attracting and immobilising monocytes and polymorphonuclear cells (PMN) on MSCs (201), as well as immunosuppressing lymphocytes (202). In contrast to *Maleki et al*, who reported the variability in percentage of CD90 expressing MSCs when isolated from Wharton's Jelly (13.47%) and the hair follicle (50.85%) (197), MSCs isolated from Is, Va, Ad, BM and UC (Wharton's Jelly) showed a similar percentage of CD90 expressing cells (>81%). CD105 -otherwise known as Endoglin- is a cell surface glycoprotein expressed on lineages within the vascular system and endothelial cells as well as MSCs. It functions as an accessory receptor for members of the TGF-ß superfamily (203) and is involved in blood vessel development, implicated in erythropoiesis and myelopoiesis (204), as well as representing a powerful marker for neovascularisation (205). Notably, culture medium has been shown to have an effect on expression levels of CD105, where serum free medium had reduced cellular CD105 expression compared to serum-supplemented medium. Importantly, serum-supplemented media contains growth factors such as TGF-ß that could induce CD105 expression and subsequently downregulating its surface expression (206). Similarly, Ad MSCs have been reported to have varying expression levels of CD105 (207), however, here a consistent expression of CD105 was demonstrated, not only among Ad MSCs, but also among all other tissue sources of MSCs – Is, Va, BM and UC.

CD73 (ecto-5'-nucleotidase) was also expressed evenly on >97% of all tissue source MSCs, To my knowledge, there have been no studies comparing the expression of CD73 on MSCs maintained under various conditions or comparing the expression of CD73 on MSCs isolated from different tissue sources. This could be due to the lack of literature surrounding its function on the surface of MSCs, with the exception of one study which suggests that it plays a role in MSC

migration (208). As a membrane bound glycoprotein, its function is to metabolise adenosine 5'-monophosphate (AMP) to adenosine. However, it is also known to play an important role in ion fluid transport, tissue injury and acts as an adhesion molecule for lymphocytes binding to the epithelium (209, 210). Moreover, CD73, and its product adenosine, have been implicated as an innate mechanism to inhibit excessive, harmful aspects of neutrophil activation and accumulation (211). In addition, CD73 is involved in tumour-associated immune suppression, where the production of adenosine has been shown to limit anti-tumour T cell immunity (211, 212). If CD73 functions like the aforementioned on the surface of MSCs, this suggests an immunomodulatory role of the marker, which MSCs isolated from Is, Va, Ad, BM and UC MSCs could all mediate due to their uniform expression of CD73. Although MSC expression of CD90, CD105 and CD73 allows for rapid identification of MSCs, the expression of these markers is not limited to MSCs and therefore lack of expression of other markers such as CD14 and CD45 assures that MSC cultures are not confounded by other cells likely to be isolated along with MSCs. As CD90 and CD73 are found on hematopoietic stem cells and T cells respectively, ensuring that MSCs lack the pan-leukocyte marker, CD45, will account for any potential contamination of these cells within MSC culture. Additionally, CD14, the co-receptor for TLR4 allows for identification of monocytes and macrophages which are the most likely hematopoietic cell types to be found in MSC culture (95). MSCs isolated from Is, Va, Ad, BM and UC show only small percentages of cells positive for CD45 (<3%) and CD14 (<1.16%).

With the three MSC criteria stated by ISCT being satisfied for Is, Ad and Va MSCs and two out of three of the criteria being satisfied for UC and BM MSCs, it may be concluded that the cells isolated from these tissue sources can be regarded as MSCs.

As MSCs are being evaluated as treatments for inflammatory conditions and diseases, understanding their phenotype during these conditions, and if tissue source of MSC has any affect, is important because it could suggest preferential tissue sources for MSC isolation for therapeutic use (213).

Human leukocyte antigens (HLA) ABC and DR, are vital in regulating the immune system and encode the major histocompatibility complex in humans – MHC I and

MHC II, respectively. This system is designed to bind self and foreign antigens from within (MHC I) and out with the cell (MHC II) and present them on the cell surface in the form of peptides. MHC I is expressed on all nucleated cells within the body and interacts with cytotoxic T lymphocytes (CD8), whereas MHC II is normally expressed on antigen presenting cells and allows interactions with CD4 T cells. With respect to transplant rejection, MHC molecules can themselves act as antigens in the recipient and thus aid in transplant rejection. MSCs are known to elicit a weak cellular and humoral allogeneic immune response, due to low expression of HLA antigens and co-stimulatory molecules (214, 215). For this reason, their use within transplant settings has been popular (216). In line with the literature, Is, Va, and Ad MSCs express HLA-ABC under homeostatic conditions which was upregulated under inflammatory conditions (217). In the current study, Is, Va and Ad MSCs markedly upregulated HLA-ABC expression under inflammatory stimulation, which was reflected in a substantial increase in MFI. Importantly, Ad MSCs showed the largest increase in MFI (increase of 37.4) from homeostatic to inflammatory conditions, whilst also displaying the highest HLA-ABC MFI (60.03) compared to Is (47.17) and Va (51.85) MSCs. Notably, the expression of HLA-DR on Is, Va and Ad MSCs was very low in homeostatic conditions but this was slightly upregulated under inflammatory conditions. This upregulation of HLA-DR on MSCs under inflammatory stimulation is well documented within the literature (218). Little variation in HLA-DR expression on MSCs under inflammatory conditions was observed, with Ad MSCs being the highest expressers and Is and Va expressing similar levels. Despite the expression of HLA-ABC and HLA-DR (after stimulation) on MSCs being documented within the literature, to my knowledge, this is the first report of MSCs isolated from these locations, being compared and showing subtle variations in the expression levels of these markers.

As well as HLA-ABC and HLA-DR being upregulated on MSCs under inflammatory stimulation, CD166 (ALCAM), which plays an important role in adhesion of epithelial cells to T cells via CD6, was also upregulated in Is, Ad and Va MSCs under inflammatory conditions (219). As mentioned, CD166 has variable levels of expression depending on where MSCs are isolated from (194, 197), therefore understanding if its expression varies between Is, Va and Ad MSCs under homeostatic and inflammatory conditions was of interest. During homeostatic

conditions, the expression of CD166 fluctuated largely within one tissue source, with Is MSCs showing the largest variability. Due to such large variabilities within one tissue source of MSC, no solid comparison or conclusion can be made from comparing the percentage of CD166 expressing MSCs across tissue sources. However, there is a trend of upregulation when Is, Va and Ad MSCs were maintained under an inflammatory environment, perhaps suggesting that under inflammatory conditions, MSCs are able to adhere to T cells more readily. Similar to CD166, CD146 (MCAM) is a marker of MSCs which varied in expression depending on MSC tissue origin (194). Its expression has been observed to fluctuate within one tissue source of MSC (BM) where an increase in age of BM donor was linked to a decreased expression (195). In line with the literature, Is, Va and Ad MSCs exhibited large variations in the proportion of CD146 expressing cells. This large variation was exaggerated when MSCs were maintained under inflammatory conditions, however the overall trend of MSC CD146 expression was to upregulate when stimulated.

Consistency and pattern of CD271 expression was not similar to the other markers tested, as a uniform pattern of upregulation or downregulation was not observed when MSCs were inflamed. Despite being classified as a universal marker for MSCs, studies suggest that CD271 is useful for identifying a particular fraction of BM MSCs, where other studies have shown UC blood MSCs to have no expression, and MSCs from the Wharton's jelly have varied CD271 expression (96, 220, 221). In agreement with the literature, we found that Ad MSCs expressed CD271 (193). Importantly, CD271 expression was highest on Ad MSCs, and this was the only source of MSCs to upregulate their expression under inflammatory conditions. Is and Va MSCs downregulated CD271 expression when maintained under inflammatory conditions, and showed large variability in expression, suggesting that differences were due to donor variation. Studies have suggested that age affects CD271 expression, therefore the donor variation observed here, could be due to variance in patient age (193).

As previously mentioned, CD73 is perhaps the most reliable and popular marker used in conjunction with other markers to identify MSCs. Interestingly, no difference in expression levels was observed between Is, Va and Ad MSCs under homeostatic and inflammatory conditions. There was a slight trend for all MSCs

to slightly downregulate CD73 expression. Due to the lack of knowledge surrounding its function on MSCs, why MSCs would do this is unknown. One study suggests that CD73 is involved in MSCs migration, therefore its downregulation could be a mechanism used by MSCs to minimise their migration and be retained within the inflammatory site (208).

Overall, the expression of the commonly used markers CD90, CD105 and CD73 remained consistent amongst Is, Va, Ad, UC and BM. CD14 and CD45 were absent in all MSC cultures. Expression levels of CD166 and CD146 varied within one tissue source of MSC, suggesting donor variability. The upregulation of CD166 on the surface of Is, Ad and Va MSCs under inflammatory conditions, could be a mechanism used by MSCs to adhere T cells to their surface via CD6 to ensure the immunomodulation of T cells in close proximity during inflammation (219, 222, 223). In addition, CD146 is also an adhesion molecule thought to be involved in leukocyte transendothelial migration, therefore its upregulation on MSCs during inflammation could be a mechanism to allow leukocytes to gain access to the site of damage (224). Importantly, HLA-ABC and HLA-DR were upregulated under inflammatory conditions, with Ad MSCs showing the highest expression levels for both markers. As these molecules are involved in transplant rejection, these data have implications when considering which tissue source of MSC might be most suited in a transplant setting.

As well as MSCs being used within transplants, they are also used within other inflammatory diseases (225), with one route of delivery being intravenous infusion into patients (226). One limitation of this route of delivery is the lack of targeted delivery of MSCs to the site of inflammation. Mouse models suggest that MSCs become trapped in the lung after infusion (148, 227), however their anti-inflammatory capacity is not affected by this (228). As the literature suggested pre-stimulation of MSCs before patient infusion in order to upregulate anti-inflammatory mediators secreted by MSCs (229), MSC phenotype during inflammation was assessed. One parameter which might affect the likelihood of MSC entrapment is their size, thus the size of MSCs from Is, Va, Ad, UC and BM was compared under homeostatic and inflammatory conditions where no differences were observed. One particular phenotype which was different from source to source, and which drastically changed under inflammation, was MSC

granularity. Granules are small vesicles within cells that contain a variety of molecules important in immunity for example histamine which is important in vasodilation, CXCL8, a chemokine stored pre-made in Wiebel-Palade bodies important for attracting immune cells, Von Willebrand factor which is important in blood clotting and angiopoietin-2, vital in angiogenesis allowing vascular remodelling (230, 231). Therefore granules hold many factors that contribute to inflammation, homeostasis and angiogenesis. With respect to the functions of the mediators within granules we can conclude that upon MSC de-granulation, MSCs isolated from the Is have the potential to be less inflammatory and less angiogenic under both homeostatic and inflammatory conditions when compared to Va, Ad, UC and BM MSCs. However, this assumption would have to be further tested in order to confirm this conclusion.

All of the data in this chapter show that MSCs isolated from the Is, Va, Ad, BM and UC can confidently be considered MSCs. Additionally, markers that are typically used to identify MSCs that might also have the potential to play a role in MSCs immunomodulatory capacity, CD73, CD90 and CD105, were all expressed at similar levels on all MSCs. MSCs from all sources tested were of similar size and this did not change under inflammatory conditions. The expression of HLA-ABC and HLA-DR on Is, Va and Ad MSCs was upregulated post inflammation, where Is MSCs showed the least expression of both markers. In addition to this, Is MSCs were the least granular out of Ad, BM, UC and Va MSCs. Thus, MSCs isolated from different anatomical locations do not show variation in markers involved in MSC identity, however molecules which may have an impact in a transplant setting such as HLA-ABC and HLA-DR, show subtle variation between MSC sources.

Chapter 4

Transcriptional profiling of MSC chemokine and chemokine receptor expression

Chapter 4 Transcriptional profiling of MSC chemokine and chemokine receptor expression

4.1 Introduction and aims

Work described in the previous chapter led to the conclusion that MSCs isolated from the Islet (Is), visceral adipose surrounding the pancreas (Va), adipose (Ad), bone marrow (BM) and Wharton's jelly within the umbilical cord (UC) satisfied the ISCT MSC classification criteria.

Overall, the aim of this thesis was to assess the potential *in vivo* behaviour of Is, Va, Ad, BM and UC derived MSCs by assessing their response to an inflammatory environment and their interaction with immune cells. As chemokines and their receptors govern the *in vivo* migration and interaction of cells under homeostatic and inflammatory conditions, the transcriptional expression of chemokine and chemokine receptors by MSCs was assessed. Importantly, understanding if MSCs isolated from different tissues exhibit differences in their chemokine and chemokine receptor expression, could highlight a preferential MSC for use within specific diseases.

In addition to MSCs being co-transplanted with e.g. organs, they are being investigated for use within a wide variety of clinical applications such as liver fibrosis and kidney repair (232, 233). For the majority of these applications, systemic infusion (intravenous or intra-arterial) of MSCs is the preferred route of delivery as it is less invasive than local administration (intracoronary injection or direct injection into the tissue of interest). Moreover, as they are infused into arteries or veins, this ensures that they are in close contact to oxygen and nutrient-rich blood vessels (234). However intravenous infusion (IV) is not unproblematic as IV delivered MSCs often results in MSCs getting trapped within the lung (148). Despite pulmonary passage being a challenge for targeted MSC infusion, MSCs have been observed in other tissues such as the gastrointestinal tract, kidney, liver, thymus and spleen following IV injection (148, 235). Whether these observations reflect specific migration of MSCs to these organs or whether the MSCs become entrapped is unknown. Evidence to support the specific migration of MSCs *in vivo* has been published, where MSCs have been

observed to specifically migrate towards tumour microenvironments, wound sites and islets (236-238). Despite the *in vivo* migration of MSCs not being fully understood, it is assumed that it is similar to leukocyte migration and therefore thought to involve the expression of specific chemokine receptors. MSCs isolated from the BM have been shown to bear functional CCR3, CCR4, CCR5 and CXCR4. Interestingly, Ad derived MSCs have been shown to express CXCR4 but at differential levels in comparison to BM derived MSCs, highlighting that MSCs isolated from different sources exhibit differential chemokine receptor expression, suggesting potential variations in MSC migratory potential, which could impact clinical outcome in various clinical settings (98, 182).

Similarly, chemokine secretion by MSCs has been demonstrated to attract leukocytes and thus understanding which chemokines MSCs secrete will allow us to predict which type of leukocytes MSCs might attract towards them in an *in vivo* setting (239-241).

Therefore, this chapter aimed to identify; i) which chemokines and chemokine receptors MSCs expressed at a transcriptional level, ii) whether MSC tissue source influences the expression of these molecules and iii) how inflammation alters MSC chemokine and chemokine receptor transcription.

Due to the large number of samples within this study, initial investigations focussed on establishing a suitable assay. Several factors were considered, including; array sensitivity and accuracy, cost efficiency, reproducibility, high quality controls, appropriate targets and time effectiveness.

After consideration, two arrays were of interest; the TaqMan Low Density Array (TLDA) (Applied Biosystems) and the RT^2 profiler PCR Array (RT^2 Array) (QIAGEN). Despite the TLDA being more time efficient and perhaps more sensitive than the RT^2 array due to the design of the array and its probes, respectively, the inclusion of a built in panel of 5 housekeeping genes (HKG) and 3 internal controls on each plate, resulted in the RT^2 Array being used in these studies. The internal controls on the RT^2 array allowed for the assessment of PCR reproducibility on an intra and inter-plate basis, satisfying the requirements of the controls needed for a large study. Notably, a built-in genomic DNA contamination well on the RT^2 array allowed for the detection of non-specific

DNA amplification, an important factor when dealing with human samples. Moreover, this control monitored the specificity of the primers within the assay, as primers that are not exon-spanning can result in the amplification of genomic DNA.

Therefore, initial experiments focussed on the reliability and reproducibility of the RT^2 arrays.

Following that, the RT^2 arrays were used to transcriptionally profile the expression of chemokines and their receptors by Is, Va, Ad, UC and BM MSCs. See Section 2.3 for specific methodology of the RT^2 arrays.

Results

4.2 Assessment and validation of RT^2 Arrays for transcriptional analysis of chemokine and chemokine receptor expression by MSCs

Due to the large numbers of samples tested under both homeostatic and inflammatory conditions, it was expected to appreciate that not all samples could be collected, prepared and tested at one time. Therefore sample preparation would occur over several weeks, resulting in transcriptional analysis of MSC chemokine and chemokine receptor expression being carried out on different days. To ensure that this approach was feasible and accurate, a ruler sample (human skin fibroblast isolated induced pluripotent stem cells (IPSCs) differentiated into MSCs (IPMSC), provided by Dr Jo Mountford – University of Glasgow was spread over three RT^2 arrays. A ruler sample is a sample which is an example of your cell type and allows for cross-array comparison. Using the ruler sample over three plates and running them on different days allowed us to detect possible discrepancies of cDNA conversion, cDNA storage and machine laser alignment on separate days.

4.2.1 IPMSCs satisfy the ISCT criteria to be classified as an MSC

In order for IPMSCs to serve as an accurate ruler sample on the RT^2 arrays, experiments were carried out to ensure that they satisfied ISCT criteria of a MSC prior to transcriptional assessment.

As well as being spindle shaped (highlighted with black arrows) and adherent to plastic (**Figure 4-1** IPMSCs can be classified as MSCs**A**), flow cytometry was used to assess IPMSCs surface marker expression of typical MSC markers (**Figure 4-1 B**). 98.17% of IPMSCs expressed CD105 (B.iv), 98.06% expressed CD90 (B.iv) and 94.88% expressed CD73 (B.v). Additionally only 3.73% of IPMSCs expressed CD14 (B.v), 2.76% expressed CD45 (B.vi) and 1.82% expressed CD34 (A.vi). In order to satisfy all of ISCT's criteria, IPMSCs were also assessed for their differentiation capabilities (**Figure 4-1 C & D).** IPMSCs demonstrated successful differentiation into adipocytes using anti-fatty acid binding protein 4 (FABP4) antibody (C.i) and chondrocytes using anti-aggrecan antibody (D.i). Specificity of the antibodies was demonstrated through the lack of fluorescence in isotype (C.ii, D.ii) and no primary controls (C.iii, D.iii).

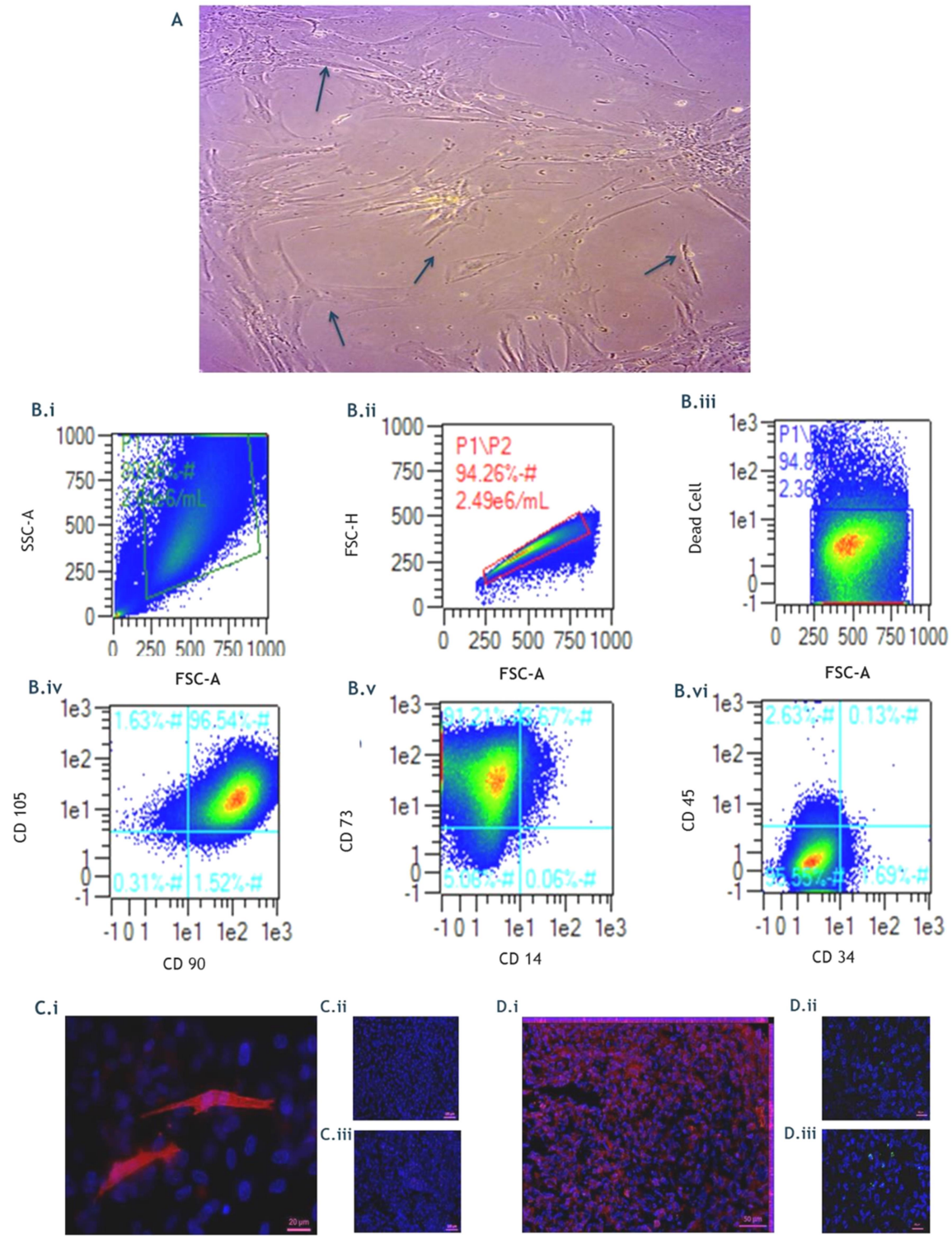


Figure 4-1 IPMSCs can be classified as MSCs
Morphologically, IPMSCs presented as spindle shaped cells(marked with black arrows) that adhered to plastic (**A).** Flow cytometry was used to assess the surface protein expression of MSC markers on IPMSCs **(B).** 96.54% of IPSCs expressed both CD105 and CD90 (**B.iv**) and 91.21% of IPSCs expressed CD73 (**B.v**). IPSCs were negative for CD14, CD45 and CD34 (**B.v, B.vi**). Fluorescence microscopy was used to assess the differentiation of IPMSCs into adipocytes **(C.i)** (detected by the specific binding of fatty acid binding protein 4 (FABP4) (RED)) and IPMSC differentiation into chondrocytes **(D.i)** (detected by the specific binding of aggrecan) (RED)). Specificity of both antibodies was demonstrated through the absence of fluorescence in isotype controls (**C.ii & D.ii**) and no primary controls (**C.iii and D.iii**). DAPI (blue) stains cell nuclei. IPMSC n=1.

Therefore, based on their spindle shaped morphology, surface marker expression and ability to differentiate into adipocytes and chondrocytes, IPMSCs could be classified as MSCs, which allowed them to serve as an accurate ruler sample on the RT^2 arrays.

4.2.2 **Standardisation of RT^2 Arrays**

4.2.2.1 **Experimental design and plate set-up**

To assess the consistency and accuracy of the RT^2 arrays, the experimental set up was designed to test for potential areas where inconsistencies might arise, such as cDNA synthesis on different days, freezing of cDNA vs immediate use of cDNA, and PCR efficiency on different days. To achieve this, RNA was extracted from one IPMSC sample, frozen and then converted into cDNA on three different days, resulting in 3 lots of cDNA from one sample of RNA. cDNA that was synthesised on day 1 was immediately put onto plate 1 and analysed. cDNA synthesised on day 2 was frozen for 1 day and run on plate 2. cDNA synthesised on day 3 was frozen for 2 days and run on plate 3 (Figure 4-2). Whilst the experimental set up was not precise enough to pin point exactly where discrepancies might be introduced, the design aimed to test the system for inherent discrepancies.

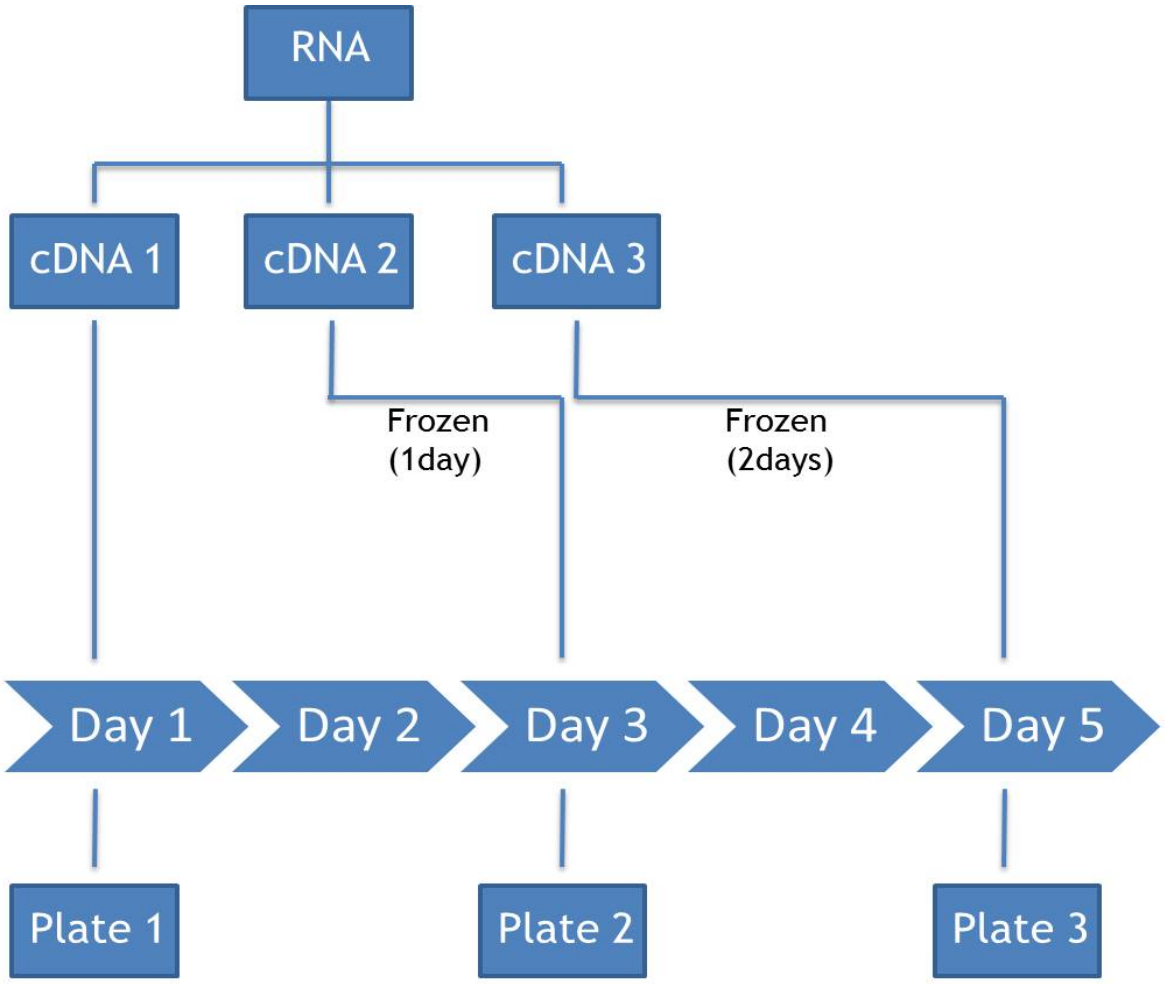


Figure 4-2 Experimental workflow outlining the timeline of events when testing the RT^2 arrays reproducibility.
3 lots of cDNA were synthesised from RNA on Day 1, Day 2 and Day 3. cDNA synthesised on day 1 was used immediately on an RT^2 array. cDNA synthesised on day 2 was frozen for one day and applied to the RT^2 array the following day. cDNA synthesised on day 3 was frozen for 2 days and run on the RT^2 arrays 48 hours later. This set up resulted in 3 plates being assayed over 5 days.

The experimental set up is further outlined in **Figure 4-3**. Each plate consisted of 94 genes, with 4 wells per gene. Thus, the most accurate and cost efficient approach was to have 2 technical replicates per sample, per gene. Therefore IPMSC cDNA was applied to half of each of the three plates and either Is, Va or Ad cDNA on the other half (**Figure 4-3 B**).

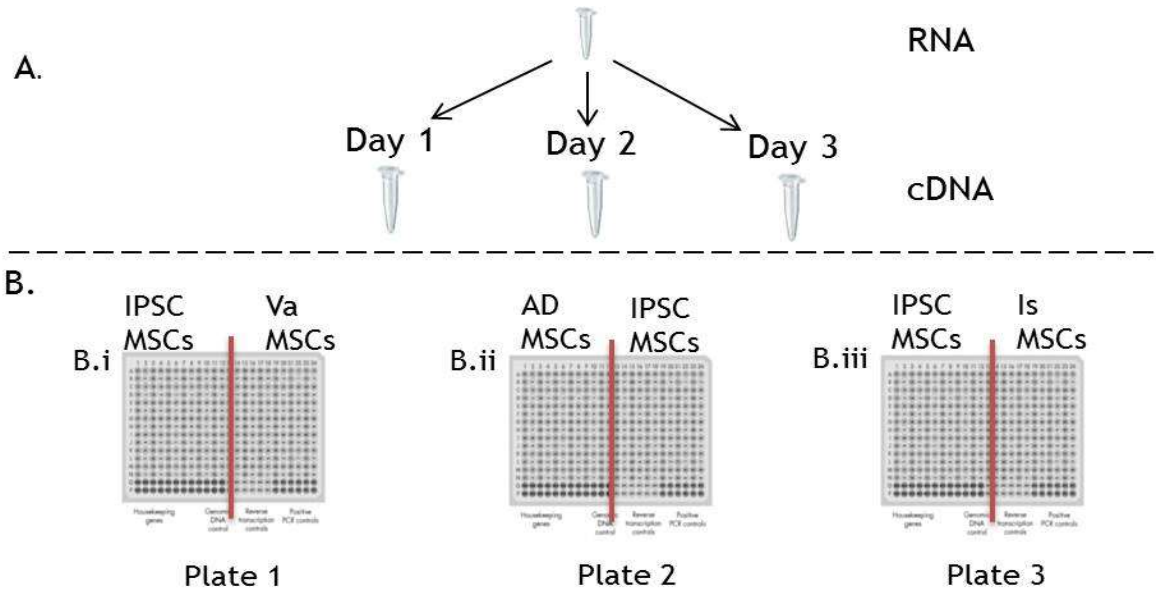


Figure 4-3 Diagrammatic illustration of the preparation of cDNA and RT^2 array set up.

To assess if cDNA synthesis on different days would result in varied, non-comparable results, three lots of cDNA were synthesised over three separate days (**A**). The IPMSC cDNA synthesised on day one was applied to one half of plate 1, with Va MSCs applied to the other half of plate 1 (**B.i**), IPMSC cDNA synthesised on day two was applied to one half of the plate 2, with Ad MSCs applied to the other half of plate 2 (**B.ii**) and IPMSC cDNA synthesised on day three was applied to one half of plate 3, with Is MSCs applied to the other half of plate 3 (**B.iii**).

4.2.2.2 **Analysis of controls to assess intra- and inter-plate reproducibility**

To measure the reproducibility of the plates on an intra- and inter- plate basis, three controls were assessed;

1. Reverse transcription control (RTC) wells
2. Positive PCR control (PPC) wells
3. Comparison of the cycle threshold (CT) results from the IPMSC cDNA.

RTC wells assessed the efficiency of the reverse transcription reaction in the cDNA synthesis step (**Figure 4-4 A**). A ΔCt (details on how ΔCt values were

calculated are in Section 2.3.3.5) value less than 5 signified successful reverse transcription (http://www.sabiosciences.com/pcrarraycontrols.php), therefore the reverse transcription step for the cDNA synthesis of plate 1 (ΔCt = 1.74), plate 2 (ΔCt = 1.13) and plate 3 (ΔCt=1.43) was successful. Importantly, similar RTC ΔCt values on each plate suggested that although cDNA was synthesised on different days for each plate, this has no effect on the reverse transcription reaction and each reaction performed to a comparable level (Figure 4-4 **B**).

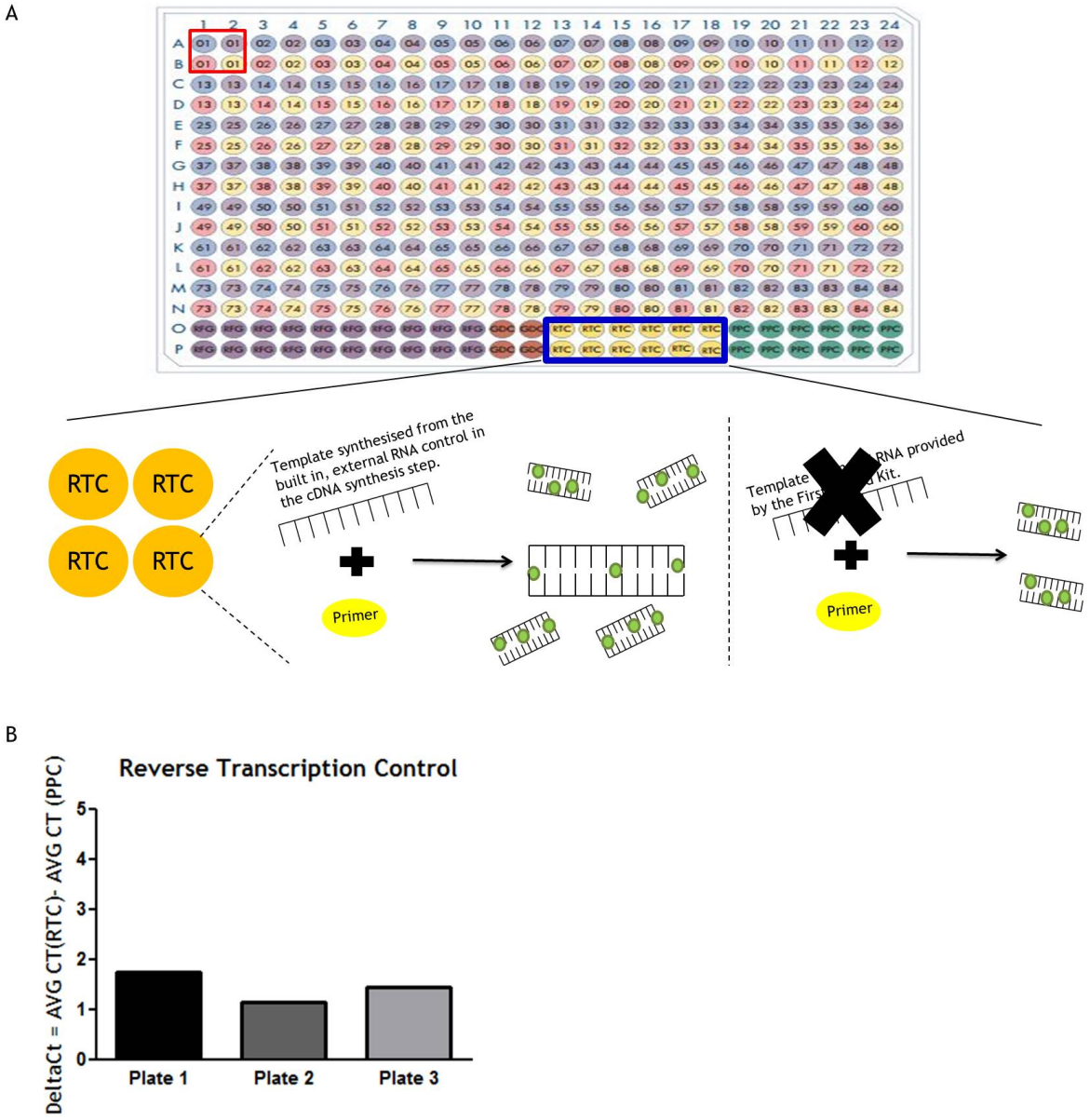


Figure 4-4 Internal controls on the RT^2 array suggested accurate reverse transcription over several days.
A schematic of the RT^2 array is included for reference and clarity (**A**). The schematic illustrates the setup of the genes in quadruplicate (indicated by the red box) and internal controls at the bottom of the plate (rows O + P). These internal controls include; 5 housekeeping genes (purple wells), Genomic DNA contamination control wells (red wells), RTC wells (indicated by the blue box) and PPC wells (green wells). The experimental set up was the same as in Figure 4-3 where IPMSC cDNA was applied to three plates in order to assess the reproducibility of the RT^2 arrays. Due to patented technology in the control wells, information on their make-up and execution is limited, thus the schematics represent the control wells starting materials and methods of detection in a very simplistic manner. The reverse transcription control wells detected a template synthesised by an RNA template provided by the RT^2 first strand kit (cDNA synthesis kit). If there were enzyme inhibitors, low incorporation of SYBR into the newly synthesised DS DNA from the internal RNA template will result in very high CT values **(A)**. The RTC from all of the wells containing IPMSC cDNA on one plate (6 wells) is calculated and graphed as either plate 1 (1.74), plate 2 (1.13) or plate 3 (1.43) (**B**). For more details on RTC controls, visit: http://www.sabiosciences.com/pcrarraycontrols.php.

Positive PCR control wells (PPC) assessed the efficiency of the PCR reaction (**Figure 4-5 A**). The PCR efficiency for each sample was monitored by this internal control. A C_T of 20±2 suggested that the PCR reaction was optimal (http://www.sabiosciences.com/pcrarraycontrols.php). Plate 1 (C_T=21.16), Plate 2 (C_T=21.81) and Plate 3 (C_T=21.33) demonstrated successful PCR reproducibility for the IPMSC sample on an intra-plate basis (**Figure 4-5 B**). Notably, this value did not vary more than 0.34 of a Ct between plates, indicating that PCR reproducibility was also precise and successful on an inter-plate basis.

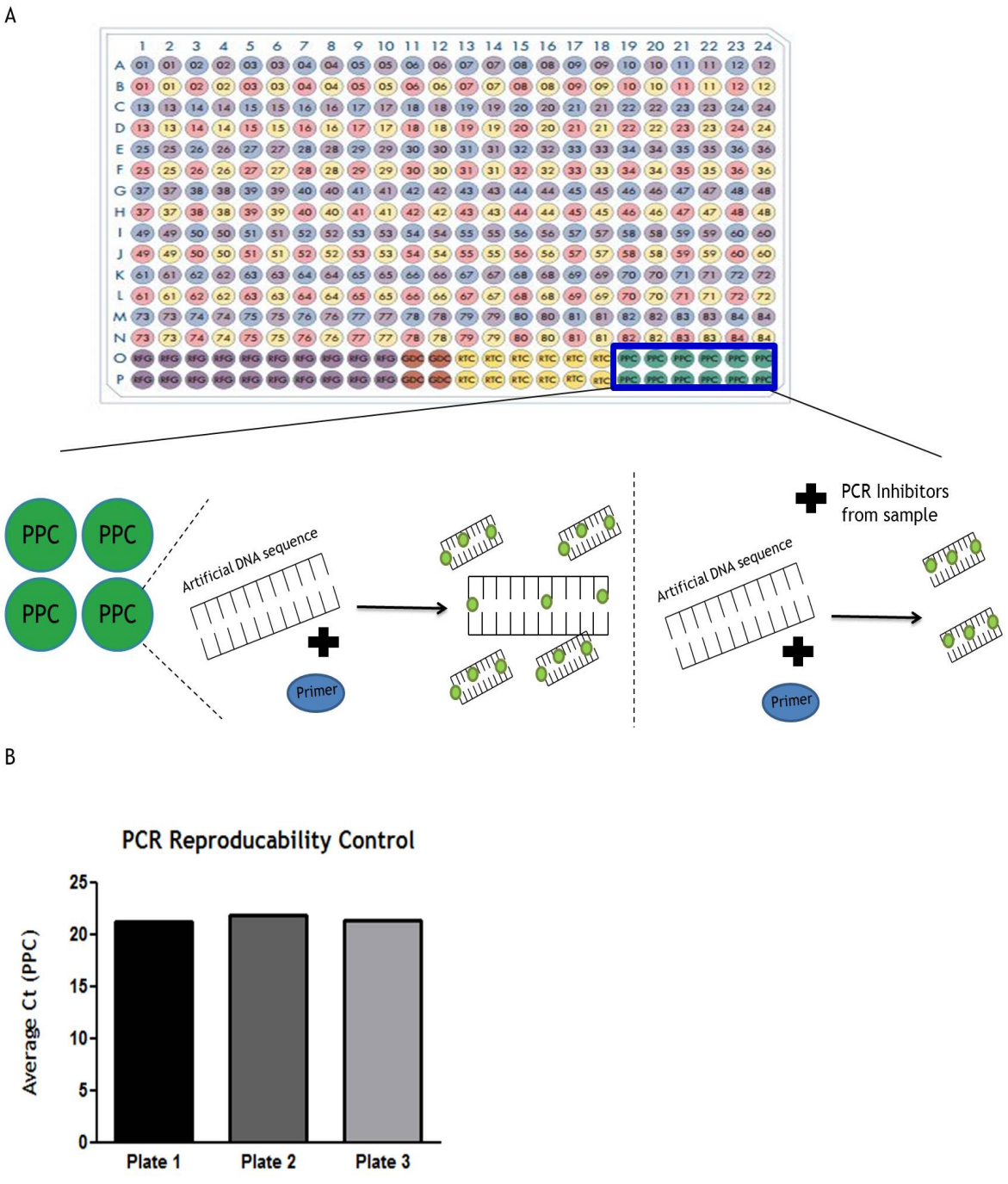


Figure 4-5 Internal controls on the RT^2 PCR profiler array suggested accurate PCR reproducibility over several days.
Experimental set up and design was as shown in Figure 4-3.

PPC wells are highlighted by the blue box at the bottom right hand side of the 384 well plate (**A**). They consisted of an artificial DNA sequence and the assay which detects it (Primer in blue) (**A**). If PCR inhibitors exist in your sample / in a well then PCR efficiency will be reduced and will result in low CT values or very high CT values – depending on which type and quantity of PCR inhibitors are present. Reverse transcription control value (ΔC_t) was calculated by ΔC_t = AVG C_T^{RTC}- AVG C_T^{PPC}. The PPC was calculated as the average CT value across the 6 wells. IPMSCs average CT values from plate 1 (21.16), plate 2 (21.81) and plate 3 (21.33) are plotted (**B**). For more details on PPC controls, visit: http://www.sabiosciences.com/pcrarraycontrols.php.

To further assess the inter-plate consistency of the RT^2 arrays, the raw CT values from the IPMSC samples for each of the 84 genes were graphed to assess the correlation between each of the plates **(Figure 4-6).** Plate 1 vs plate 2 and plate 1 vs plate 3 showed high levels of correlation, with an R^2 value of 0.9493 (**Figure 4-6 A**) and 0.9359 (**Figure 4-6 B**), respectively. Plate 2 vs plate 3 displayed the largest correlation with an R^2 value of 0.9771 (**Figure 4-6 C**).

Reproducibility of the results was further examined by statistically assessing the IPMSC CT values of 10 genes. The cycle threshold values (Ct) of 5 housekeeping genes and 5 genes with a Ct near that of undetectable (Ct>35) were graphed and analysed to ensure that there were no significant differences between them. A Ct of 35 or over was the recommended "undetectable" value on the RT^2 arrays provided by QIAGEN.

Ct values of the house keeping genes on all three plates were exceptionally close with an average standard deviation of 0.32 (**Figure 4-6 D**). As expected, the CT values of genes near the limit of no detection were slightly more spread, with an average standard deviation of 0.46 (**Figure 4-6 E**). No significant differences were calculated between the CT values of the house keeping genes, or the genes with high CT values.

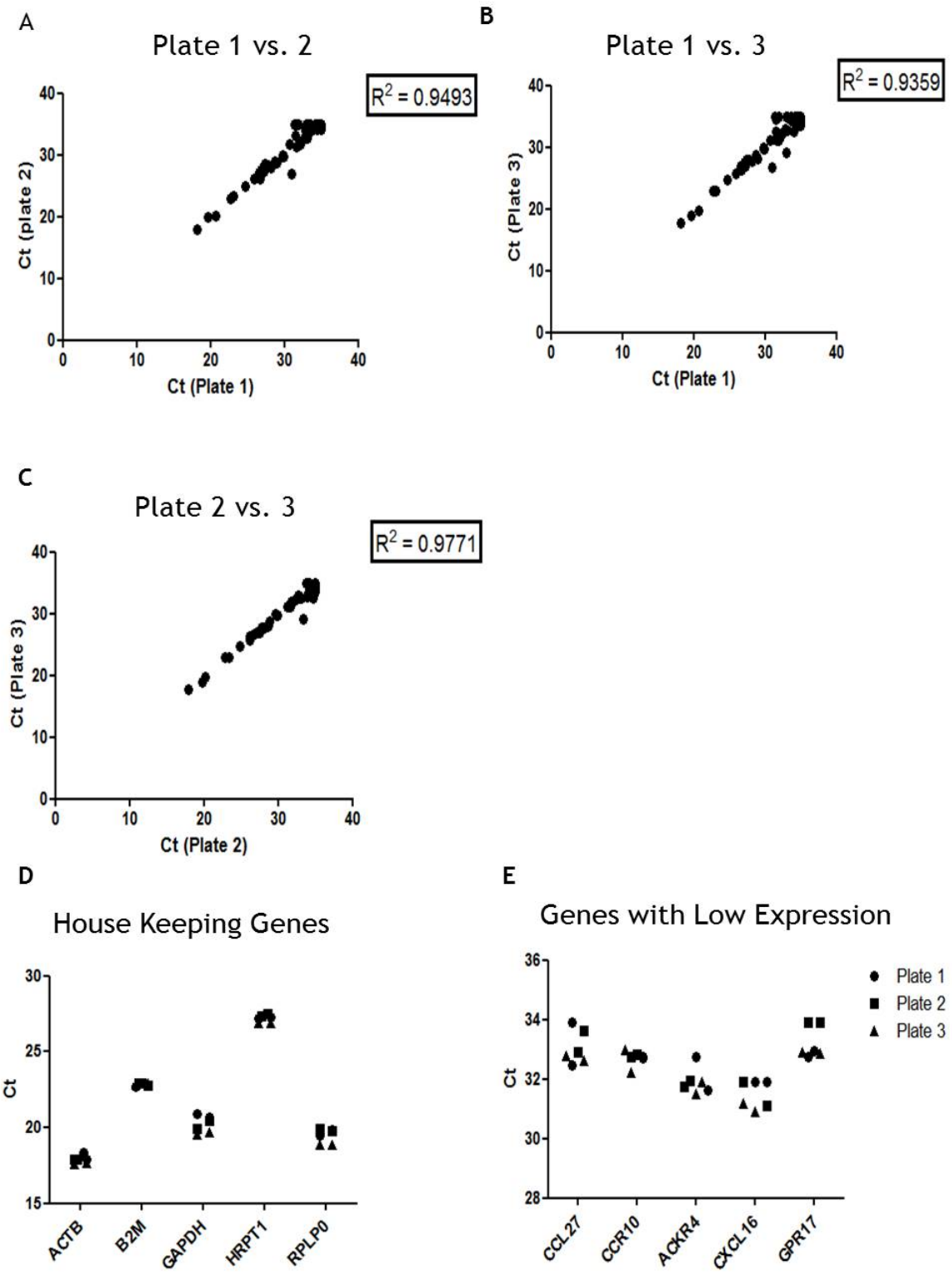


Figure 4-6 Strong correlations of RT^2 arrays suggested that inter-plate analysis was comparable.

IPMSCs were used as a ruler sample to compare the reproducibility of the arrays. The IPMSC Ct values of all the genes present on the plate (94) were compared to each other across the three plates, and the correlation (R^2) was calculated. Comparison of all plates illustrated a high correlation value of >0.93 (**A-C**). Housekeeping genes **(D)** and 5 genes with very little to no mRNA expression (**E**) (based on CT value), were graphed and analysed to determine if there were any significant differences between the CT values from each plate. No significant differences were observed when comparing the raw IPMSC CT values over three plates.

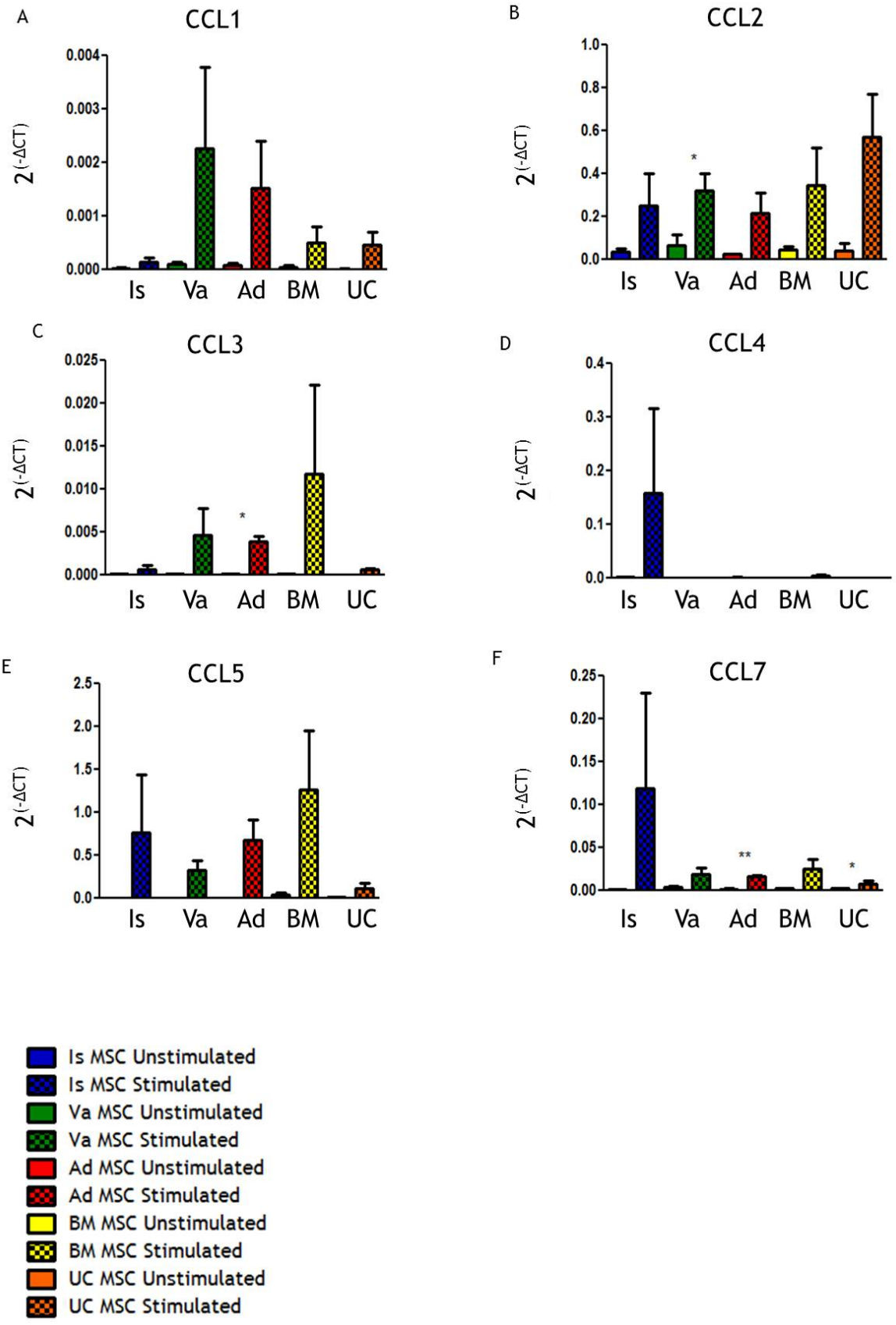
Taken together, successful RTCs, PPCs and high correlation of all Ct values on the three plates ($R^2 > 0.93$) suggested that although cDNA synthesis, plate preparation and plate reading occurred on different days, the reproducibility of the array was suitably robust to allow intra- and inter-plate analysis. Moreover, no significant differences were observed between genes of high or low CT value, therefore concluding that the RT^2 arrays were consistent over different PCR runs and were therefore suited for this large study.

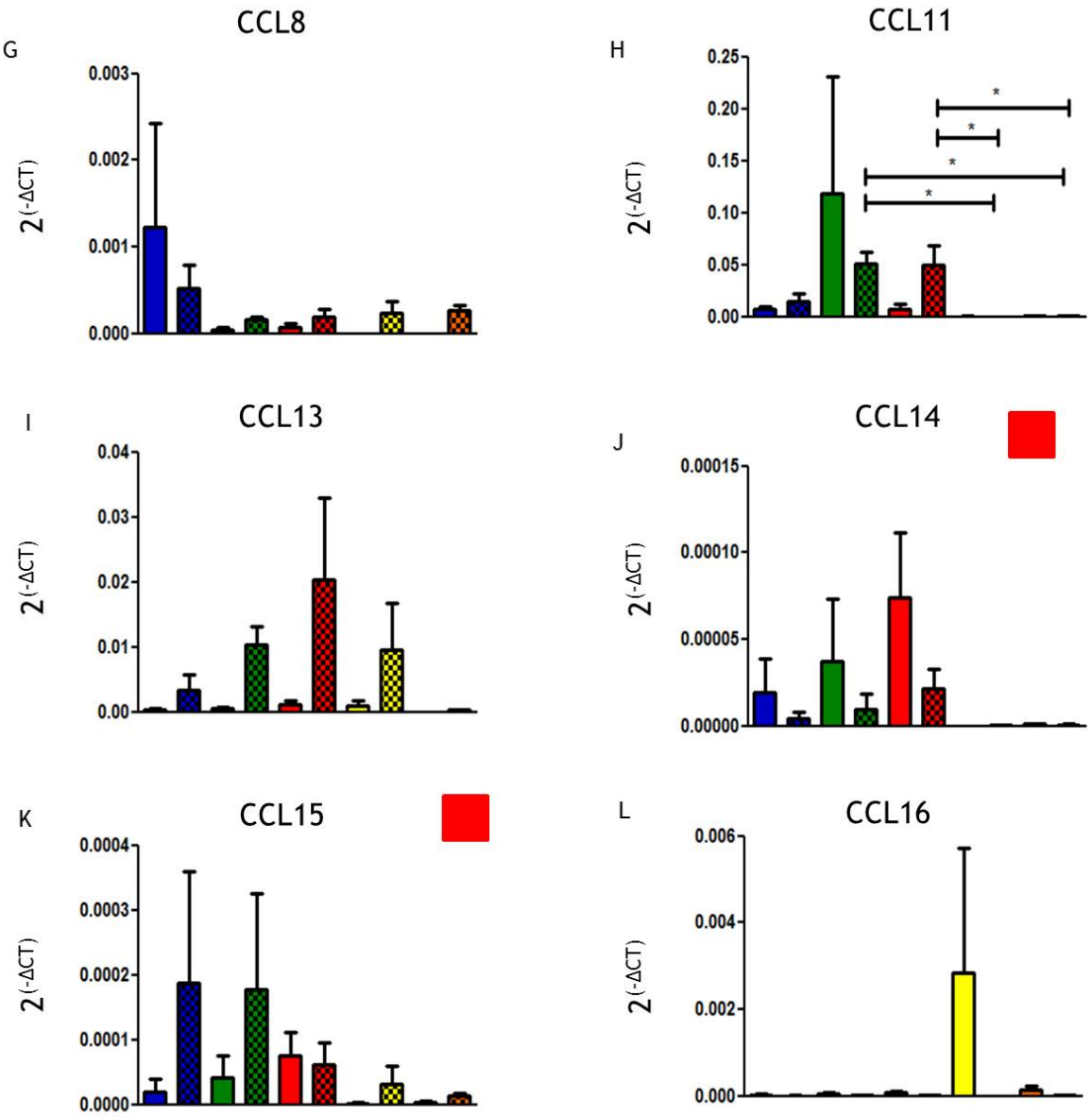
4.3 Transcriptional analysis of chemokine and chemokine receptor expression by Is, Va, Ad, UC and BM MSCs under homeostatic and inflammatory conditions.

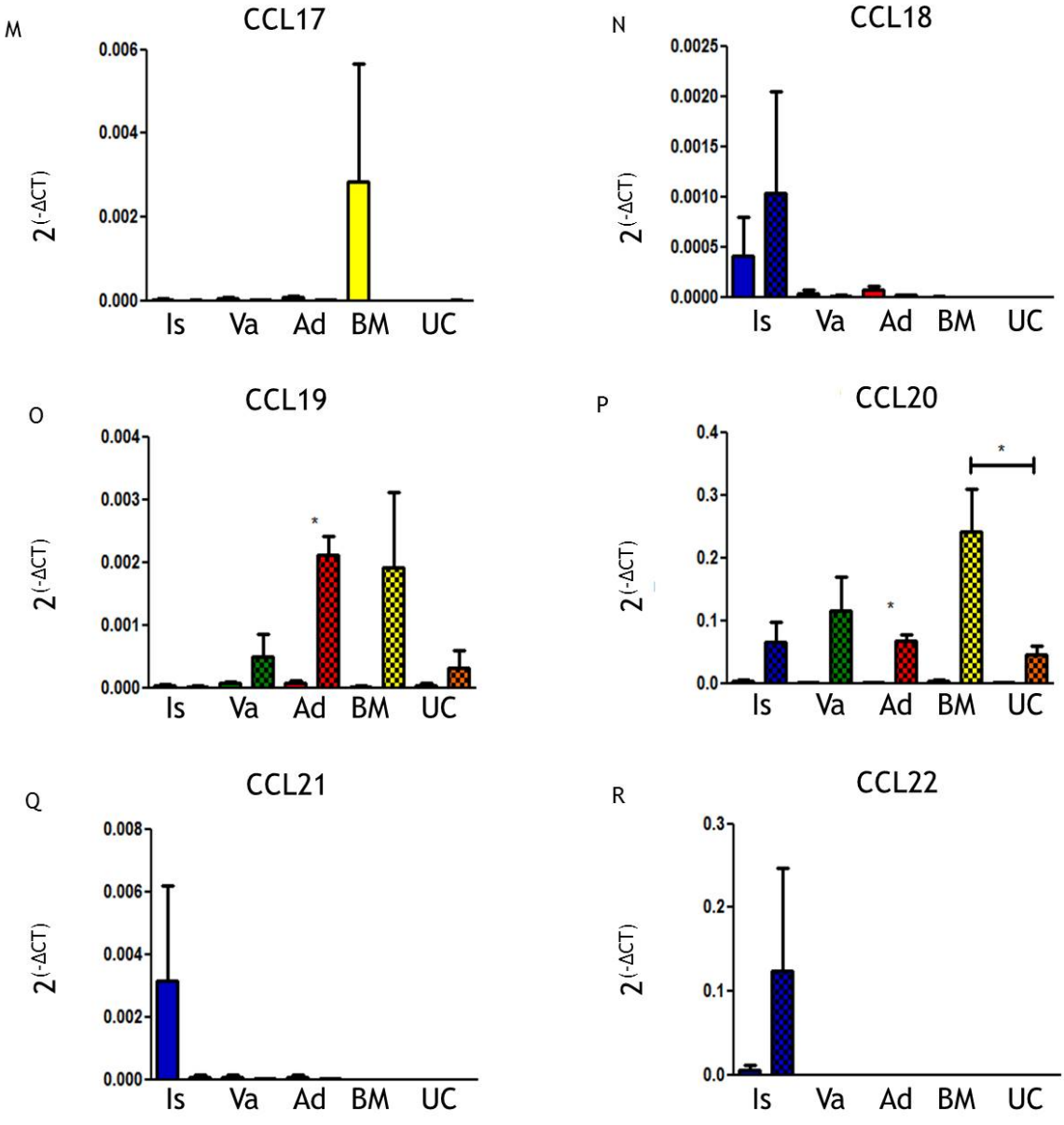
To understand how MSCs might migrate and interact with the immune system, it was important to elucidate their chemokine and chemokine receptor expression. To do this, transcriptional analysis of all the chemokines and their receptors, along with other molecules important in migration and interaction with the immune system were carried out. Is, Va, Ad, UC and BM MSCs were transcriptionally evaluated under homeostatic and inflammatory conditions to determine whether MSC tissue source and/or inflammatory conditions altered the MSC chemokine and chemokine receptor transcriptional profile.

4.3.1 Transcriptional analysis of CC chemokines under homeostatic and inflammatory conditions.

The results of the transcriptional analysis of the CCL chemokines (and all other genes) are graphed as $2^{(-\Delta CT)}$ (**Figure 4-7**). All transcriptional data throughout this chapter was normalised to the HKG beta-2 microglobulin (B2M). As this study did not have a reference sample (i.e. we did not want to visualise an upregulation or downregulation in specific genes relative to one specific MSC sample), the data is represented as $2^{(-\Delta CT)}$ as opposed to $\Delta\Delta$Ct. This representation of the data allows one to visualise the 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 by MSCs.


A
CCL1
2^(-ΔCT)
0.004
0.003
0.002
0.001
0.000
Is
Va
Ad
BM
UC
B
CCL2
1.0
0.8
0.6
0.4
0.2
0.0
*
C
CCL3
0.025
0.020
0.015
0.010
0.005
0.000
*
D
CCL4
0.4
0.3
0.2
0.1
0.0
E
CCL5
2.5
2.0
1.5
1.0
0.5
0.0
F
CCL7
0.25
0.20
0.15
0.10
0.05
0.00
**
*
Is MSC Unstimulated
Is MSC Stimulated
Va MSC Unstimulated
Va MSC Stimulated
Ad MSC Unstimulated
Ad MSC Stimulated
BM MSC Unstimulated
BM MSC Stimulated
UC MSC Unstimulated
UC MSC Stimulated


G
CCL8
2 (-ΔCT)
0.003
0.002
0.001
0.000
H
CCL11
2 (-ΔCT)
0.25
0.20
0.15
0.10
0.05
0.00
*
*
*
*
I
CCL13
2 (-ΔCT)
0.04
0.03
0.02
0.01
0.00
J
CCL14
2 (-ΔCT)
0.00015
0.00010
0.00005
0.00000
K
CCL15
2 (-ΔCT)
0.0004
0.0003
0.0002
0.0001
0.0000
L
CCL16
2 (-ΔCT)
0.006
0.004
0.002
0.000


M
CCL17
0.006
0.004
0.002
0.000
2(-ΔCT)
Is Va Ad BM UC
N
CCL18
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
2(-ΔCT)
Is Va Ad BM UC
O
CCL19
0.004
0.003
0.002
0.001
0.000
2(-ΔCT)
*
Is Va Ad BM UC
P
CCL20
0.4
0.3
0.2
0.1
0.0
2(-ΔCT)
*
*
Is Va Ad BM UC
Q
CCL21
0.008
0.006
0.004
0.002
0.000
2(-ΔCT)
Is Va Ad BM UC
R
CCL22
0.3
0.2
0.1
0.0
2(-ΔCT)
Is Va Ad BM UC

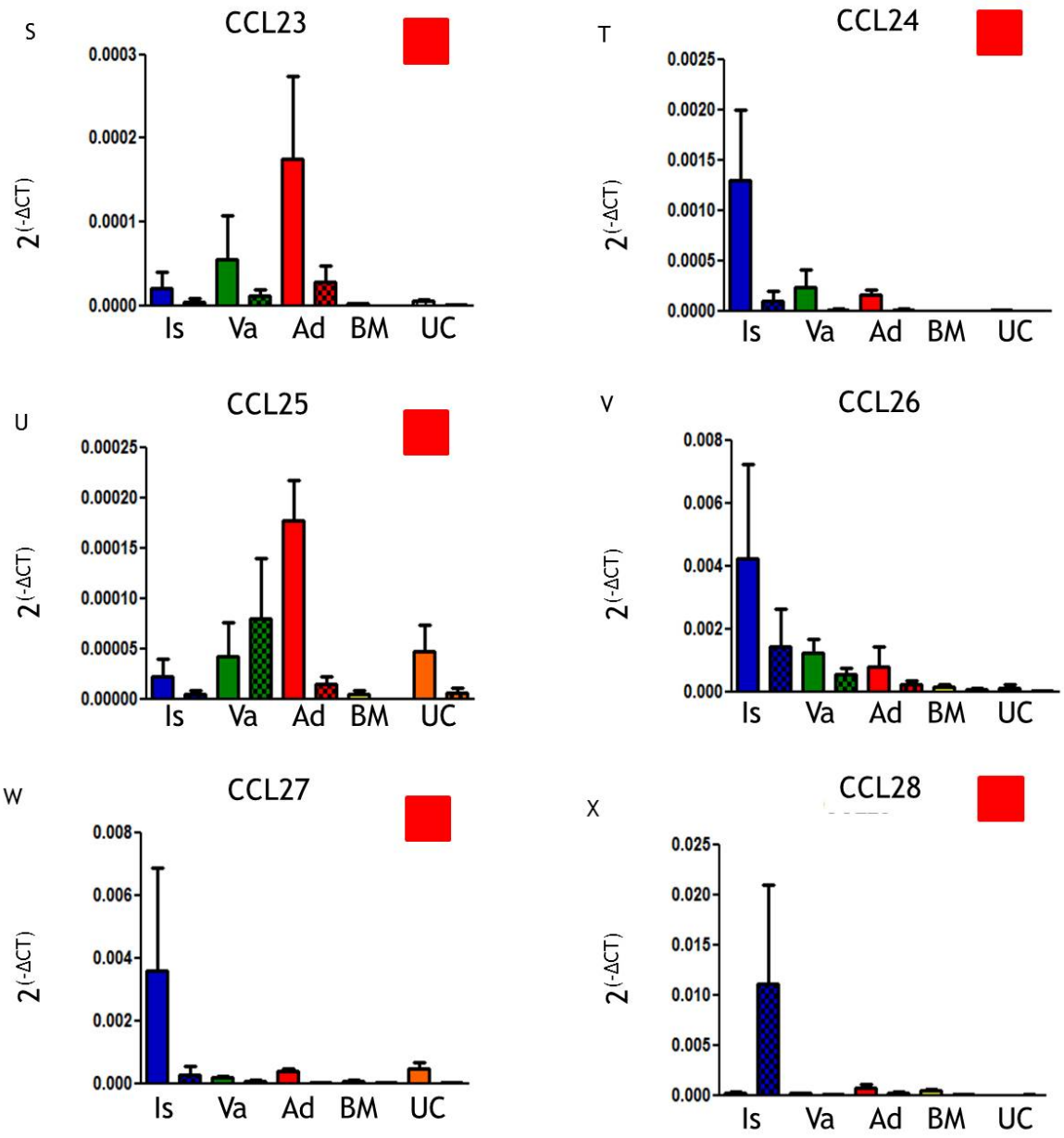


Figure 4-7 Inflammation and MSC tissue origin impacts CC chemokine transcript levels in MSCs.
MSCs from all sources were grown to passage 3. MSCs isolated from each donor (n of 3 for each MSC tissue source) were split into two T75 flasks. Once MSCs reached 80% confluency, MSCs were either cultured under homeostatic conditions or treated with 10ng/mL IFN-gamma, TNF-alpha and IL-1beta in MSC medium for 24 hours. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate CCL transcripts in Is, Va, Ad, BM and UC MSCs under homeostatic and inflammatory conditions. Bars represent mean ± SEM (n=3). Data are normalised to the HKG B2M and expressed as $2^{(-\Delta CT)}$. Appropriate statistical analysis was performed and includes Students paired T test between one MSC tissue source (Homeostatic vs Inflammatory Conditions) and One Way ANOVA with Tukey's multiple comparisons post-test to compare all MSC sources. Significant differences are marked with the appropriate number of asterisks and P values are stated throughout the text.

Under homeostatic conditions, Is, Va, Ad, BM and UC 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 4-7 B**).

A pattern of transcriptional upregulation after stimulation in all MSCs was observed in CCL1 (**Figure 4-7 A**), CCL2 (**B**), CCL3 (**C**), CCL5 (**E**), CCL7 (**F**), CCL13 (**I**), CCL19 (**O**) and CCL20 (**P**). Noticeably, these chemokines were upregulated differentially, depending on MSC tissue source:

Va and Ad MSCs exhibited the largest transcriptional upregulation of CCL1, whereas Is, BM and UC MSCs showed little upregulation (**Figure 4-7 A**). As mentioned, CCL2 was transcriptionally expressed under homeostatic conditions at fairly substantial levels in all MSCs with Va MSCs expressing the most. Inflammatory stimulation resulted in a substantial and significant upregulation of CCL2 transcripts by Is, Ad, BM and UC MSCs and a significant upregulation by Va MSCs (P=0.0129). UC MSCs had the highest levels of CCL2 transcripts, whereas Is MSCs have the lowest under inflammatory stimulation (Figure 4-7 **B**). CCL3, transcribed at similar levels to CCL1, showed a large spread between sources. Like CCL1, expression levels of CCL3 varied between sources under inflammation with Va, Ad and BM expressing the most transcripts and Is and UC MSCs expressing relatively little (**Figure 4-7 C**).

CCL5 was the most transcribed CCL in BM, Ad and Is MSCs. Under homeostatic conditions, CCL5 was transcribed at low levels by BM MSCs only. Inflammatory stimulation resulted in all MSCs upregulating CCL5 transcription. BM MSCs produced the most CCL5 transcripts, an 11 fold upregulation compared to UC MSCs which transcribed the least CCL5 (**Figure 4-7 E**).

Transcript levels of CCL7 were very low during homeostatic conditions. Under inflammatory stimulation CCL7 was significantly upregulated by Ad (P=0.0018) and UC MSCs (P=0.0142), however, overall transcription levels still remained relatively low. One Is MSC donor exhibited high CCL7 transcripts during inflammatory conditions, accounting for the large error bar (**Figure 4-7 F**).

CCL19 was transcribed at low levels in all MSC sources during homeostatic conditions. Following inflammatory stimulation, all MSCs (with the exception of

UC MSCs) upregulated CCL19. Transcriptional levels of CCL19 varied between sources with Ad MSCs transcribing 107 fold more than Is MSCs (**Figure 4-7 O**).

During homeostatic conditions, CCL20 was transcribed at similar levels to CCL19 during inflammation. Inflammatory stimulation resulted in a substantial upregulation of CCL20 in BM MSCs, resulting in BM MSCs producing significantly higher CCL20 transcripts than UC MSCs ($P \leq 0.05$) (Figure 4-7 **P**).

Other CCL chemokines do not show the same pattern of upregulation under inflammatory stimulation. CCL8 (**Figure 4-7 G**), CCL11(**H**), CCL14(**J**), CCL15(**K**), CCL16(**L**), CCL17(**M**), CCL18(**N**), CCL21(**Q**), CCL22(**R**), CCL23(**S**), CCL24(**T**), CCL25(**U**), CCL26(**V**), CCL27(**W**) and CCL28(**X**) showed variable patterns of upregulation or downregulation under inflammatory stimulation between MSC tissue sources. Transcripts of all of these chemokines, with the exception of CCL11, were very low (as marked with a red box).

During homeostatic conditions, CCL11 was transcribed at low levels by Is and Ad MSCs and high levels by Va MSCs. BM and UC MSCs did not transcribe CCL11 during homeostatic conditions. Inflammatory stimulation resulted in a pattern of CCL11 transcriptional upregulation by all MSC sources except Va MSCs, where CCL11 was downregulated. Tissue origin of MSCs also had an influence on CCL11 transcription under inflammatory conditions, where Va transcribed significantly more CCL11 than BM and UC MSCs, as did Ad MSCs ($P \leq 0.05$ in all cases) (**Figure 4-7 H**).

In summary, MSCs from all sources expressed a variety of CCL chemokines, suggesting that they could attract immune cells such as monocytes, macrophages, NK cells, eosinophils and B cells. During homeostatic conditions, MSCs transcribed very low levels of CCLs, with the exception of CCL2. Conversely, inflammatory stimulation resulted in a marked upregulation of CCL transcription by all tissue sources of MSCs. Importantly, the level of CCL2 transcript production varied with source.

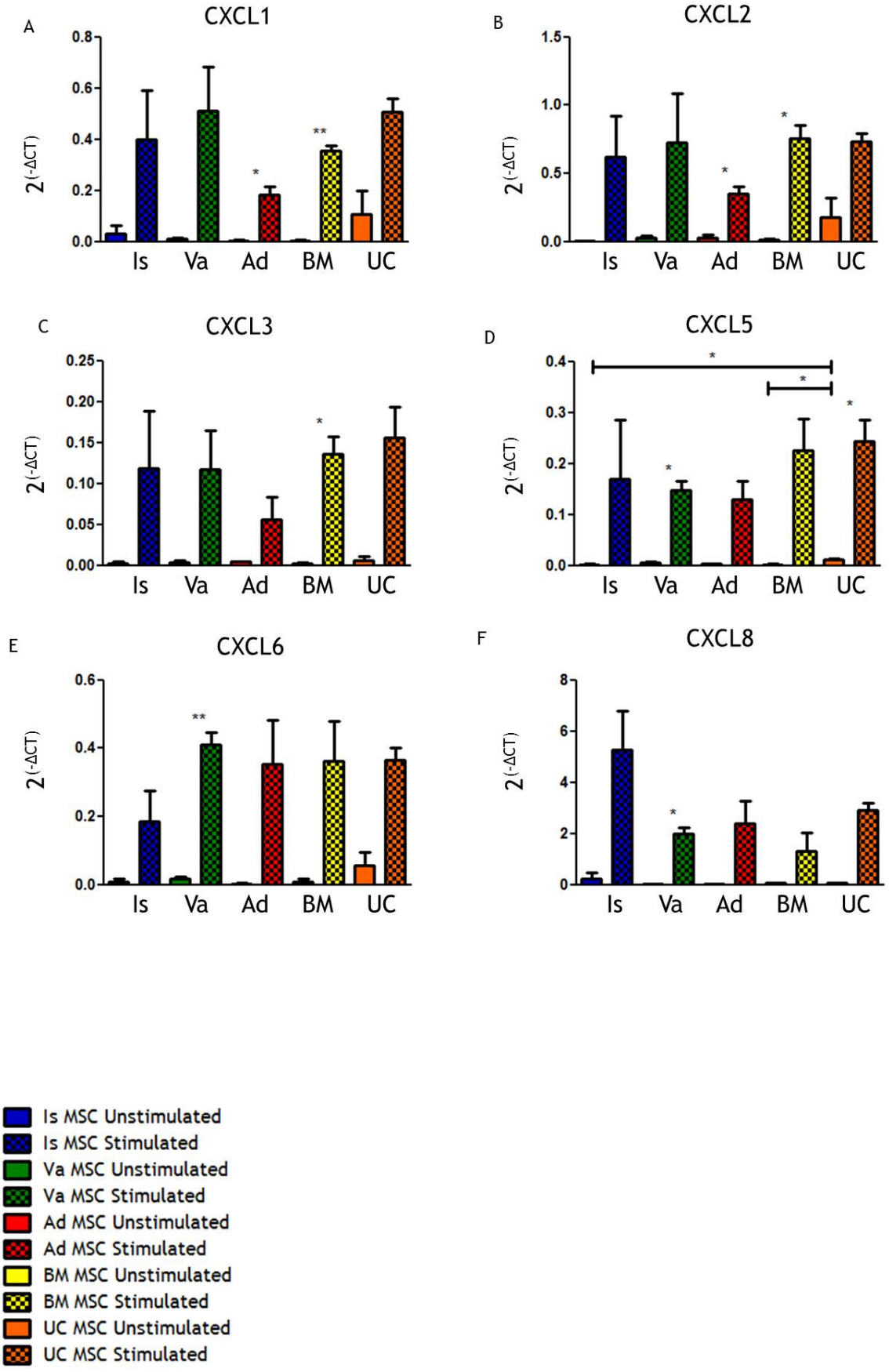
4.3.2 **Transcriptional analysis of CXC chemokines under homeostatic and inflammatory conditions.**

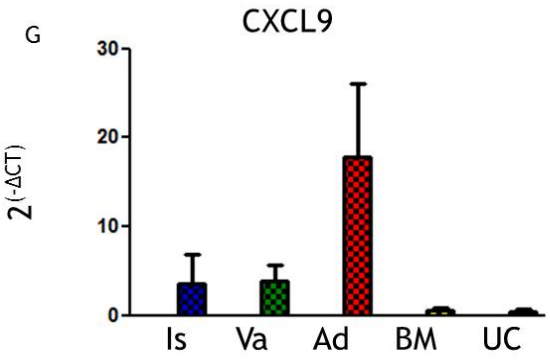
During homeostatic conditions, CXCL transcripts in all MSC sources were low with the exception of UC MSCs which transcribed CXCL1 (**Figure 4-8 A**), CXCL2 (**Figure 4-8 B**), and CXCL6 (**Figure 4-8 E**) at slightly higher levels than other MSC sources. UC MSCs also transcribed significantly higher quantities of CXCL5 in homeostatic conditions when compared to BM MSCs ($P \leq 0.05$) and Is MSCs ($P \leq 0.05$) (**Figure 4-8 D**).

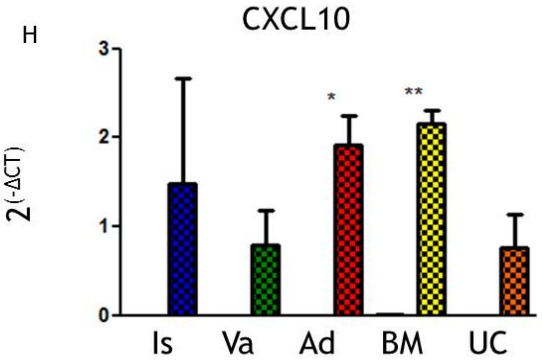
Under inflammatory stimulation, CXCLs 1, 2, 3, 5, 6, 8, 9, 10 and 11 transcripts were substantially upregulated by all MSC sources. CXCL1 transcripts were significantly upregulated by Ad MSCs ($P=0.0262$) and BM MSCs ($P=0.0043$) when compared to homeostatic conditions. CXCL1 transcript levels varied slightly between MSC tissue sources under inflammatory conditions, where Va MSCs transcribed 2.7 fold more than Ad MSCs (**Figure 4-8 A**). Similarly, CXCL2 was also significantly upregulated by Ad ($P=0.0130$) and BM MSCs ($P=0.0204$) after 24 hours of inflammatory stimulation. Transcriptional levels of CXCL2 differed very slightly between MSC tissue sources where Ad MSCs transcribed the least and Is, Va, UC and BM MSCs transcribed comparable levels (**Figure 4-8 B**). BM MSCs significantly upregulated CXCL3 transcripts ($P=0.0240$) under inflammatory stimulation, however, despite not reaching significance Is, Va, Ad and UC MSCs also markedly upregulated CXCL3 transcripts, therefore resulting in no differences in CXCL3 transcription between MSC sources (**Figure 4-8 C**). CXCL5 transcripts were significantly upregulated by Va ($P=0.0172$) and UC ($P=0.0435$) MSCs as well as being substantially upregulated by all other MSC sources under inflammatory stimulation. Despite differences in CXCL5 transcript levels between MSC sources maintained under homeostatic conditions, upon inflammatory stimulation, MSCs from all tissue sources transcriptionally upregulated CXCL5 to similar levels (**Figure 4-8 D**). Likewise, CXCL6 was transcriptionally expressed at equivalent levels across all MSC tissue sources under inflammatory conditions. Slight differences were observed between Is MSCs and Va MSCs, where Va MSCs expressed a $2(^{-\Delta CT})$ 2 fold higher than that of Is MSCs (**Figure 4-8 E**).

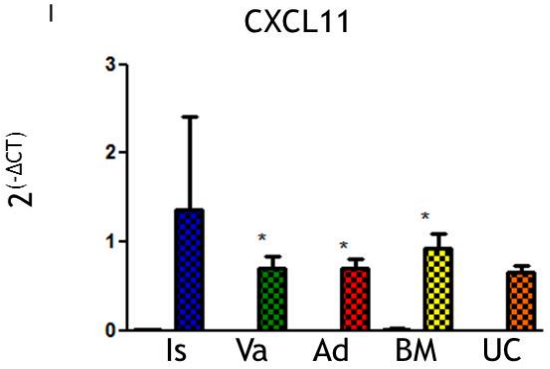
During homeostatic conditions, CXCL8 was transcribed at moderate levels by Is MSCs only. When comparing homeostatic to inflammatory conditions, fold changes of up to 21 were seen in Is MSCs CXCL8 $2^{(-\Delta CT)}$, along with significant upregulation of CXCL8 transcripts by Va MSCs (P=0.0124). Additionally, transcript levels of CXCL8 differed depending on MSC tissue source under inflammatory stimulation, where Is MSCs exhibited a $2(^{-\Delta CT})$ 2 fold higher than Va, Ad, UC and BM MSCs. Moreover, CXCL8 was the highest transcribed gene by Is and UC MSCs on the RT^2 array (**Figure 4-8 F**). Conversely, CXCL9 was the highest transcribed gene by Ad and Va MSCs on the RT^2 array. MSCs from all sources did not transcribe CXCL9 under homeostatic conditions, however fold changes of up to 120660 by Ad MSC CXCL9 $2(^{-\Delta CT})$ were observed going from homeostatic to inflammatory conditions (**Figure 4-8 G)**. Similar to CXCL9, CXCL10 was not transcribed under homeostatic conditions by any tissue source of MSC. Inflammatory stimulation resulted in a substantial upregulation of CXCL10 transcripts by Is, Va and UC MSCs, whereas Ad and BM MSCs significantly upregulated CXCL10 transcripts (Ad: P=0.0286. BM: P=0.0055). CXCL10 was the highest transcribed gene by stimulated BM MSCs on the profiling array (**Figure 4-8 H).** CXCL11 was not transcribed by any tissue source of MSC during homeostatic conditions. Inflammatory stimulation resulted in a substantial upregulation of CXCL11 transcription by Is and UC MSCs and a significant upregulation by Va (P=0.0373), Ad (P=0.0220) and BM (P=0.0321) MSCs. CXCL11 transcription did not significantly vary between MSC tissue source (**Figure 4-8 I**).

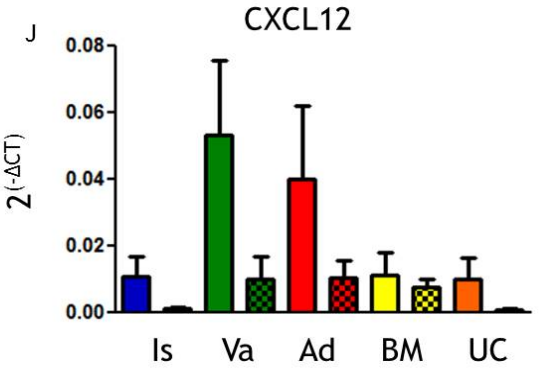
CXCL12 was transcribed at moderate levels by Va and Ad MSCs, whereas Is, BM and UC MSCs transcribed 3 fold less than Va and Ad MSCs in homeostatic conditions. Inflammatory stimulation resulted in downregulation of CXCL12 transcripts by all tissue sources of MSCs, where Va, Ad and BM MSCs transcribed similar levels and Is and UC MSCs transcribed more than 9 fold less than Va, Ad and BM MSCs (**Figure 4-8 J**).

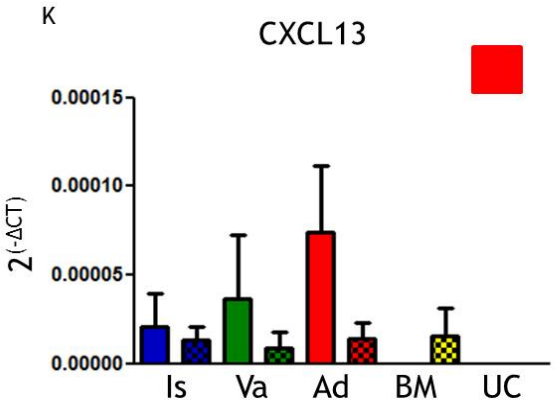

A
CXCL1
2^(-ΔCT)
0.8
0.6
0.4
0.2
0.0
Is
Va
Ad
BM
UC
B
CXCL2
1.5
1.0
0.5
C
CXCL3
0.25
0.20
0.15
0.10
0.05
0.00
D
CXCL5
0.4
0.3
0.2
0.1
E
CXCL6
0.6
F
CXCL8
8
6
4
2
Is MSC Unstimulated
Is MSC Stimulated
Va MSC Unstimulated
Va MSC Stimulated
Ad MSC Unstimulated
Ad MSC Stimulated
BM MSC Unstimulated
BM MSC Stimulated
UC MSC Unstimulated
UC MSC Stimulated

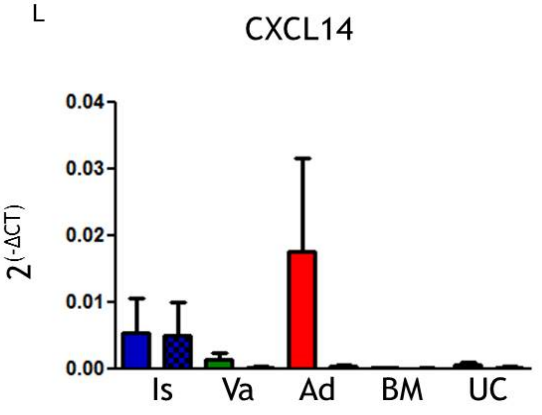

G
CXCL9
2(-ΔCT)
30
20
10
0
Is
Va
Ad
BM
UC


H
CXCL10
2(-ΔCT)
3
2
1
0
*
**
Is
Va
Ad
BM
UC


I
CXCL11
2(-ΔCT)
3
2
1
0
*
*
*
Is
Va
Ad
BM
UC


J
CXCL12
2(-ΔCT)
0.08
0.06
0.04
0.02
0.00
Is
Va
Ad
BM
UC


K
CXCL13
2(-ΔCT)
0.00015
0.00010
0.00005
0.00000
Is
Va
Ad
BM
UC


L
CXCL14
2(-ΔCT)
0.04
0.03
0.02
0.01
0.00
Is
Va
Ad
BM
UC

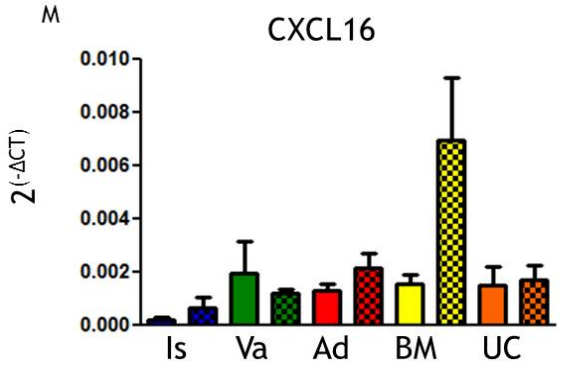

M
CXCL16
2(-ΔCT)
0.010
0.008
0.006
0.004
0.002
0.000
Is
Va
Ad
BM
UC

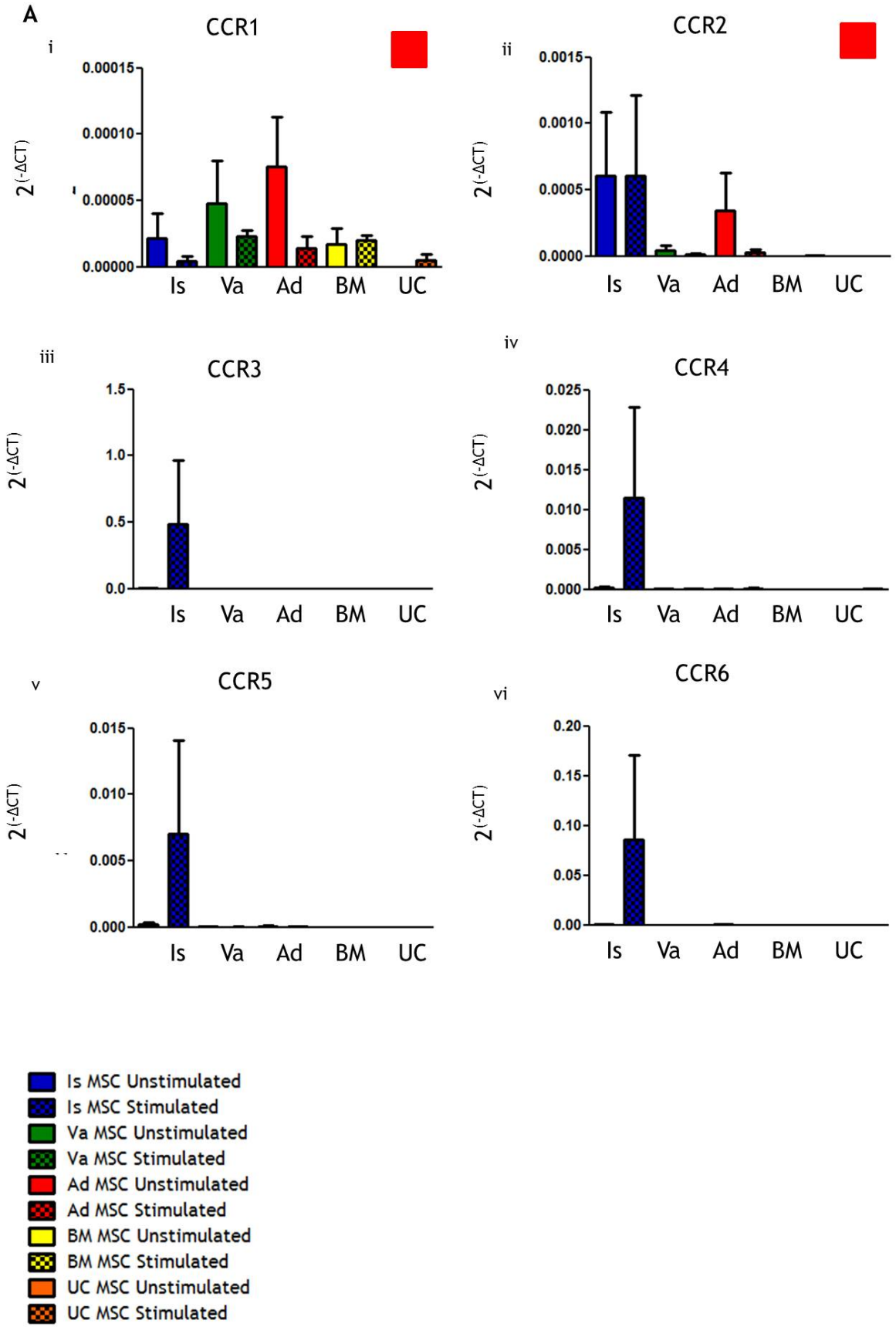
Figure 4-8 Inflammation and MSC tissue origin impacts CXC chemokine transcript levels in MSCs.
Experimental set up was identical as described previously (Figure 4-3). MSCs from all sources were grown to passage 3. MSCs from each donor (n of 3 for each MSC tissue source) were split into two T75 flasks. Once MSCs reached 80% confluency, MSCs were either left in homeostatic conditions or treated with 10ng/mL IFN-gamma, TNF-alpha and IL-1beta for 24 hours. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate CXCL transcripts in Is, Va, Ad, BM and UC MSCs under homeostatic and inflammatory conditions. Bars represent mean± SEM (n=3). Appropriate statistical analysis was performed and includes Students T test between one MSC tissue source (Homeostatic vs Inflammatory Conditions) and One Way ANOVA with Tukey's multiple comparisons post-test to compare all MSC sources. Significant differences are marked with the appropriate number of asterisks and P values are stated throughout the text.

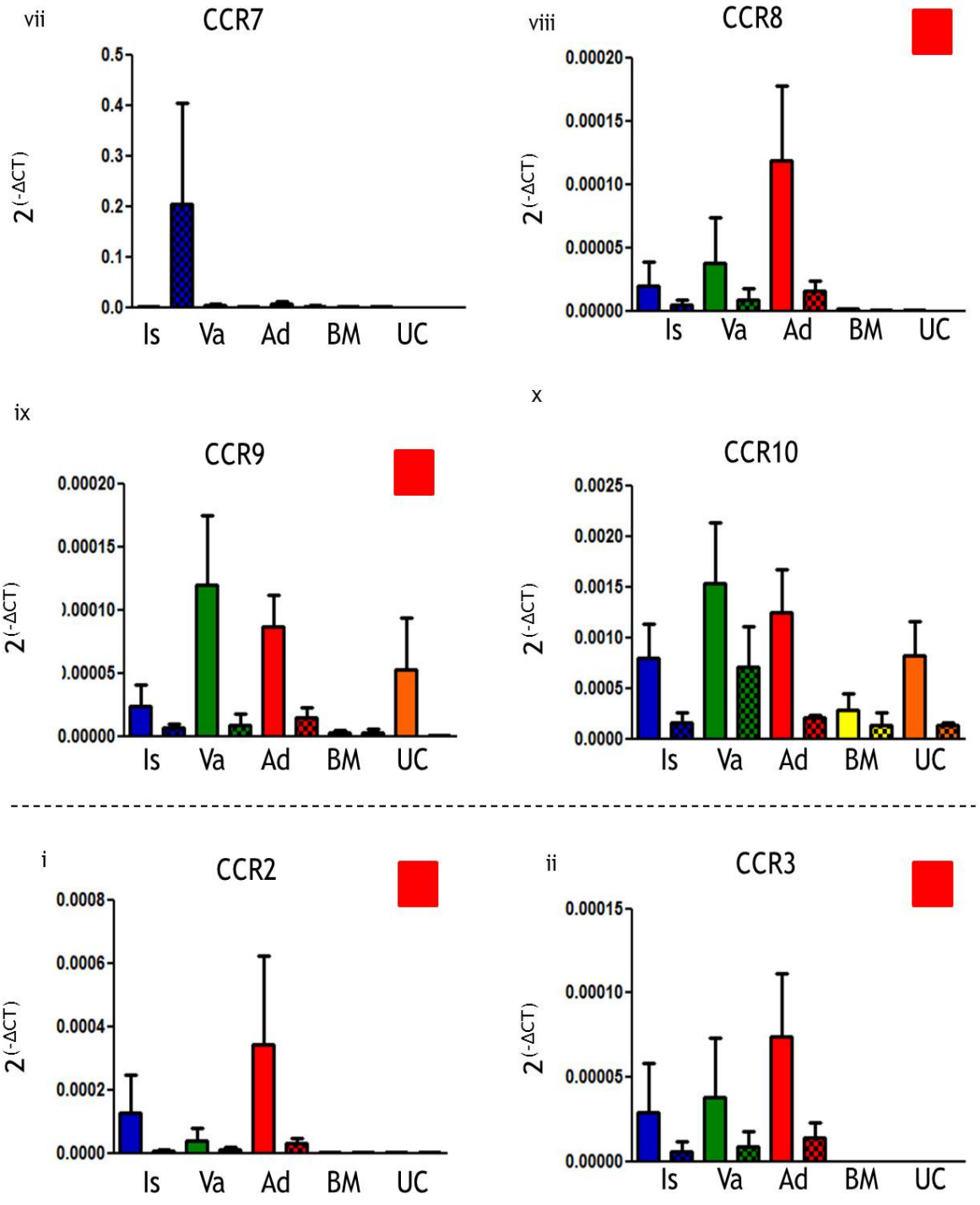
To summarise, the CXC chemokines were transcribed at relatively low levels during homeostatic conditions with the exception of UC MSCs which transcribed CXCL1, 2 and 6 at slightly higher levels. Notably, under inflammatory stimulation the CXC chemokines were the highest transcribed genes on the RT^2 arrays by all MSCs. MSC tissue origin dictated which CXCL was most highly transcribed, with CXCL8 being the greatest transcribed by Is and UC MSCs, CXCL9 by Va and Ad MSCs and CXCL10 by BM MSCs. These findings highlight the effect of inflammatory stimulation and the difference that MSC tissue origin has on CXC chemokine transcription in MSCs. Additionally, these findings suggest that MSCs might attract different immune cells depending on MSC tissue origin, where Is and UC MSCs should preferentially attract neutrophils (CXCL8), and Va, Ad and BM MSCs should attract T cells, NK cells and dendritic cells (CXCL9, CXCL10). Moreover, the exceedingly high levels of CXC chemokine transcription highlights target genes for protein analysis.

4.3.3 Transcriptional analysis of CC chemokine receptors under homeostatic and inflammatory conditions.

In comparison to CCL and CXCL chemokines, CC chemokine receptors are transcribed at extremely low levels. Despite low levels of transcription, CCR1 (**Figure 4-9 A.i**), CCR2 (**A.ii**), CCR8 (**A.vii**), CCR9 (**A.ix**) and CCR10 (**A.xi**) follow a trend of downregulation by all MSC tissue sources after inflammatory stimulation. Due to low transcript levels, differences between MSC sources are not measurable. Transcriptional expression of CCR3 (**Figure 4-9 A.ii**), CCR4 (**A.iv**), CCR5 (**A.v**), CCR6 (**A.vi**) and CCR7 (**A.vii**) were high for 1 sample of Is MSCs, accounting for the large error bars observed. Using a Grubbs' test, this sample was deemed an outlier and was excluded from the appropriate analysis and results are graphed in **Figure 4-9 B**. Exclusion of this sample allows for

better visualisation of CCR4, 5, 6 and 7 transcript levels by all MSC tissue sources.


A
i
CCR1
0.00015
0.00010
0.00005
0.00000
2(-ΔCT)
Is
Va
Ad
BM
UC
ii
CCR2
0.0015
0.0010
0.0005
0.0000
iii
CCR3
1.5
1.0
0.5
0.0
iv
CCR4
0.025
0.020
0.015
0.010
0.005
0.000
v
CCR5
0.015
0.010
0.005
0.000
vi
CCR6
0.20
0.15
0.10
0.05
0.00
Is MSC Unstimulated
Is MSC Stimulated
Va MSC Unstimulated
Va MSC Stimulated
Ad MSC Unstimulated
Ad MSC Stimulated
BM MSC Unstimulated
BM MSC Stimulated
UC MSC Unstimulated
UC MSC Stimulated


vii
CCR7
viii
CCR8
ix
CCR9
x
CCR10
B
i
CCR2
ii
CCR3
2(-ΔCT)
Is
Va
Ad
BM
UC

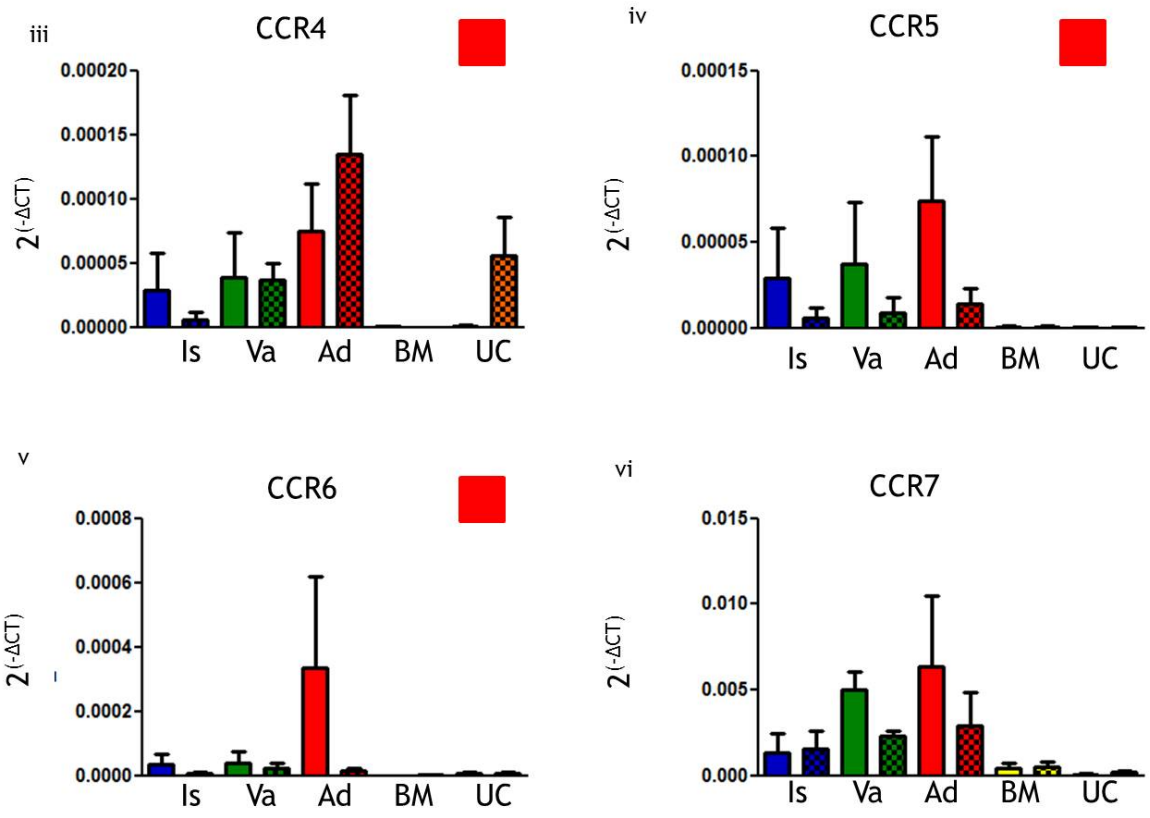


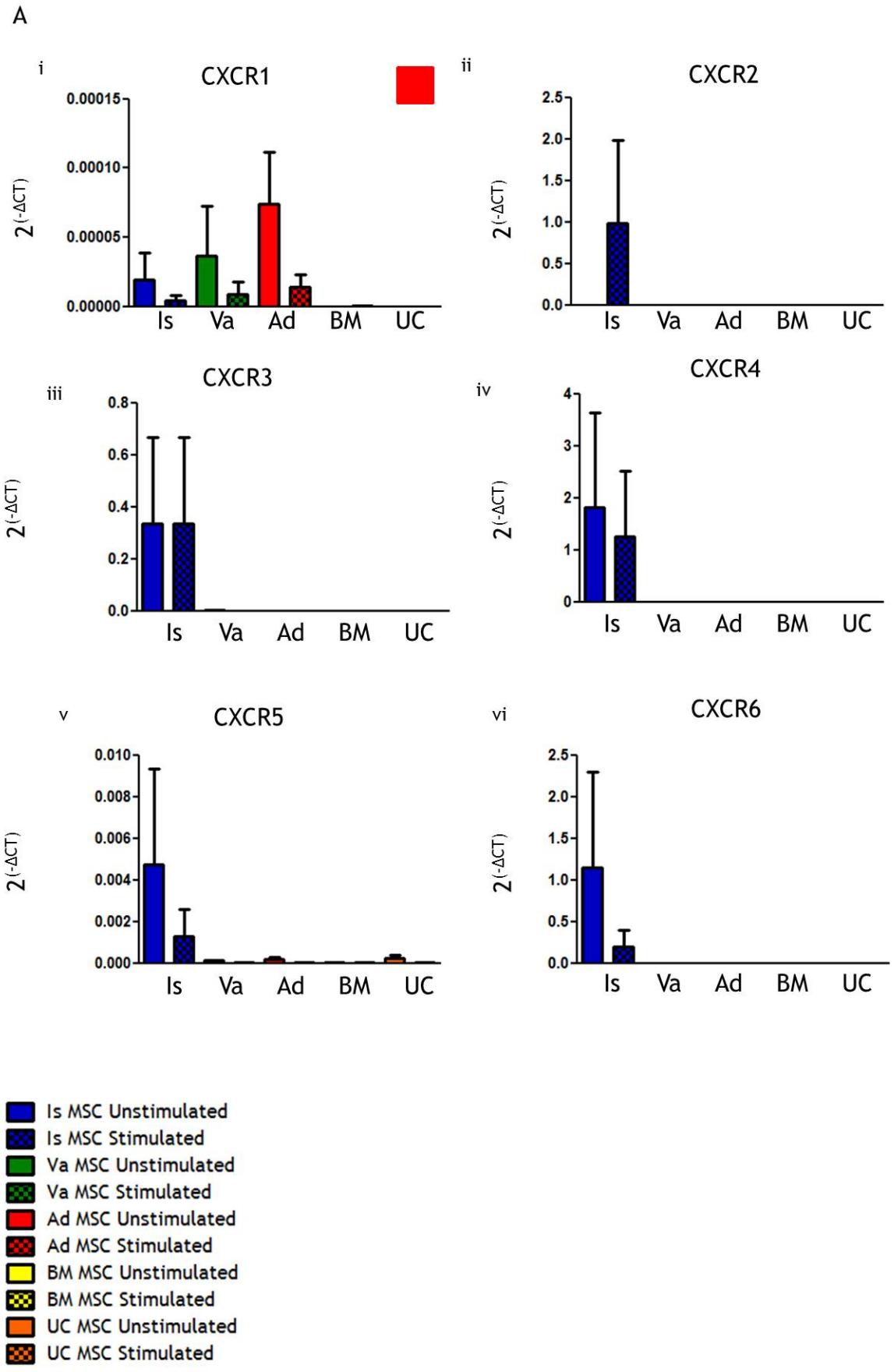
Figure 4-9 CC receptor transcript levels in MSCs under homeostatic and inflammatory conditions.
Experimental set up was identical as described previously (Figure 4-3). MSCs from all sources were grown to passage 3. MSCs from each donor (n of 3 for each MSC tissue source) were split into two T75 flasks. Once MSCs reached 80% confluency, MSCs were either left in homeostatic conditions or treated with 10ng/mL IFN-gamma, TNF-alpha and IL-1beta for 24 hours. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate CCR transcripts in Is, Va, Ad, BM and UC MSCs under homeostatic and inflammatory conditions. Graphs above the dotted line represent all data collected **(A)**, graphs below the dotted line represent the data, removing the one Is MSC outlier (assessed with Grubbs' test) **(B)**. Bars represent mean± SEM (n=3). Appropriate statistical analysis was performed and includes Students T test between one MSC tissue source (Homeostatic vs Inflammatory Conditions) and One Way ANOVA with Tukey's multiple comparisons post-test to compare all MSC sources. Where relevant, significant differences are marked with the appropriate number of asterisks and P values are stated throughout the text.

To summarise, CCRs were expressed at very low levels. Inflammatory stimulation resulted in a transcriptional downregulation in CCRs 1, 2, 8, 9 and 10. No differential expression of CCRs was observed between MSC tissue sources with the exception of one Is MSC sample for CCRs3, 4, 5, 6 and 7, which was deemed to be an outlier. The highest transcribed receptors for all MSC sources were CCR7 and CCR10.

4.3.4 Transcriptional analysis of CXC chemokine receptors under homeostatic and inflammatory conditions.

Similar to the CCRs, CXCRs were transcribed at very low levels. However, one Is MSC sample transcribed CXCR2 (**Figure 4-10 A.ii**), CXCR3 (**A.iii**), CXCR4 (**A.iv**), CXCR5 (**A.v**) and CXCR6 (**A.vi**) at substantially higher levels than other Is MSC samples (accounting for the large error bars) and other MSC tissue sources. This was the same Is MSC sample removed from the CCR analysis (**Figure 4-9 A+B**), possibly suggesting that receptor expression is highly variable depending on MSC donor. As before, a Grubbs' test deemed this sample an outlier and therefore it was removed from the appropriate graphs. Removing this sample allowed the visualisation of CXCR transcription by other tissue sources of MSCs where inflammatory stimulation resulted in a downregulation of all CXC chemokine receptors with the exception of CXCR4 (**Figure 4-10 B**).

As a result of low transcript levels, differences between MSC tissue sources are not measurable and all CXCRs are transcribed at comparable levels by all MSC tissue sources, with the exception of CXCR1 which was transcribed at slightly lower levels than other CXCRs.


A
i
CXCR1
0.00015
0.00010
0.00005
0.00000
2 (-ΔCT)
Is
Va
Ad
BM
UC
ii
CXCR2
2.5
2.0
1.5
1.0
0.5
0.0
iii
CXCR3
0.8
0.6
0.4
0.2
0.0
iv
CXCR4
4
3
2
1
0
v
CXCR5
0.010
0.008
0.006
0.004
0.002
0.000
vi
CXCR6
Is MSC Unstimulated
Is MSC Stimulated
Va MSC Unstimulated
Va MSC Stimulated
Ad MSC Unstimulated
Ad MSC Stimulated
BM MSC Unstimulated
BM MSC Stimulated
UC MSC Unstimulated
UC MSC Stimulated

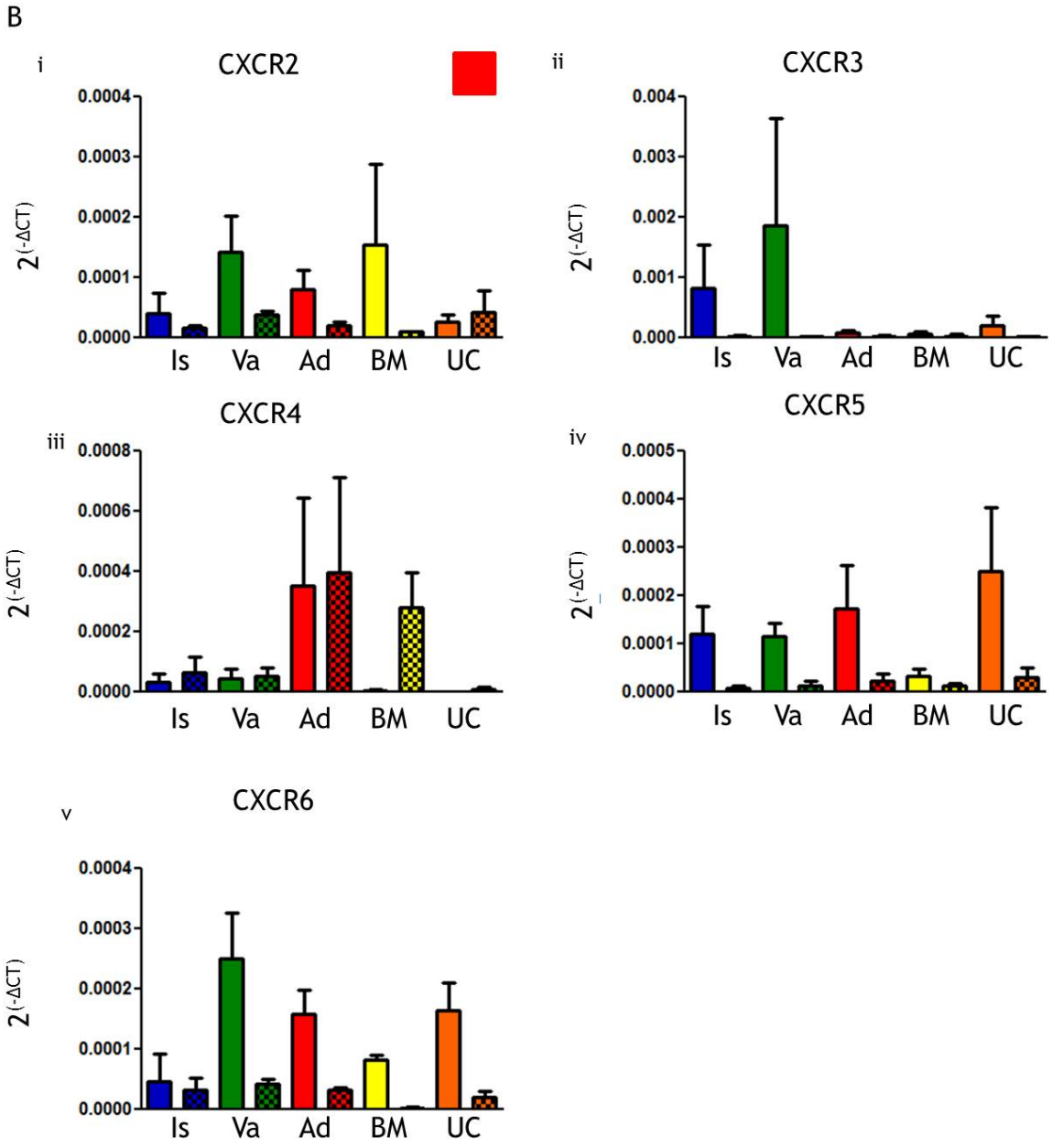


Figure 4-10 CXC receptor transcript levels in MSCs under homeostatic and inflammatory conditions

Experimental set up was identical as previously described (Figure 4-3). MSCs from all sources were grown to passage 3. MSCs from each donor (n of 3 for each MSC tissue source) were split into two T75 flasks. Once MSCs reached 80% confluency, MSCs were either left under homeostatic conditions or treated with 10ng/mL IFN-gamma, TNF-alpha and IL-1beta for 24 hours. Quantitative reverse transcription PCR (QRT PCR) was performed to evaluate CXCR transcripts in Is, Va, Ad, BM and UC MSCs in homeostatic and inflammatory conditions. Graphs in (**A**) represent all data collected, graphs in (**B**) represent the data, removing the one Is MSC 'outlier'. Bars represent mean± SEM (n=3). Appropriate statistical analysis was performed and includes Students T test between one MSC tissue source (Homeostatic vs Inflammatory Conditions) and One Way ANOVA with Tukey's multiple comparisons post-test to compare all MSC sources. Where relevant, significant differences are marked with the appropriate number of asterisks and P values are stated throughout the text

4.3.5 **Transcriptional analysis of atypical chemokine receptors under homeostatic and inflammatory conditions.**

The atypical chemokine receptors were transcribed at higher levels than the CC and CXC receptors.

Under homeostatic conditions, Va and Ad MSCs transcribed moderate levels of ACKR2, whereas Is, BM and UC MSCs transcribed substantially less. Ad MSCs – which transcribed the highest levels of ACKR2 in homeostatic conditions - transcribed 31-fold higher ACKR2 than UC MSCs – which transcribed the lowest ACKR2 in homeostatic conditions (**Figure 4-11 A.ii**). Inflammatory stimulation had differential effects on ACKR2 transcription, where Is MSCs marginally upregulated ACKR2 expression and Va, Ad, BM and UC downregulated ACKR2 expression. MSC tissue source also appeared to have an effect on ACKR2 transcription. Thus during inflammatory conditions, Is MSCs transcribed the highest levels of ACKR2.

ACKR3 (**Figure 4-11 A.iii**) was the highest transcribed atypical receptor by all MSC tissue sources, except Is MSCs which transcribed marginally more ACKR4 (**Figure 4-11 A.iv)**. During homeostatic conditions, MSC tissue source affected the transcript levels of ACKR3, where Va MSCs transcribed significantly more than Is MSCs ($P \leq 0.05$) and Ad MSCs transcribed significantly more than UC MSCs ($P \leq 0.05$). Inflammatory stimulation had differential effects on MSC ACKR3 expression depending on MSC tissue origin. ACKR3 transcripts were upregulated by Va, Ad and UC MSCs, downregulated in BM MSCs and remained the same in Is MSCs

MSCs isolated from all sources downregulated ACKR4 transcription under inflammatory stimulation. ACKR4 was highest transcribed ACKR by Is MSCs and the second highest transcribed ACKR after ACKR3 by Va, Ad, BM and UC MSCs. ACKR4 was transcriptionally expressed at similar levels by Is, Va and Ad MSCs, whereas UC and BM MSCs show very little ACKR4 transcription (**Figure 4-11 A.iv**).

Excluding the one Is MSC sample which expressed high transcription levels of ACKR1 (using Grubb's test), the remaining Is MSCs and other MSC tissue sources

showed very little transcription of ACKR1 (**Figure 4-11 B.i**), similar to that of ACKR5 transcription levels (**Figure 4-11 A.v**)

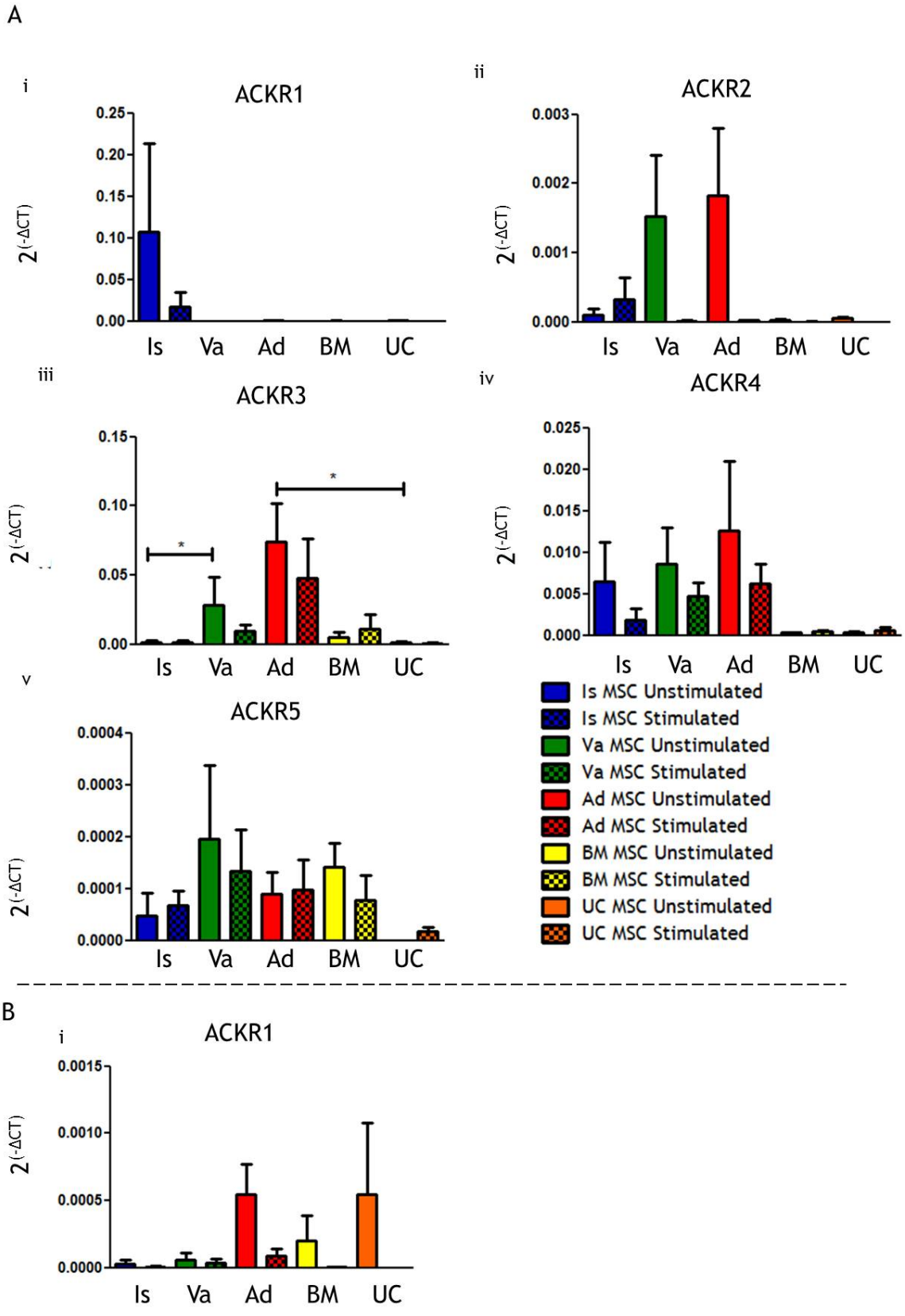


Figure 4-11 ACK Receptor transcript levels in MSCs under homeostatic and inflammatory conditions

Experimental set up was identical as previously described (Figure 4-3) MSCs from all sources were grown to passage 3. MSCs isolated from each donor (n of 3 for each MSC tissue source) were split into two T75 flasks. Once MSCs reached 80% confluency, MSCs were either left in homeostatic conditions or treated with 10ng/mL IFN-gamma, TNF-alpha and IL-1beta for 24 hours. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate ACKR transcripts in Is, Va, Ad, BM and UC MSCs in homeostatic and inflammatory conditions. Graphs above the dotted line represent all data collected **(A)**, graphs below the dotted line represent the data, removing the one Is MSC 'outlier' **(B)** Bars represent mean± SEM (n=3). Appropriate statistical analysis was performed and includes Students T test between one MSC tissue source (Homeostatic vs Inflammatory Conditions) and One Way ANOVA with Tukey's multiple comparisons post-test to compare all MSC sources. Where relevant, significant differences are marked with the appropriate number of asterisks and P values are stated throughout the text

Overall, atypical chemokine receptors were transcriptionally expressed at higher levels that CCRs and CXCRs. Stimulation with inflammatory mediators had differential effects on MSC ACKR transcription. Moreover, expression levels of AKCR3 are significantly different between MSC tissue sources. Thus inflammatory stimulation and MSC tissue source had a profound effect on MSC ACKR transcription profiles.

4.3.6 **Transcriptional analysis of XCL and CX3C chemokines, their receptors and other cytokines.**

During homeostatic conditions, CX3CL1 **(Figure 4-12 A**) and its receptor CX3CR1 (**Figure 4-12 B**) were transcribed at low levels by all MSCs with the exception of one Is MSC sample, accounting for the large error bar observed. XCL1 (**Figure 4-12 C**), XCL2 (**Figure 4-12 D**) and its receptor XCR1 (**Figure 4-12 E**) were transcribed at extremely low levels by all MSC tissue sources.

Hypoxia-inducible factor 1-alpha (HIF1A) was transcribed at substantial levels by Is, Va and Ad MSCs, whereas UC and BM MSCS transcribed considerably lower levels under homeostatic conditions. Upon inflammatory stimulation, Is, Va and Ad MSCs markedly downregulated HIF1A transcription, and UC and BM transcribed HIF1A at similar levels under both conditions (**Figure 4.12 F**).

Inflammatory stimulation considerably upregulated IL-1B transcription by MSC from all tissue sources and was transcribed at similar levels by all MSCs (**Figure 4-12 G**).

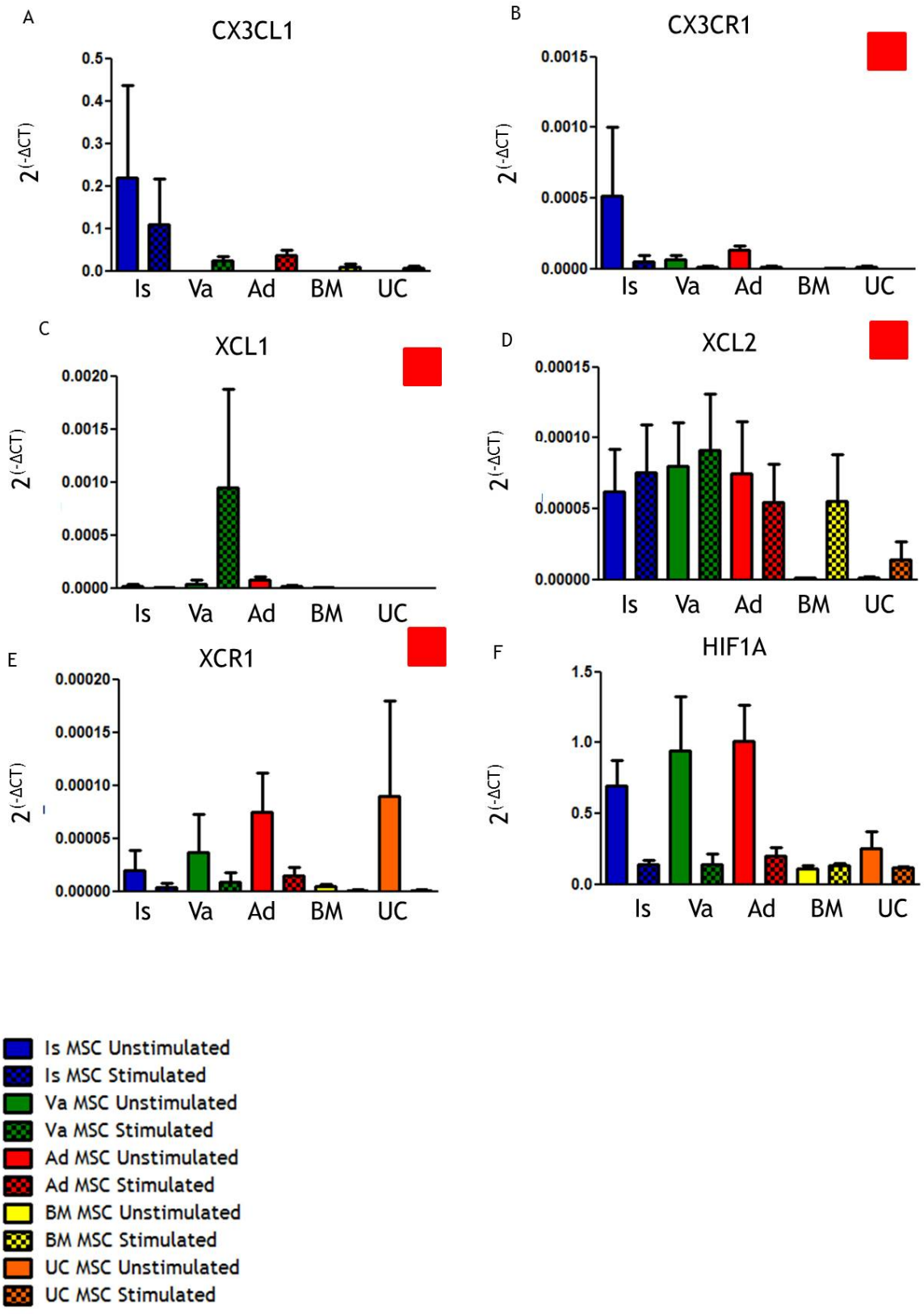
Despite being transcribed at exceedingly low levels, inflammatory stimulation resulted in MSCs from all tissue sources downregulating IL-4 transcription (**Figure**

4-12 H). Differences in IL-4 transcript levels between MSC tissue sources are hard to interpret due to such low transcript levels.

Transcription of TNF-alpha was slightly upregulated by MSCs from all tissue sources, however variations in TNF-alpha transcription within one MSC population was evident, where Ad, UC and BM MSCs exhibited large spreads of data, perhaps due to donor variability (**Figure 4-12 I**).

TLR4 was transcribed at similar levels by MSCs from all tissue sources under both homeostatic and inflammatory conditions with the exception of UC MSCs which transcribed significantly less than Ad MSCs ($P \leq 0.05$) and substantially less than Is, Va and BM MSCs (**Figure 4-12 J**).

TLR2 and IL-16 were transcribed at very low levels by all MSCs with the exception of one BM MSC donor in TLR2 transcription (**Figure 4-12 K)** and one Is MSC donor in IL-16 transcription (**Figure 4-12 L),** accounting for the large spread of data observed on those graphs for MSCs from those tissue sources.


A
CX3CL1
2^(-ΔCT)
0.5
0.4
0.3
0.2
0.1
0.0
Is
Va
Ad
BM
UC
B
CX3CR1
0.0015
0.0010
0.0005
0.0000
C
XCL1
0.0020
0.0015
0.0010
0.0005
0.0000
D
XCL2
0.00015
0.00010
0.00005
0.00000
E
XCR1
0.00020
0.00015
0.00010
0.00005
0.00000
F
HIF1A
1.5
1.0
0.5
0.0
Is MSC Unstimulated
Is MSC Stimulated
Va MSC Unstimulated
Va MSC Stimulated
Ad MSC Unstimulated
Ad MSC Stimulated
BM MSC Unstimulated
BM MSC Stimulated
UC MSC Unstimulated
UC MSC Stimulated

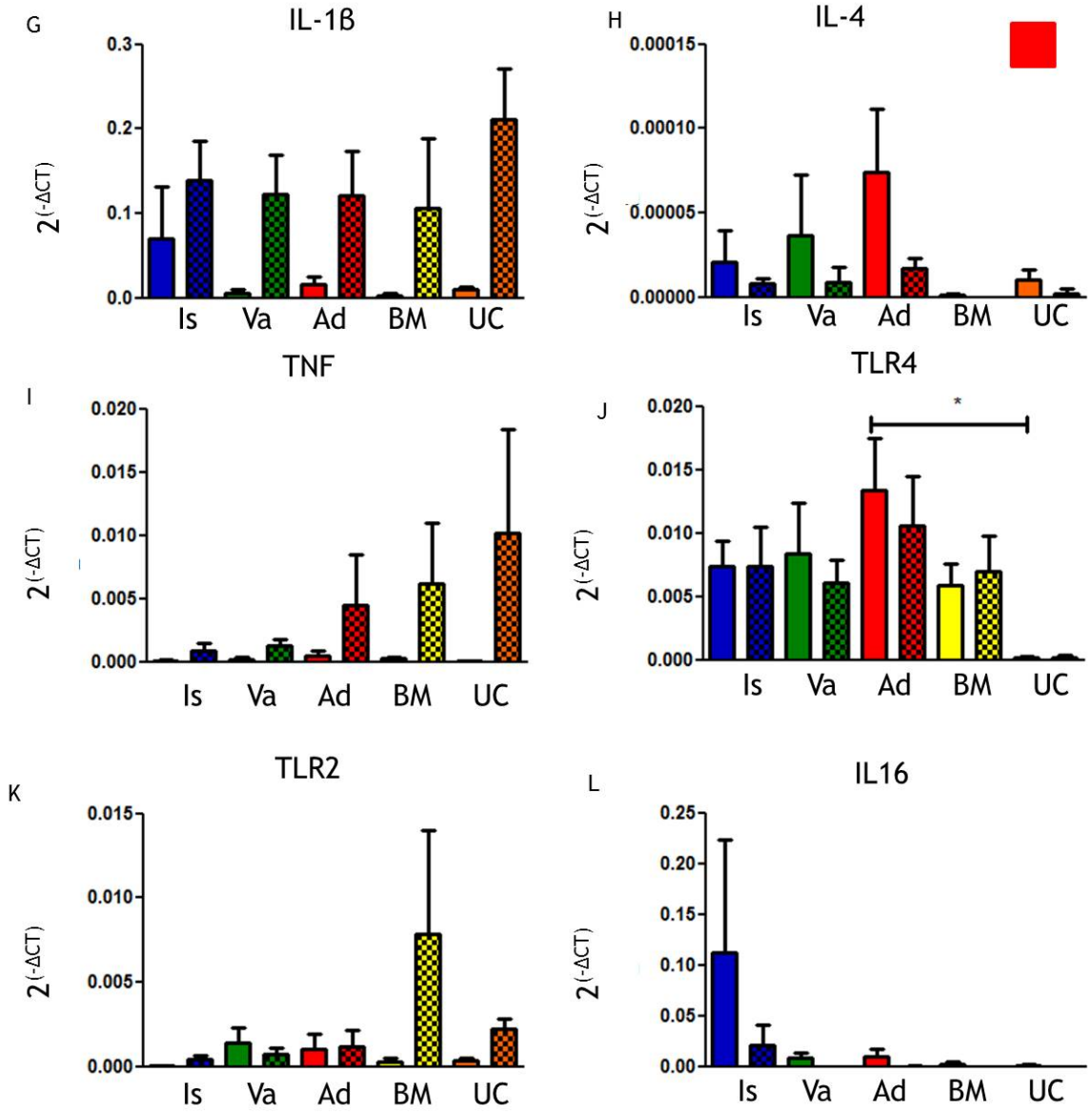


Figure 4-12 Inflammation and MSC tissue origin impacts the transcript levels of other genes important in immunity and inflammation
Experimental set up was identical as previously described (Figure 4-3). MSCs from all sources were grown to passage 3. MSCs isolated from each donor (n of 3 for each MSC tissue source) were split into two T75 flasks. Once MSCs reached 80% confluence, MSCs were either left in homeostatic conditions or treated with 10ng/mL IFN-gamma, TNF-alpha and IL-1beta for 24 hours. Quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate transcript levels of several genes in Is, Va, Ad, BM and UC MSCs in homeostatic and inflammatory conditions. Bars represent mean± SEM (n=3). Appropriate statistical analysis was performed and includes Students T test between one MSC tissue source (Homeostatic vs Inflammatory Conditions) and One Way ANOVA with Tukey's multiple comparisons post-test to compare all MSC sources. Where relevant, significant differences are marked with the appropriate number of asterisks and P values are stated throughout the text

To Summarise, CX3CR1, XCR1, XCL1 and XCL2 were all transcribed at very low levels. CX3CL1 was transcribed at similar levels by VA, Ad, BM and UC MSCs under inflammatory stimulation, suggesting MSCs potential to attract T cells and

monocytes during inflammation. Additionally, the pro-inflammatory cytokine IL-1B was transcriptionally expressed at similar levels by MSCs isolated from all tissue sources. TLR4, a molecule important in activating the innate immune system was transcribed at similar levels under homeostasic and inflammatory conditions in all MSCs with the exception of UC MSCs. HIF-1A was also transcribed at different levels depending on where MSCs were isolated from, with Is, Va and Ad MSCs transcribing similar levels under homeostasis and all MSCs transcribing similar levels after inflammatory stimulation.

Importantly, these genes are involved in the activation of the immune system. Notably, inflammatory stimulation and MSC tissue source had a variable effect on the transcript levels of these genes. Therefore MSC tissue source and inflammatory stimulation of MSCs may have differential effects on surrounding immune cells *in vivo*.

4.4 Discussion

Work presented in this chapter was designed to determine whether the RT^2 arrays were a suitable tool to analyse the transcriptional expression of chemokines and their receptors by MSCs isolated from Is, Ad, Va, BM and UC. Stringent analysis of the plate's internal controls, along with an external control allowed for the assessment of intra and inter-plate reproducibility and established that data generated using this system was accurate and reliable.

Secondly, after the establishment of a suitable assay, the transcriptional analysis of chemokines and chemokine receptors by MSCs from all tissue source maintained under homeostatic and inflammatory conditions was assessed and is summarised in **Figure 4-13**. Careful analysis of the transcript data allowed genes of interest to be identified and targeted for protein assays. Protein expression of chemokines and their receptors are examined in the next chapter to give a more thorough examination of the key molecules implicated in MSC phenotype and potential *in vivo* behaviour. To avoid repetition, a combined, in depth discussion on MSC chemokine and chemokine receptor expression at a transcript and protein level will follow in the next chapter.

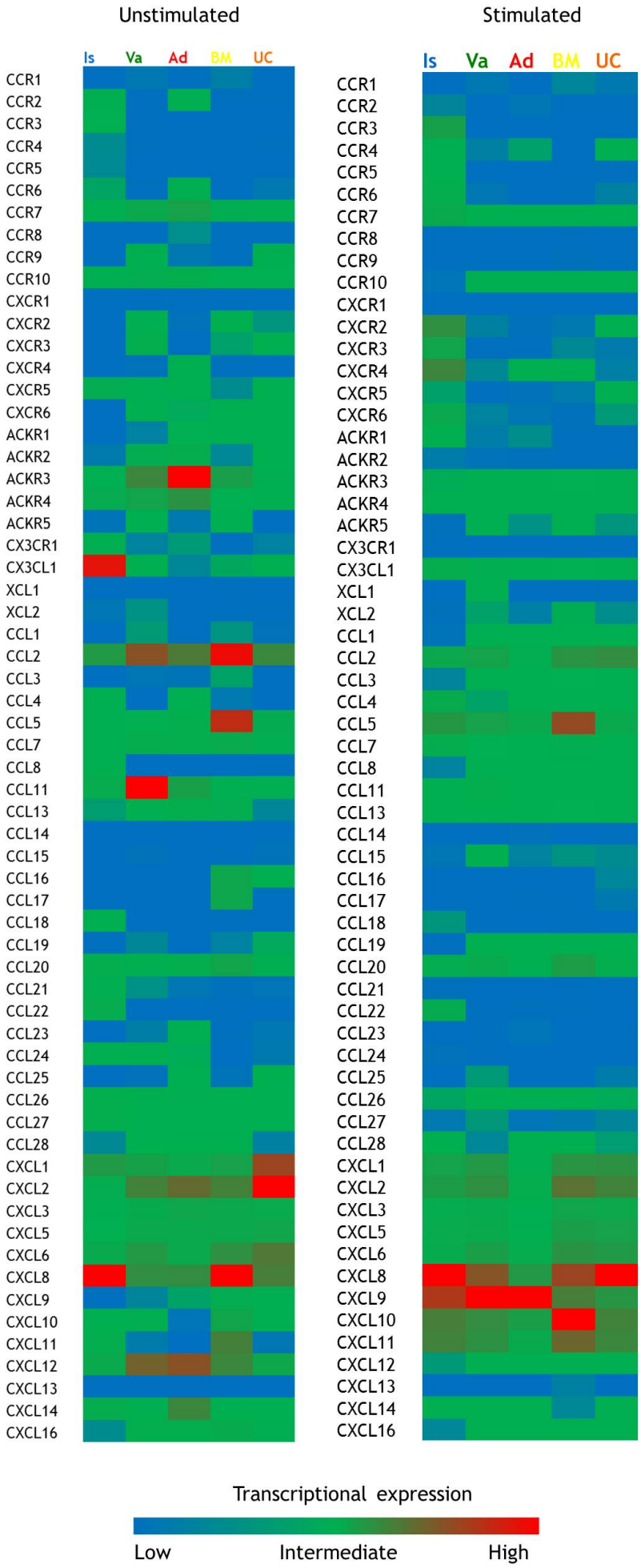

Unstimulated
Stimulated
Is
Va
Ad
BM
UC
CCR1
CCR2
CCR3
CCR4
CCR5
CCR6
CCR7
CCR8
CCR9
CCR10
CXCR1
CXCR2
CXCR3
CXCR4
CXCR5
CXCR6
ACKR1
ACKR2
ACKR3
ACKR4
ACKR5
CX3CR1
CX3CL1
XCL1
XCL2
CCL1
CCL2
CCL3
CCL4
CCL5
CCL7
CCL8
CCL11
CCL13
CCL14
CCL15
CCL16
CCL17
CCL18
CCL19
CCL20
CCL21
CCL22
CCL23
CCL24
CCL25
CCL26
CCL27
CCL28
CXCL1
CXCL2
CXCL3
CXCL5
CXCL6
CXCL8
CXCL9
CXCL10
CXCL11
CXCL12
CXCL13
CXCL14
CXCL16
Transcriptional expression
Low
Intermediate
High

Figure 4-13 Heat maps of MSC transcriptional expression of chemokines and their receptors
Data from Figure 4-7 to Figure 4-11 are combined and presented as heat maps to illustrate the chemokine and chemokine receptor transcriptional profile of MSCs maintained under homeostatic (left) and inflammatory conditions (right). The heat maps summarise each individual tissue source of MSC highest and lowest transcribed genes under homeostatic 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 green and genes with high $2^{(-\Delta CT)}$ values are highlighted in red.

4.5 **Conclusions**

To summarise, Is, Va, Ad, BM and UC MSCs differentially expressed chemokines and their receptors at a transcriptional level. Inflammatory stimulation resulted in a substantial transcriptional upregulation of CC and CXC chemokines, whereas it had differential effects on receptor transcripts. Under inflammatory conditions, MSCs appeared to have a very specific, distinct chemokine transcriptional profile with prominent transcription of the CXC chemokine family. Importantly, the tissue source of MSC dictates their chemokine and chemokine receptor transcriptional profile. The observation that the original MSC tissue source dictates the transcriptional profile of chemokines and their receptors could have major effects on MSC *in vivo* behaviour. Further investigation into the expression of these molecules by MSCs at a protein level is vital to begin to elucidate if *in vivo* behavioural differences might exist in MSCs isolated from Is, Va, Ad, BM and UC.

Chapter 5

Determining the chemokine and chemokine receptor expression by MSCs at a protein level

Chapter 5 Determining the chemokine and chemokine receptor expression by MSCs at a protein level

5.1 Introduction and aims

Work presented in the previous chapter demonstrated that MSCs isolated from the islet (Is), visceral adipose (VA), adipose (Ad), bone marrow (BM) and umbilical cord (UC) differentially expressed transcripts for chemokines and their receptors, depending on their environment (homeostatic or inflammatory) and tissue source. Chemokine receptors, in general, displayed low transcript levels, however, under both homeostatic conditions and after inflammatory stimulation, CCR7, CCR10, CXCR4, CXCR6, ACKR3 and ACKR4 were variously transcribed at higher levels. Chemokines were transcribed at much higher levels than chemokine receptors under both homeostatic conditions and following inflammatory stimulation. Homeostatically maintained MSCs transcribed moderate to high levels of CCL2, CCL5, CCL11, CCL13, CXCL1, CXCL2, CXCL5, CXCL6, CXCL8, CXCL12 and CXCL14. Upon inflammatory stimulation, the aforementioned chemokines were substantially upregulated and moderate to high levels of transcription of the following chemokines were induced; CCL1, CCL3, CCL7, CCL20, CXCL3, CXCL9, CXCL10 and CXCL11.

As mentioned previously, chemokine receptor expression by MSCs is reported in the literature but the findings are confusing and largely focus on BM MSCs. More recently, with the improved ease of access to MSCs isolated from other tissue sources, data are emerging reporting chemokine receptor expression profiled for MSCs from other sources (242, 243). Several studies have highlighted the poor homing efficiency of MSCs (244), therefore, analysing MSC chemokine receptor expression will shed light on the homing potential of MSCs to specific locations such as the bone marrow (CXCR4), sites of myocardial infarction (CXCR4) and the skin (CCR10). At these sites they can act as anti-inflammatory mediators in hematopoietic stem cell transplantation, cardiac regeneration and wound healing, respectively (234, 245-247). Some studies have focussed on attempting to upregulate chemokine receptor expression to improve the tissue-specific

homing efficiency of MSCs in order to ultimately improve therapeutic efficacy (244, 248). Due to the use of alternative isolation methods, different culture medium, different passage number and varied tissue culture techniques, the literature reporting MSC chemokine receptor expression is inconsistent. Through a set of standardised methods, the work presented in this chapter aimed to compare chemokine receptor expression of Is, Va, Ad, BM and UC MSCs under both homeostatic and inflammatory conditions. Understanding the effect of inflammation on chemokine receptor expression could inform our understanding of MSC behaviour in inflammatory settings, or provide a rationale for pre-treating MSCs before infusion into patients.

MSC secretion of several chemokines is reported in the literature, with some studies highlighting differences in chemokine secretion between MSCs isolated from different tissue sources. For example, Amable *et al*. reported that UC MSCs secreted higher levels of CXCL8 in comparison to Ad and BM derived MSCs, whereas the same group report differential chemokine secretion by MSCs cultured in platelet-rich plasma in comparison to fetal bovine serum (249-251). Importantly, the majority of studies have focused on human BM MSC chemokine secretion, with little clarification of the function of MSC secreted chemokines. Some studies suggest that MSC derived chemokines are involved in wound healing and others suggest that the secretion of chemokines is key to attracting immune cells to areas of tissue regeneration or damage (240). Through a set of standardised techniques and reagents, work described in this chapter aimed to i) compare chemokine secretion by Is, Va, Ad, BM and UC MSCs under homeostatic and inflammatory conditions and ii) to understand if the secretion of these chemokines attracts immune cells towards MSCs. Understanding if MSCs differentially secrete chemokines and therefore preferentially attract different immune cell subtypes, could highlight potential differences in *in vivo* behaviour of MSCs isolated from different sources.

Therefore, having assessed the transcription of chemokines and their receptors by MSCs under both homeostatic and inflammatory conditions, this chapter aimed to determine if these differences were also evident at the protein level. Additional aims of this chapter included assessing the functionality of MSC secreted chemokines.

Results

5.2 Surface chemokine receptor expression by MSCs

Although the majority of chemokine receptor transcripts were low for all MSC populations, a handful that were transcribed at slightly higher levels were selected for surface protein analysis – CCR7, CCR10, CXCR6, ACKR3 and ACKR4. Although CXCR4 was transcribed at lower levels, it was also included in this panel due to the extensive literature reporting its relationship with MSCs (151, 152).

To assess whether MSCs expressed chemokine receptors on their surface, and if MSC tissue source and/or inflammatory stimulation affected expression levels, flow cytometry was used to assess the surface expression of CCR7, CCR10, CXCR4, CXCR6 and ACKR3, the mean fluorescence intensity (MFI) was recorded and is graphed in **Figure 5-1.** Positive chemokine receptor expression was assessed by the MFI of receptor stained MSCs compared to the respective MFIs of isotype controls (black bars). Due to the lack of reliable flow cytometry antibodies for ACKR4, IHC was used to identify the presence of ACKR4 on Is, Va and Ad MSC (**Figure 5-2**).

In agreement with the chemokine receptor transcript data, broadly speaking, surface chemokine receptor expression by MSCs was low. CXCR6 - an important receptor involved in NK and NKT cell patrolling and retention within the liver (252-254) was barely expressed above background levels (isotype controls) by MSCs from all sources under homeostatic and inflammatory conditions (**Figure 5-1 B).** Conversely, CXCR4 was expressed at moderate levels above background by Va MSCs under homeostatic conditions, whereas Is, Ad, BM and UC MSCs minimally expressed CXCR4 above background fluorescence levels **(Figure 5-1 C)**. Statistically speaking, CXCR4 expression was only affected by inflammatory stimulation on BM MSCs, where they were the only tissue source of MSC to significantly upregulate CXCR4 expression comparing homeostatic to inflammatory conditions (P=0.0453), however both Va and BM MSCs expressed CXCR4 at moderate levels above background stained controls. Similarly, CCR7, an important receptor for DC trafficking to the lymph node (255), was moderately expressed by Va and BM MSCs under both homeostatic and

inflammatory conditions. Inflammatory stimulation did not appear to have an impact on CCR7 expression **(Figure 5-1 D)**.

CCR10, a receptor important in immune cell homing to the skin was barely expressed by any MSC populations above background levels under homeostatic and inflammatory conditions **(Figure 5.1 E),** suggesting that this receptor is likely not expressed on the surface of MSCs.

The atypical receptor ACKR3, is a scavenging receptor which binds and degrades CXCL11 and 12 and is constantly recycling. MSCs isolated from Va and the BM were the only MSC populations to stain positively for ACKR3 above background levels when maintained under homeostatic conditions and upregulated ACKR3 expression under inflammatory conditions **(Figure 5-1 F).** Is and Ad MSCs barely expressed ACKR3 above background controls under homeostatic and inflammatory conditions, suggesting an absence of this receptor on these MSC populations. It is however, important to note that this is an atypical receptor, therefore is constantly recycling and is intracellularly stored within endocytic vesicles. Despite experiments being carried out at 4^{o}C, which slows receptor cycling, it is likely that a large proportion of ACKR3 will be intracellular and therefore surface staining might not be an accurate representation of ACKR3 expression.

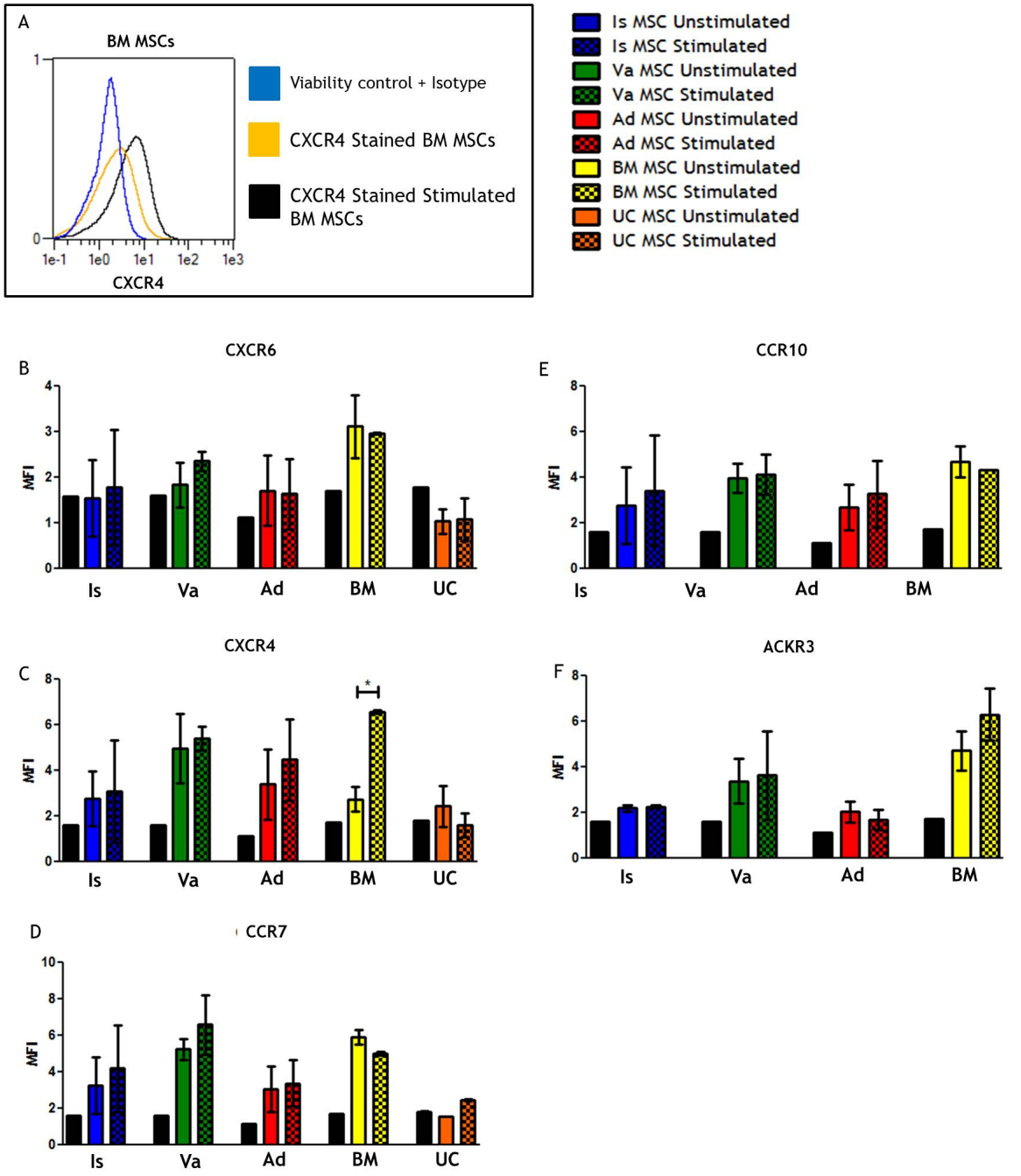


Figure 5-1 Flow cytometry analysis of surface chemokine receptor expression by MSCs
(**A**) A representative histogram showing P3 BM MSCs CXCR4 staining, under homeostatic (yellow) or inflammatory (Black) conditions - isotype control in dark blue.

(**B-F**) Graphs show mean fluorescence intensity of CXCR6, CXCR4, CCR7, CCR10 and ACKR3. **Black bar represents isotype controls.**

Each bar represents an n of 3 ± SEM. ONE WAY ANOVA with Tukey's post test was used to statistically analyse receptor expression between MSC sources. Students paired T test was used to compare between homeostatic and inflammatory conditions within one MSC tissue source. Significance is marked in where applicable.

At P3, Is (A), Va (B) and Ad (C) MSCs stained positively for ACKR4 (Orange) (**Figure 5-2 A-D**).

The lack of signal in isotype controls (D) suggests that positive staining was specific for ACKR4. Moreover, the punctate staining pattern (highlighted in B with a white arrow) is typical staining you would expect to see for an atypical chemokine receptor because they reside in vesicles within the cell. This further suggests true ACKR4 staining.

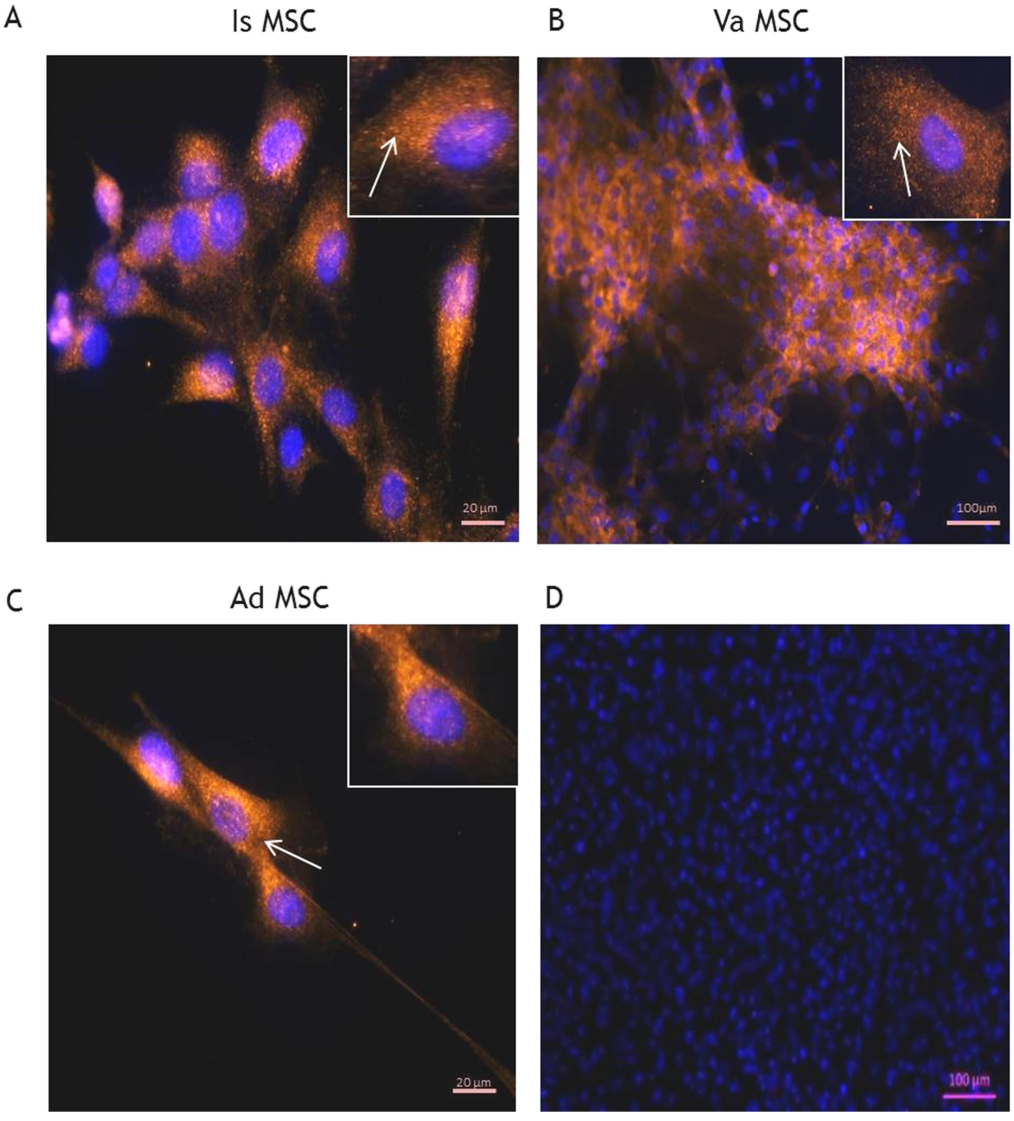


Figure 5-2 Immunohistochemistry showing ACKR4 expression by Is, Va and Ad MSCs
Is (**A**), Va (**B**) and Ad (**C**) MSCs were grown to passage 3 and stained with anti-ACKR4 (Orange (**A-C**)) or isotype control (**D**) antibodies. Thumbnail inserts show zoomed images of ACKR4 stained cells and white arrows point to punctate ACKR4 staining

5.3 Chemokine secretion by MSCs

To assess the chemokines secreted by MSCs, a LUMINEX assay was performed to measure the concentration of an array of chemokines present in MSC conditioned medium (CM) after 24 hours under homeostatic or inflammatory conditions. Due to UC and BM MSCs being tested on separate plates a substantial time apart, it was important to establish that the reagents, kits and the machine could still perform similarly between experiments. To assess this, a sample (Is MSC CM) used on the original plates with Is, Va and Ad MSC samples, was also used on the BM and UC MSC plate and the concentrations of each analyte were compared to ensure that they matched in both runs. Some analytes displayed very strong matches over the plates, suggesting that these analytes were consistent with the previous plate and therefore the results for UC and BM samples were accurate. Conversely, others showed dissimilar concentrations between runs (CXCL10 and CCL20 highlighted in red box) perhaps suggesting that these analytes were not performing as successfully as the previous run and therefore the results for BM and UC MSCs could not be directly compared to Is, Va and Ad MSCs (**Figure 5-3 A).** Thus, to serve as a means of comparison between Is, Va and Ad MSCs LUMINEX results vs. BM and UC MSCs LUMINEX results, a dilution value was calculated for specific analytes which differed between the two LUMINEX runs (**Figure 5-3 B).** Where appropriate, BM and UC values were divided by the calculated dilution factor and graphed. As this is a crude assessment of comparison, UC and BM chemokine secretion values have to be interpreted with caution. Chemokines where BM and UC values are likely to be accurate, graphs are marked with an asterisk (*). Conversely, chemokines where BM and UC samples were generated by the division of a dilution factor, graphs are marked with a red box.

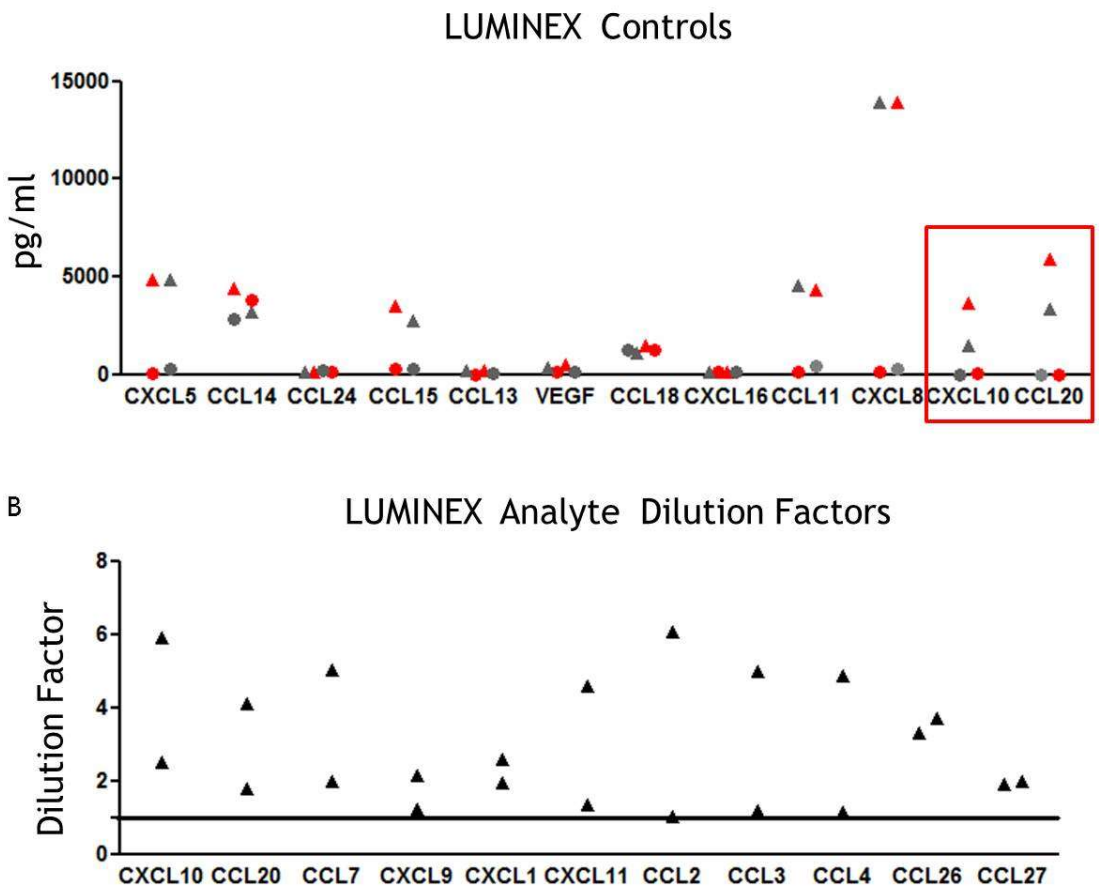


Figure 5-3 Comparison of LUMINEX analytes over two separate runs
Chemokines in conditioned medium (CM) of Is, Va, Ad, BM and UC MSCs were assessed via LUMINEX. BM and UC CM were assessed on the same plates at a substantial time apart from Is, Va and Ad CM. Therefore, to assess the reproducibility and comparability of the LUMINEX plates, 2 Is MSC CM samples used on the previous LUMINEX plates was run along with UC and BM MSC CM to assess the reproducibility and sensitivity of the assay. To fully test the range of sensitivity of the assay, homeostatically maintained Is MSC CM was used (circles) coupled with an inflamed Is CM (triangles). The results from the recent run (red) were compared to results of the previous run (grey). The concentration (pg/mL) of reproducible and 2 non-reproducible (red box) analytes are graphed in A.

For analytes where sample 1 and sample 2 values were substantially higher or lower than the previous run, a dilution factor was generated to evaluate the spread of results and graphed in B. Analytes where symbols are close to 1 (black bar) – CXCL9 and CXCL1 – represent more reliable readings for BM and UC MSCs as they did not differ higher than 2 fold. Analytes with a larger dilution factor – CXCL10 and CCL2 –are relatively unreliable concentrations for UC and BM MSC CM.

5.3.1 **CC Chemokines present in MSC conditioned medium**

Concentrations (pg/mL) of the CC chemokines assayed are graphed in **Figure 5-4**. As MSCs were cultured in 10% human AB pooled plasma, it was likely that this may contain chemokines and skew the results. Therefore MSC culture medium alone was run on the plate to serve as a background control. This control is plotted as a black bar for **Figure 5-4** and **Figure 5-5**. When the concentrations of chemokine present in MSC conditioned medium was at similar

levels to background controls, it was assumed that MSCs did not secrete these chemokines. Generally, when CC chemokines were secreted at substantial levels by MSCs, there were no significant differences in chemokine secretion between different MSC populations. This difficulty in obtaining statistical significance between MSC populations is likely due to the extensive donor variability in the chemokine concentrations that we observed. However significant differences were observed between homeostatic to inflammatory conditions (**Figure 5-4**).

CCL2, an inflammatory chemokine important in monocyte chemotaxis, was one of the top secreted chemokines out of the CC and CXC families by all MSCs under homeostatic and inflammatory conditions (**Figure 5-4 A**). The levels of CCL2 secreted by MSCs under homeostatic conditions were surprising as MSCs are notoriously anti-inflammatory cells. Although not significant, slight differences in the secretion levels of CCL2 under homeostatic conditions between MSC populations were observed, where Ad and Is MSCs secreted the most, followed by Va, BM and then UC MSCs. Not surprisingly, under inflammatory stimulation, Is and Va MSCs substantially upregulated CCL2 secretion, however this did not reach significance likely due to donor variability. Ad MSCs however, significantly upregulated CCL2 secretion ($P=0.0435$). BM and UC CCL2 secretion values were generated with a dilution factor and therefore are not fully reliable.

Similar to CCL2, CCL3 is also an inflammatory chemokine. Under homeostatic conditions, there were significant differences between the levels of CCL3 in the conditioned medium of MSCs, where Va MSCs and Ad MSCs had significantly more than Is MSCs ($P\leq0.001$) (**Figure 5-4 B**). These observations are of note as Va and Ad MSC CCL3 secretion were similar to control serum levels of CCL3, whereas Is MSC CM had significantly less ($P\leq0.001$) than background serum levels. This could suggest that Is MSCs may express high levels of receptors which would bind and internalise CCL3 such as ACKR2, however, as discussed, receptor expression by MSCs was minimal and therefore it is more likely that Is MSCs are secreting significantly more proteases capable of degrading chemokines than other tissue sources of MSCs. All populations of MSCs upregulated CCL3 secretion after inflammatory stimulation, however no statistical differences were observed between MSC populations or above background serum controls.

CCL11, a chemokine important in eosinophil chemoattraction, was not secreted by any population of MSC at substantial levels above background serum levels under homeostatic conditions (**Figure 5-4 D).** A marked increase in CCL11 secretion was observed going from homeostatic to inflammatory conditions, however this only reached significance in BM (P=0.04) and UC (P=0.01) derived MSCs as a result of very low basal level of secretion by these cells. Notably however, despite inflamed Is, Va and Ad MSCs secreting substantially more CCL11 than BM and UC MSCs (which secreted no more than background controls), there were no significant differences in the levels of secretion between these populations, again likely due to the donor variability.

CCL13 was secreted at much lower levels compared to CCL2 and CCL11. Under homeostatic conditions, MSCs from all tissue sources had similar levels of CCL13 in their conditioned medium as background controls, suggesting that this chemokine is not secreted **(Figure 5-4 E)**. After inflammatory stimulation, Ad MSCs significantly upregulated CCL13 secretion (P=0.0413), whereas Is, Va and BM MSCs upregulated CCL13 to similar levels, but the upregulation failed to reach significance due to donor variability. UC MSCs did not secrete CCL13 above background levels after inflammatory stimulation, suggesting that the secretion of CCL13 under inflammatory conditions was dependent on the tissue sources of the MSCs. Similarly, the secretion of CCL15 (**Figure 5-4 G**) also appeared to be dependent on MSCs source under inflammatory conditions as BM MSCs secreted substantially more than any other population of MSC.

CCL20, a chemokine involved in Th17 cell recruitment, was not secreted by MSCs in large quantities above background serum controls under homeostatic conditions (**Figure 5-4 I**). Strikingly, under inflammatory stimulation, Is, Va, BM and UC MSCs substantially upregulated CCL20 and Ad MSCs significantly upregulated (P=0.0490) CCL20 secretion. All populations of MSCs secreted similar levels of this chemokine. This could suggest that T cell recruitment is a universal mechanism of MSC action during inflammatory insult.

Other CCL chemokines assayed (CCL4 (C), CCL14 (F), CCL18 (H), CCL21 (J), CCL24 (K), CCL26 (L) and CCL27 (M)) are graphed in Figure 5-4, however they were not secreted at substantial levels above background levels by any of the MSCs tested.

Although not a part of the chemokine family, vascular endothelial growth factor (VEGF) was included as an analyte on the LUMINEX plate. VEGF is an important signalling protein involved in angiogenesis and was secreted at moderate levels above background controls by Is, Va and BM MSCs under homeostatic conditions **(Figure 5-4 N**). BM MSCs VEGF secretion was particularly enhanced under inflammatory stimulation in comparison to all other populations of MSCs, perhaps suggesting a tissue specific role of VEGF in the bone marrow in an inflammatory environment.

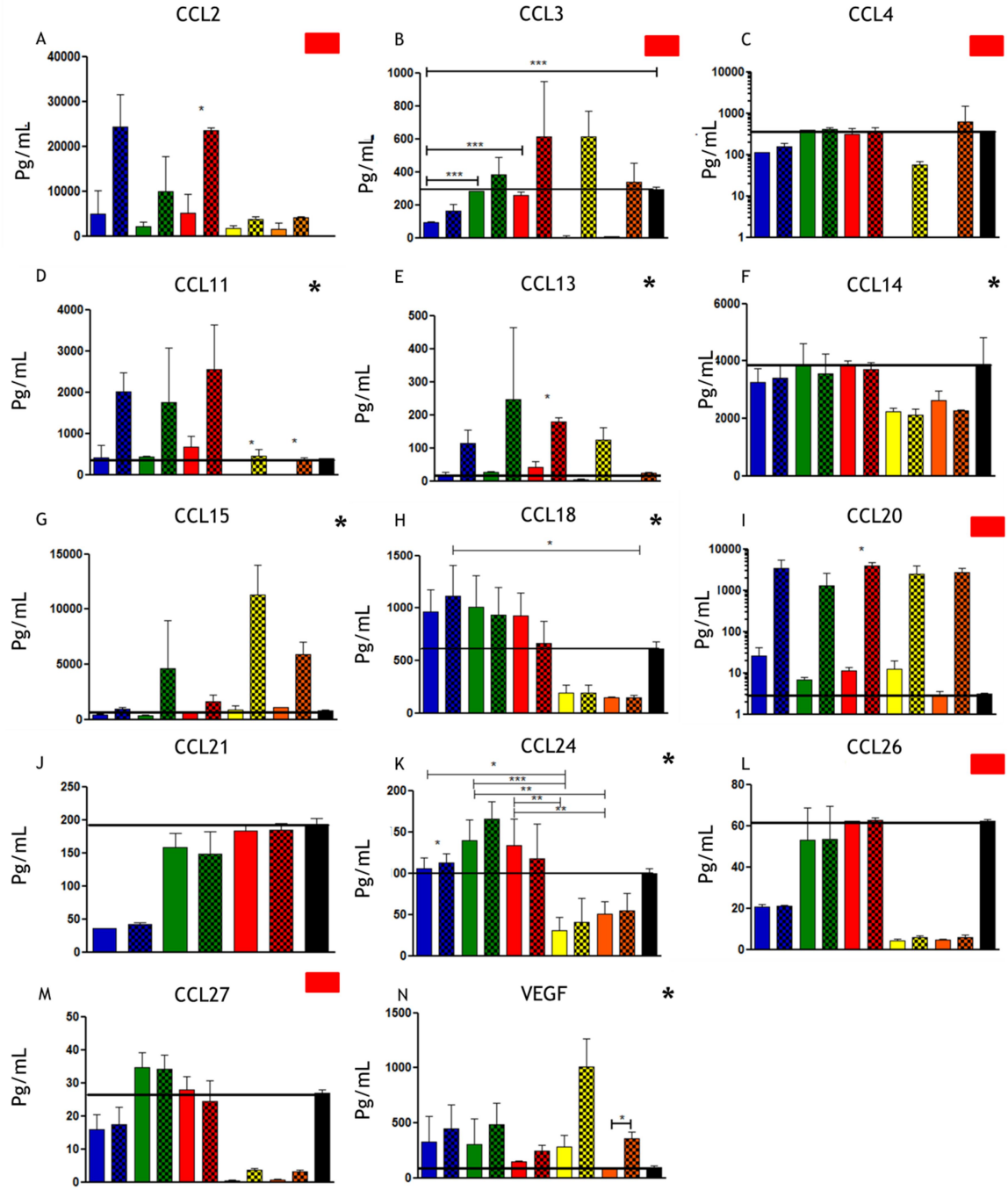


Figure 5-4 LUMINEX analysis of CC chemokines
P3 MSCs at 80% confluence were washed with warmed PBS before the addition of culture medium alone or culture medium + inflammatory cytokines (10ng/mL of TNF-α, IL-1β and IFN-Y). Cells were left for 24 hours before conditioned medium was collected, diluted 1 in 2 and added to the LUMINEX plates. Results are graphed as pg/mL. **Black bar represents the background volumes of chemokine present in human AB pooled plasma within the medium**. Each aliquot of AB pooled plasma consisted of 7-9 plasma donors, therefore each black bar represents 21-27 donors. Red boxes outline analytes where UC and BM values have been generated from a dilution factor and therefore are not directly comparable to Is, Va and Ad MSCs.

Each bar represents an n of 3 ± SEM. ONE WAY ANOVA with Tukey's post test analysis was carried out when comparing Is, Va and Ad MSCs *=P<0.05, **=P<0.001, ***=P<0.0001. Students paired T test was used to asses statistical differences between homeostatic and inflammatory conditions within one MSC source. Significance is marked on the graphs where applicable and P values are stated in the text throughout.

Overall, CCL2 was the highest secreted CC chemokine by Is, Va and Ad MSCs and the only one to be secreted at levels substantially above background controls under homeostatic conditions, suggesting that Is, Va and Ad MSCs could attract monocytes prior to any inflammatory insult, however BM and UC MSCs would not (BM and UC values for CCL2 secretion have to be interpreted with caution). As CCL2 secretion was upregulated, this suggests that monocyte migration towards CCL2 secreting MSCs would also be enhanced. In addition to this, CCL20 was secreted at very similar levels by all populations of MSCs during inflammatory conditions. The combined secretion of CCL2 and CCL20 highlights that regardless of the tissue source, MSCs could potentially be programmed to attract both monocytes and T cells under homeostatic and inflammatory conditions. Conversely, other molecules such as CCL15 and VEGF were secreted specifically BM MSCs under inflammatory stimulation, potentially suggesting that not all behaviours of MSCs are universal and that tissue origin could impact *in vivo* MSC behaviour.

5.3.2 **CXC Chemokines present in MSC conditioned Medium**

CXC chemokines were assayed in the same way as discussed above. Results are graphed similarly- BM and UC values have to be interpreted with caution. Due to substantial upregulation of CXC chemokines, some graphs have a $\log_{10}$ Y-axis in order to visualise all the data clearly. Due to large donor variation, significant findings were minimal.

The secretion of CXCL1 under homeostatic conditions was specific to UC MSCs and up to 16 fold differences were observed in CXCL1 secretion between MSC populations **(Figure 5-5 A).** Under inflammatory stimulation, all MSC populations upregulated their secretion of CXCL1, where Ad MSCs significantly upregulated CXCL1 305 fold (P=0.0327), resulting in Ad MSCs secreting the most CXCL1, 2 fold higher than BM MSCs which secreted the least CXCL1.

Like CXCL1, CXCL5 is a strong neutrophil chemoattractant. CXCL5 was not secreted above background levels by any MSC populations when they were homeostatically maintained **(Figure 5-5 B).** Under inflammatory stimulation, UC MSCs upregulated CXCL5 secretion 52 fold, Is MSCs 34 fold, BM MSCs 16 fold, Va

10 fold and Ad MSCs 7 fold. Although no significant differences were observed, CXCL5 secretion was affected by the tissue origin of MSCs where UC MSCs secreted 27 fold higher quantities than Is MSCs under inflammatory conditions. This could suggest that UC MSCs might induce the migration of higher quantities of neutrophils in comparison to Is MSCs.

Similar to CXCL1, UC MSCs did not need any prior stimulation to secrete CXCL6, this was secreted when they were maintained homeostatically. Ad MSCs also secreted CXCL6 under homeostatic conditions, resulting in CXCL6 secretion being specific to these tissue sources **(Figure 5-5 C).** Under inflammatory stimulation, MSCs from all sources substantially upregulated CXCL6 secretion, where BM MSCs upregulated CXCL6 secretion 181 fold, Va MSCs 34 fold, Is MSCs 20 fold, UC MSCs 14 fold and Ad MSCs significantly (P=0.0391) upregulated CXCL6 secretion 6 fold. Large variations in CXCL6 secretion were observed between Ad and Is MSCs, where Ad MSCs secreted 2.7 fold higher volumes of CXCL6 under inflammatory conditions than Is MSCs.

CXCL8 is a strong neutrophil chemoattractant. Strikingly, it was secreted at substantial levels by all MSCs above background under homeostatic conditions. Ad MSCs secreted the largest amounts of CXCL8, 16 fold higher than Va MSCs which secreted the lowest amount of CXCL8 during homeostasis (**Figure 5-5 D**). When MSCs were maintained under inflammatory conditions, CXCL8 secretion was markedly enhanced by all MSCs, however, only reaching significance in BM (P=0.0062) and UC MSC (P=0.0034) populations. The consistent levels of secretion of CXCL8 by all MSC populations, irrespective of tissue origin suggests that CXCL8 plays a central and universal role in MSC function perhaps by attracting neutrophils.

CXCL9, 10 and 11 are strong T cell chemoattractants and were not secreted by any MSC populations when they were maintained in homeostatic conditions (**Figure 5-5 E, G and F**). Not surprisingly, the secretion of these IFN-ϒ activated genes – CXCL9, 10 and 11 – was increased by all populations of MSCs following inflammatory stimulation, therefore suggesting that this is a mechanism MSCs use in inflammatory environments to perpetuate the inflammatory response, perhaps by attracting T cells and/or NK cells which bear the cognate receptor – CXCR3- to these chemokines.

Other CXC chemokines which were not produced by any MSC in either homeostatic or inflammatory conditions are graphed in Figure 5-5 and include CXCL12 (H), CXCL14 (I) and CXCL16 (J).

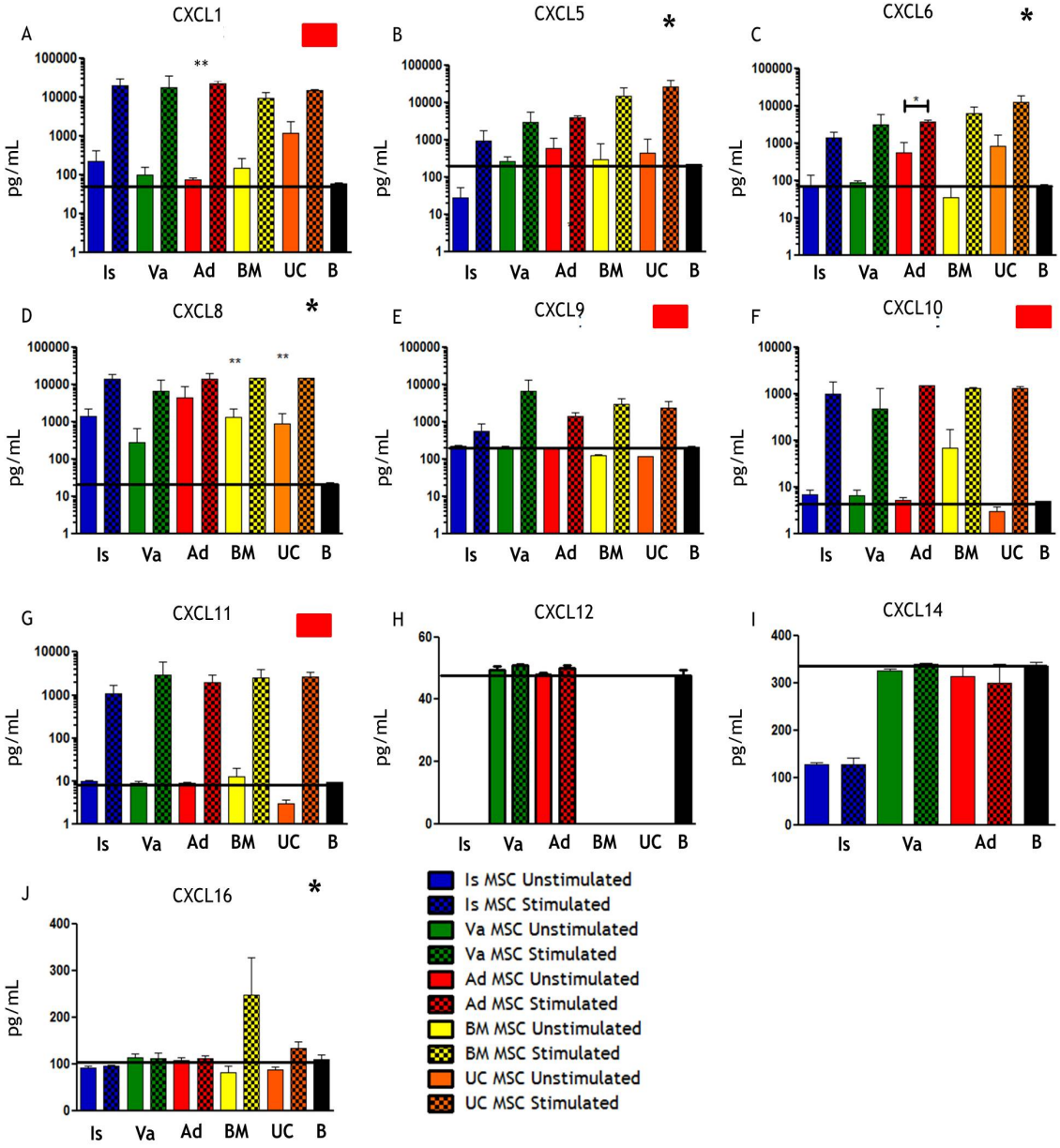


Figure 5-5 Luminex analytes of CXC chemokines
As before-

P3 MSCs at 80% confluence were washed with warmed PBS before the addition of culture medium alone or culture medium + inflammatory cytokines (10ng/mL of TNF-α, IL-1β and IFN-Y). Cells were left for 24 hours before conditioned medium was collected, diluted 1 in 2 and added to the LUMINEX plates. Results are graphed as pg/mL**. Black bar represents the background volumes of chemokine present in human AB pooled plasma within the medium**. Each aliquot of pooled plasma consisted of 7-9 plasma donors, therefore each black bar represents 21-27 donors. Red boxes outline analytes where UC and BM values have been generated from a dilution factor and therefore are not directly comparable to Is, Va and Ad MSCs.

Each bar represents an n of 3 ± SEM. ONE WAY ANOVA with Tukey's post test analysis was carried out when comparing Is, Va and Ad MSCs *=P<0.05, **=P<0.001, ***=P<0.0001. Students

paired T test was used to asses statistical differences between homeostatic and inflammatory conditions within one MSC source, P values are stated in the text throughout. Significance is marked on the graphs where applicable and P values are stated in the text throughout.

To summarise, CXC chemokine secretion by MSCs was differentially affected depending on MSC tissue source and inflammatory stimulation. During homeostatic conditions CXCL8 was the highest secreted CXC chemokine by Is, Va, Ad and BM MSCs, whereas UC MSCs secreted more CXCL1 than any other CXC chemokine assayed. After inflammatory stimulation, Is, Va and Ad MSCs secreted the largest amounts of CXCL1 compared to all other CXC chemokines, whereas UC and BM MSCs secreted the largest amounts of CXCL5 and CXCL8, respectively. This specific CXC chemokine secretion profile of all MSCs is surprising as this would suggest that all MSCs would specifically attract large numbers of neutrophils.

To review, chemokine secretion by all MSC populations, among the CC chemokine family, monocyte (CCL2) and T cell (CCL20) chemoattractants were secreted with little variability between MSC populations. A specific signature of CXC chemokines was differentially secreted by MSCs depending on MSC tissue origin, where UC MSCs appeared to secrete the majority of neutrophil chemoattractans without prior stimulation, however CXCL8, was secreted by all MSC populations when homeostatically maintained. Inflammatory stimulation resulted in all neutrophil chemoattractants (CXCL1, 5, 6 and 8) and T and NK cell chemoattractants (CXCL9, 10 and 11) being secreted by all MSC populations. This specific chemokine secretion profile of MSCs isolated from all sources suggests that MSCs would specifically attract monocytes and neutrophils under homeostatic conditions. The chemoattraction of NK and T cells would only be induced after inflammatory stimulation. Importantly CCL2 and the CXC chemokines may also be involved in angiogenesis, which could provide reason for the secretion of these molecules by MSCs. Additionally, the chemoattraction of monocytes, macrophages and neutrophils (and perhaps T cells and NK cells under inflammatory stimulation) appear to be a universal MSC *in vivo* function. Understanding if MSCs attract immune cells and, if there is any differential chemoattraction of these immune cells, would provide greater insight into the potential *in vivo* behaviours of MSCs.

5.4 Characterising the immune cell attraction profiles of MSCs

5.4.1 Optimisation of the transwell assays

To assess if the chemokines secreted by MSCs were functional and attracted the relevant immune cells, transwell assays were carried out to evaluate the ability of MSCs to attract white blood cells through a 5µm pore membrane. Before commencing transwell experiments, the assay was optimised to establish the ideal experimental procedure, which included ensuring that white blood cell separation was satisfactory, ensuring pore size of the transwell allowed migration of all immune cells and testing to see if MSC conditioned medium could be used as a substitute to MSCs in order to store samples and assay them through the same experimental time period.

To ensure that no major populations of white blood cells (WBCs) were excluded after the white blood cell isolation technique (Section 2.5.1), flow cytometry was used to assess the cell populations present in $1x10^6$ WBCs via SSC and FSC (**Figure 5-6 A**). Distinct lymphocyte (red box), monocyte (yellow box) and granulocyte (orange box) populations were present after the cell separation, indicating that this method of cell separation was suitable. Following this, it was important to establish an appropriate cell number to place on the top of the transwell insert that would include all white blood cell types identified in Figure 5-6 A and not clog the membrane. $5.5x10^5$ whole white blood cells (input population) were assessed via flow cytometry to ensure the presence of lymphocyte, granulocyte and monocyte portions and highlighted that the reduced number of start WBCs still represented the main WBC populations (**Figure 5-6 B**). Additionally, the ability of WBCs to migrate through the membrane towards MSCs was assessed, under homeostatic vs inflammatory conditions, to understand if $5.5x10^5$ cells allowed for the free migration of cells through the pores without clogging. In homeostatic conditions, all fractions of white blood cells migrated through the pores towards MSCs (**Figure 5-6 C**). However, under inflammatory stimulation, the monocyte population failed to migrate freely through the pores (**Figure 5-6 D**- yellow box), likely due to these cells becoming activated, resulting in increased adherence to the top of the transwell rather than migration through the pores.

Due to inflammatory cytokines remaining in the conditioned medium, investigating whether these cytokines (as opposed to the chemokines produced by MSCs) were affecting WBCs migration was important. To test this, the protocol for inflammatory stimulation was altered and compared. Prior to the protocol alteration, MSCs were washed with PBS and then left in 500uL of normal medium or 500uL of medium + 10ng/mL IL-1β, TNF-α and IFN-Ɣ for 24 hours. Protocol alteration required the removal of the inflammatory cytokines from the MSC medium. Therefore, MSCs + control wells (no MSCs) were left in normal medium or inflammatory medium for 24 hours. After 24 hours, all conditioned medium was removed and MSCs + control wells were washed thoroughly with warmed PBS. Fresh medium was added and MSCs + controls were left for a further 24 hours prior to the addition of WBCs on top of the insert. $5.5x10^5$ WBCs were then placed on the insert above and left for 3 hours to migrate through to the bottom wells. Lymphocytes, monocytes and granulocytes were able to freely migrate under both homeostatic (**Figure 5-6 E**) and inflammatory conditions (**Figure 5-6 F**). The increased numbers of migrating monocytes observed in the new protocol (inflammatory cytokines removed) (Figure 5-6 F) compared to the old protocol (inflammatory cytokines present) (Figure 5-6 D) suggests that the presence of inflammatory cytokines within the CM was causing monocytes to adhere to the upper chamber. Therefore, in all transwell experiments, inflammatory cytokines were removed from MSC conditioned medium to allow the migration of monocytes towards MSCs.

To compare the effectiveness of conditioned medium alone to MSCs grown on the bottom of the transwells, the total number of WBCs migrating towards MSC conditioned medium (without MSCs) under homeostatic and inflammatory conditions was compared to the total number of WBCs migrating towards conditioned medium + MSCs under homeostatic and inflammatory conditions (**Figure 5-6 G**). These were then compared to background migration of WBCs. Background migration of WBCs was measured by recording the number of WBCs which migrated (or fell) through the pores towards plastic (graphed as grey bars). Additionally, to account for any residual cytokines that might be adhering to the plastic wells, an inflammatory background control was set up to measure the number of WBCs which had migrated (or fallen) through the plastic towards wells that had been treated the same way as MSCs under inflammatory

conditions (10ng/mL of TNF-alpha, IL-1beta and IFN-gamma in MSC medium for 24 hours, washed with PBS and fresh non-stimulatory MSC medium added for 24 hours) (**Figure 5-6 G**).

In homeostatic conditions, MSCs grown on the bottom of the wells attracted WBCs above background migration levels, whereas MSC conditioned medium attracted fewer WBCs than background migration. Under inflammatory stimulation, MSCs grown on the bottom of the well and stimulated CM attracted WBCs above background stimulation controls. Importantly, the presence of MSCs appeared to have a stronger chemotactic effect than MSC conditioned medium alone as demonstrated through the larger WBC counts. Moreover, the stimulatory control well also attracted marginally more WBCs than the medium alone control, demonstrating that residual cytokines were adhering to the plastic, therefore confirming that this is an appropriate and essential control.

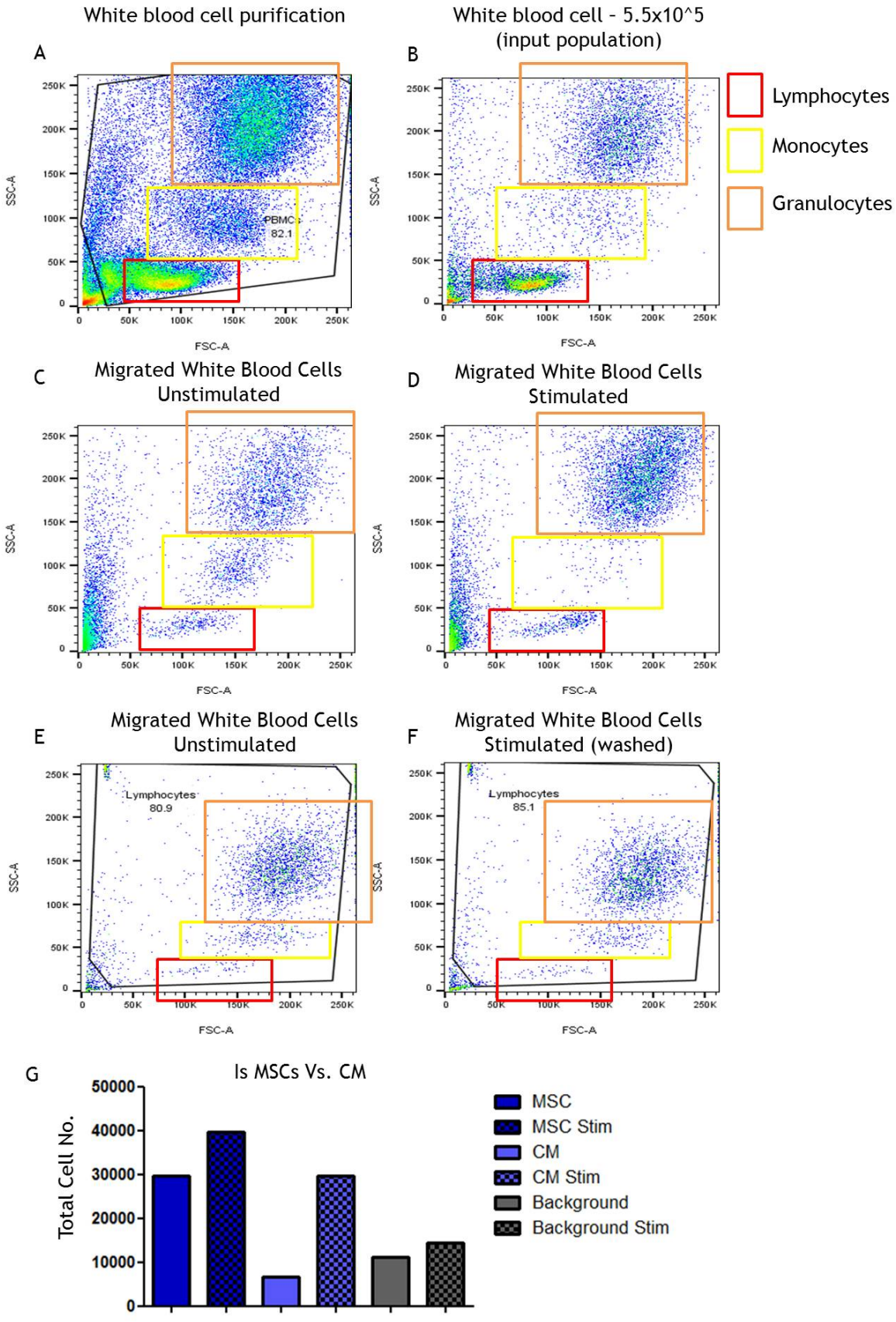


Figure 5-6 Optimisation of transwell assays using flow cytometry
To ensure that the transwell protocol was optimal before commencing all transwells, the white blood cell purification step was assessed via flow cytometry. SSC and FSC were used to ensure that all white blood cell populations were present – lymphocytes (red box), monocytes (yellow box) and granulocytes (orange box) (**A**). $5.5x10^5$ WBCs were assessed for their WBC composition (**B**),

and their ability to migrate through the 5µm pores under homeostatic (**C**) and inflammatory (**D**) conditions. To avoid leftover inflammatory cytokines in the conditioned medium of MSCs affecting WBC migration, MSCs were either left under homeostatic conditions for 24 hours (**E**) or stimulated with 10ng/mL of IFN-Ɣ, TNF-α and IL-1β for 24 hours (**F**), medium removed and MSCs washed with PBS and left for a further 24 hours in normal MSC medium before $5.5x10^5$ WBCs were added to the top of the insert. To compare the chemoattraction ability of Is MSCs grown on the bottom of the well to Is MSC CM, the total number of WBCs migrated towards Is MSCs (dark blue), Is MSC CM (light blue) and background migration under homeostatic (grey bars) or inflammatory (checked bars) conditions were counted (**G**).

To summarise, flow cytometry results from the white blood cell purification demonstrated that the isolation process was sufficient to allow assessment of the migration of all immune cells towards MSCs. Moreover, slight alteration of the stimulation protocol, and the use of $5.5x10^5$ WBCs, allowed for migration of lymphocytes, monocytes and granulocytes through the membrane without any major evidence of clogging. Lastly, the comparison of the total numbers of WBCs migrating through the membranes towards conditioned medium alone or MSCs in conditioned medium under homeostatic and inflammatory conditions, highlighted that the presence of MSCs had a larger chemotactic effect on WBCs. Therefore, for the following transwell experiments, MSCs were grown on the bottom of the transwells, stimulated or left in homeostatic conditions for 24 hours, washed and replenished with medium alone for 24 hours. $5.5x10^5$ WBCs were placed on top of the 5µm pores and incubated for 3 hours. A full outline of methods is provided in Section 2.5

5.4.2 **Flow cytometry gating strategies for transwell assays**

To assess the immune cell attraction profile of MSCs, WBCs which had migrated through the membrane towards MSCs or plastic (controls) were collected and assessed via flow cytometry with the following markers; CD4, CD8, CD19, CD1c, CD56, Siglec 8, CD14, CD16, HLA-DR and CD66b. Additionally, due to the observation of WBCs sticking to MSCs (**Figure** 5-7 **A-D**), the adherent WBCs and MSCs were removed using TryplE and stained with a different panel of antibodies; CD4, CD8, CD14, CD16 and CD45. A simpler panel was used here as adherent cell numbers were minimal and it was important to generate a clear phenotype of immune cells interacting with MSCs whilst avoiding cell loss in assay set-up.

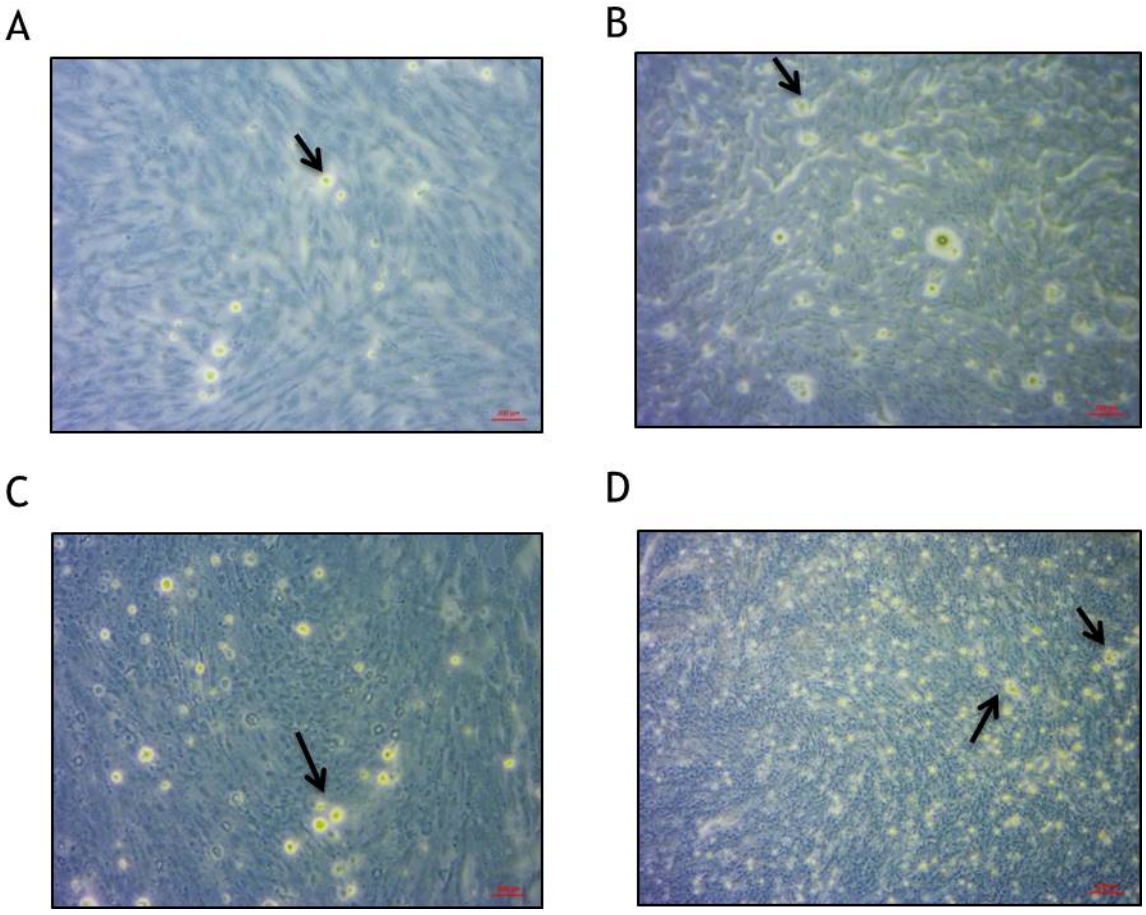
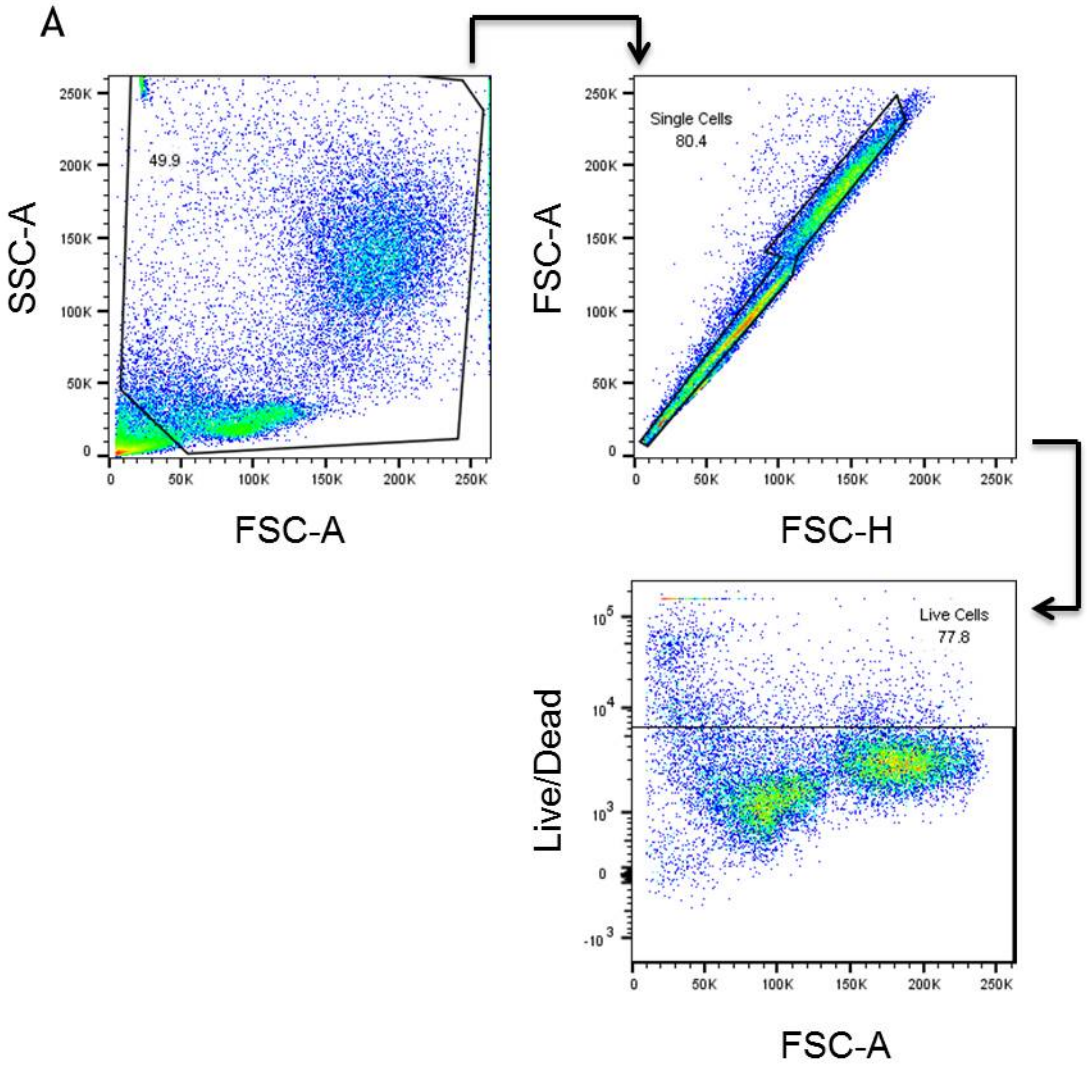
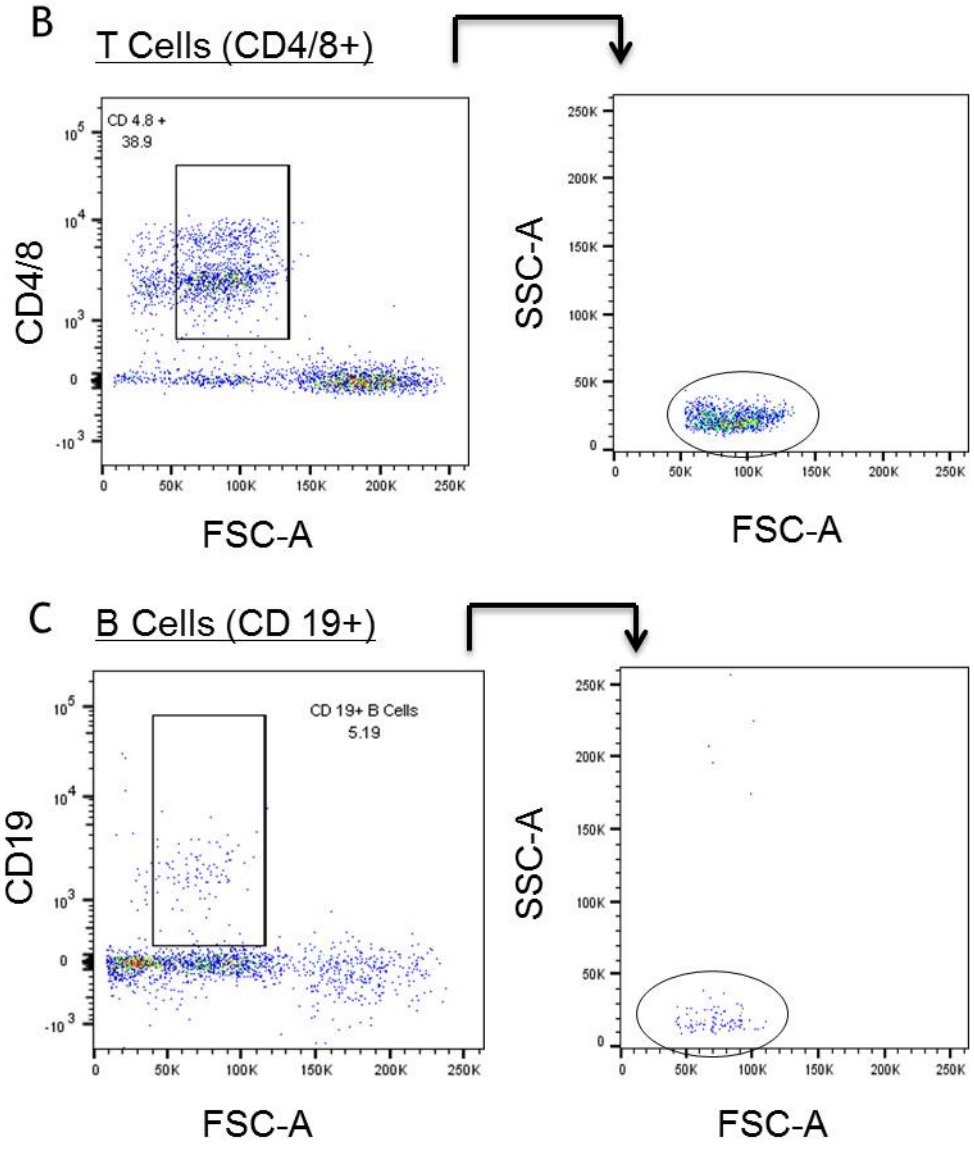


Figure 5-7 WBCs adhering to MSCs post-transwell
MSCs were grown in a monolayer at the bottom of the transwell. $5.5x10^5$ WBCs were added and allowed to migrate for 3 hours. Migrated WBCs were pipetted off and MSCs were washed with warmed PBS. WBCs adhering to MSCs in unstimulated (A-B) and stimulated (C-D) conditions were observed using a phase-light microscope. Black arrows point to adherent WBCs.

Several gating strategies were used to identify T cells, B cells, NK cells, neutrophils, eosinophils, monocytes and dendritic cells present in the supernatant in the bottom well (**Figure 5-8 A-G**). To ensure all cells analysed were live with doublets excluded, all leukocytes were positively gated and doublets excluded using a tight gate around the cells present in the FSC-H, FSC-A channels. From here, cells negative for live/dead stain were gated **(Figure 5-8 A).** This population of cells was then further analysed for its immune cell composition. To identify T cells (CD4+ and CD8+), a gate was drawn around CD4/8+ cells, being careful to exclude debris (FSC<50K). From here, FSC-A and SSC-A were used to ensure that T cells were of correct size and granularity (**Figure 5-8 B**). To identify B cells, live CD19 + cells were gated on, debris excluded (FSC<50K) and FSC-A and SSC-A were used to ensure cells were of

correct size and granularity (**Figure 5-8 C**). To identify NK cells, CD56+ cells were gated on and FSC-A, SSC-A were used to ensure cells were of correct size and granularity **(Figure 5-8 D).** To identify neutrophils and eosinophils, CD66b+ granulocytes were gated on, siglec 8+ cells were considered eosinophils and siglec 8 –ve cells were considered neutrophils **(Figure 5-8 E**). To identify DCs, CD19-ve cells were gated upon to exclude any potential CD1c+ B cells. CD1c+ cells were selected and CD16+ CD14-ve to intermediate cells were gated on (**Figure 5-8 F**). These cells were considered DCs. To assess the monocyte composition within the supernatant, CD66b-ve cells were gated upon to exclude granulocytes, CD19 –ve cells were selected to exclude any CD16+ B cells. Cells of correct size and granularity were gated on and classical ($CD16^{low}CD14++$), intermediate (CD16+CD14++) or non-classical (CD16++CD14+) (**Figure 5-8 G**) populations of monocytes were defined.

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CD 4.8 +
38.9
CD4/8
FSC-A
SSC-A
FSC-A
C
B Cells (CD 19+)
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CD19
FSC-A
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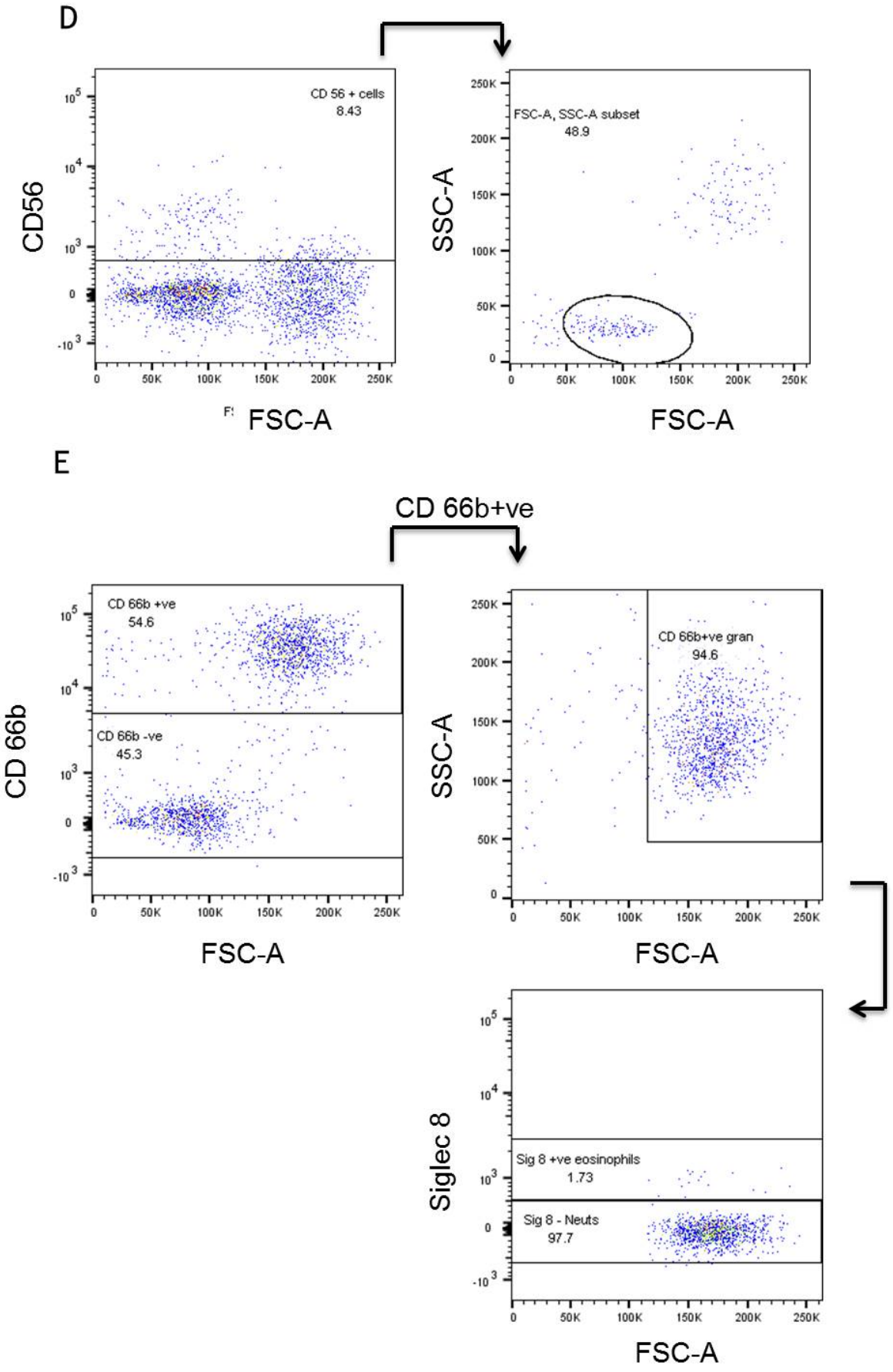
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CD 56 + cells
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FSC-A
SSC-A
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48.9
FSC-A
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CD 66b -ve
45.3
FSC-A
SSC-A
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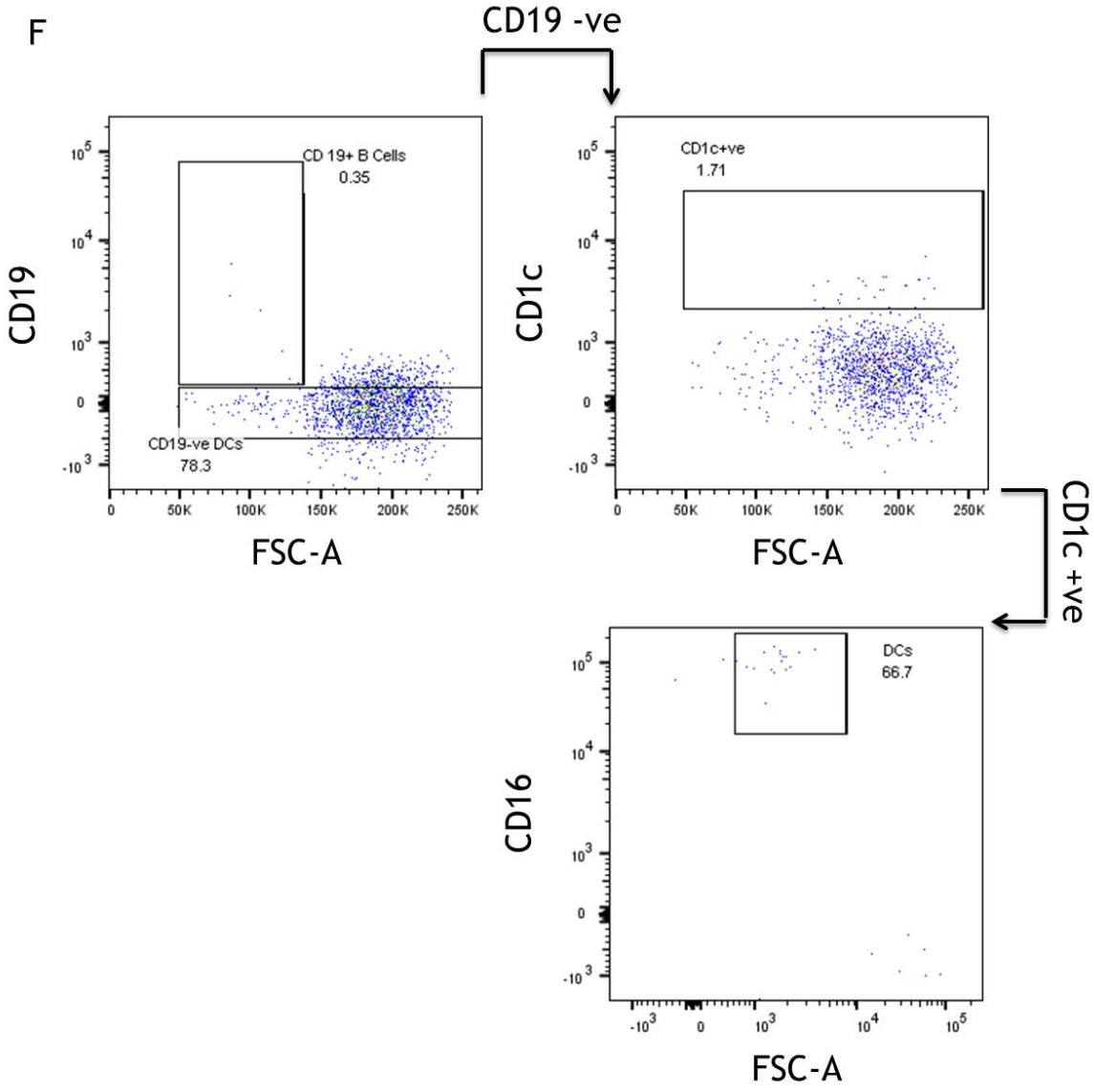
Sig 8 - Neuts
97.7
FSC-A

F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CD19
CD 19+ B Cells
0.35
CD19-ve DCs
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FSC-A

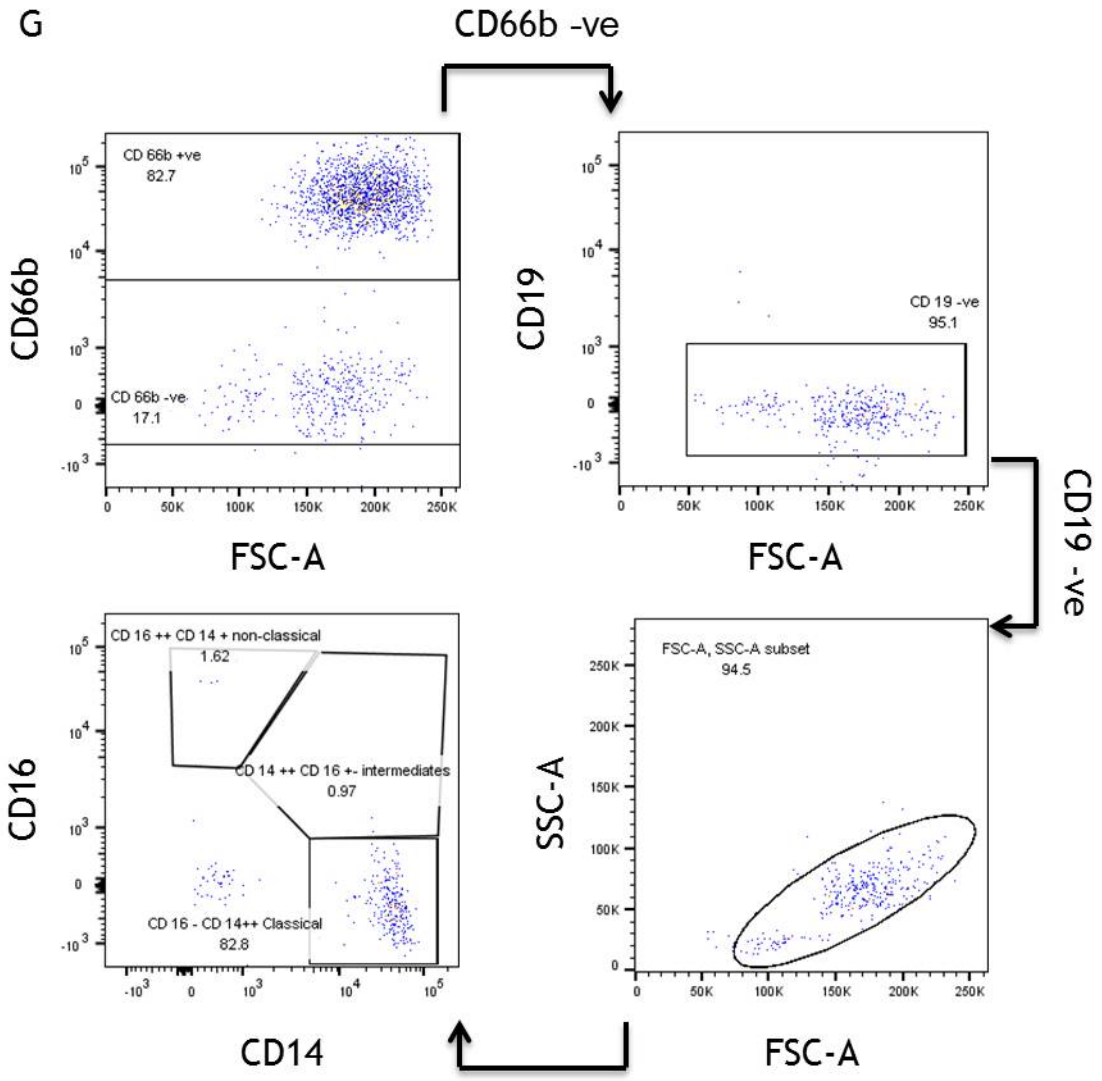


Figure 5-8 Gating strategies of migrated WBCs using flow cytometry
Flow cytometry was used to analyse the cells migrating through the transwells (**A-G**). Initial steps included gating on all leukocytes, with doublets excluded and live cells selected **(A).** From there, a specific gating strategy was used (detailed throughout the text) to isolate T cells (**B**), B cells (**C**), CD 56+ NK cells (**D**), eosinophils and neutrophils (**E**), DCs (**F**) and classical, intermediate and non-classical monocytes (**G**).

For the analysis of immune cells stuck to MSCs or plastic (controls) on the bottom of the wells, slightly different gating strategies were used to identify CD4 T cells, C8 T cells and monocytes.

The same gating strategy as above was used to isolate live cells (**Figure 5-9 A-C).** From here, CD45+ cells were selected to ensure the exclusion of MSCs **(Figure 5-9 D).** To assess monocytes, CD14 and CD16 were used to identify Classical ($CD16^{low}$ CD14++), intermediate (CD16+CD14++) or non-classical (CD16++CD14+) monocytes (**Figure 5-9 E**). CD4 was used to assess CD4 T cells and CD8 was used to assess CD8 T cells (**Figure 5-9 F**).

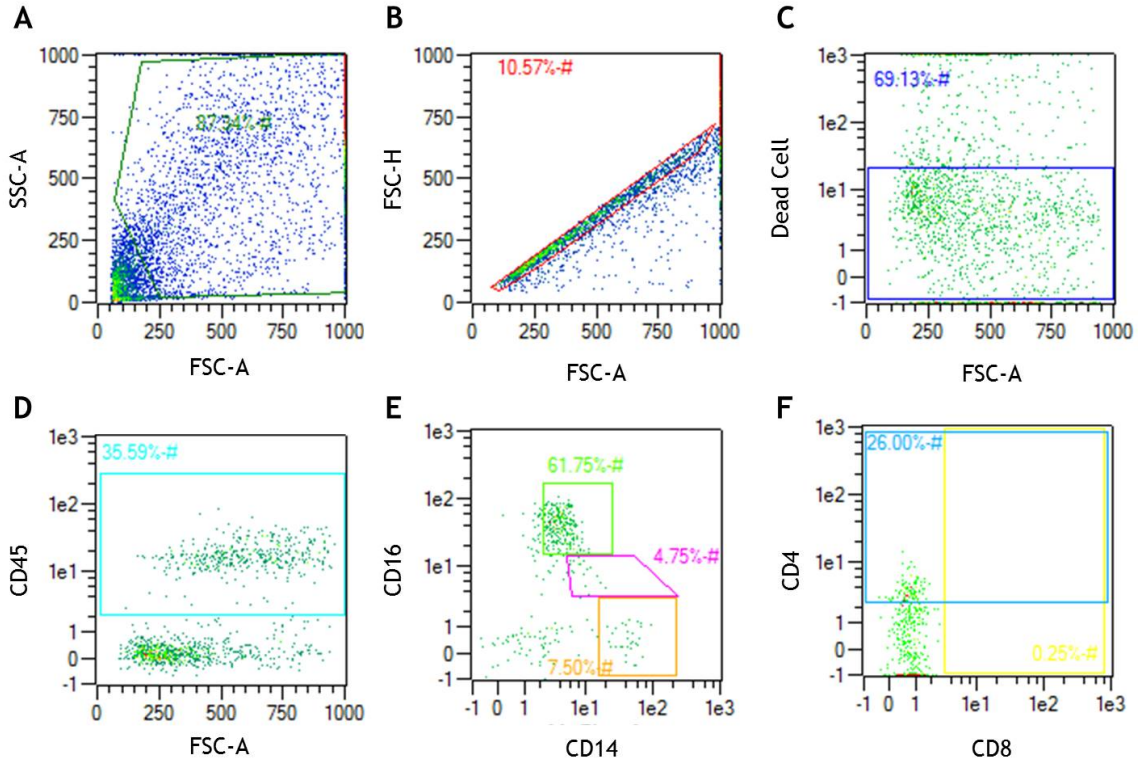


Figure 5-9 Gating strategy of migrated WBCs adhered to MSCs using flow cytometry
Using flow cytometry, cells of correct size and granularity were selected (**A**), doublets were excluded (**B**) and live cells were gated on (**C**). To ensure that MSCs were excluded from the analysis, CD45+ve cells were gated on (**D**). From here, monocytes were assessed by their expression of CD16 and CD14 – non-classical CD16++CD14+) in the green box, intermediates (CD16+CD14++) in the pink box and classical ($CD16^{low}$ CD14++) in the orange box (**E**). T cells were assessed via their expression of CD4 and CD8 (**F**).

To ensure that the gating strategies described above were accurate, the immune cell composition of the "input population" (5.5x10^5) was assessed (Figure 5-10**).** The composition of the leukocytes is graphed as percentages, with T cells making up 42.39% (± 4.81%) of the population, B cells making up 4.65% (± 1.50%), neutrophils making up 39.51% (± 6.55%), eosinophils making up 5.87% (±5.46%), NK cells making up 3.27% (± 0.72%), DCs making up 1.13% (± 0.38%) and monocytes making up 3.19% (± 0.63%) (Classical monocytes $CD16^{low}$ CD14++ = 2.04% (± 0.63%), Intermediate monocytes CD16+ CD14++ = 0.36% (±0.16%), Non-Classical monocytes = 0.78% (±0.16%)) of the WBC input population.

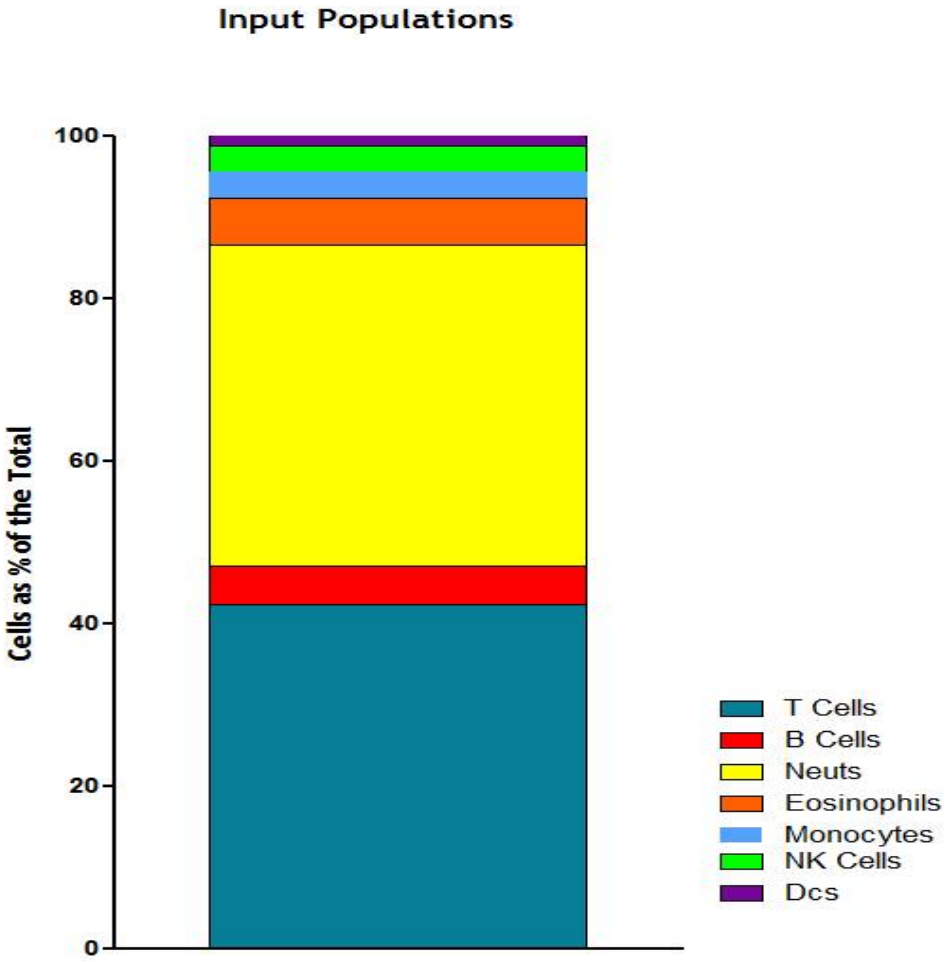


Figure 5-10 Assessment of the composition of the transwell input population
To ensure that the aforementioned gating strategies (Figure 5-8) were correct, the input populations ($5.5x10^5$) were assessed for their composition of WBCs and graphed as percentage. Graph ordering from the bottom; T cells (~ 42.39%-Blue), B cells (~ 4.65% -Red), Neutrophils (~ 39.51%-yellow), Eosinophils (~5.87% - orange), NK cells (~3.26%- green), DCs (~ 1.13% - purple), monocytes (~3.19% - light blue). N=6.

5.4.3 Analysis of leukocytes migrating towards MSCs in the transwell assays

Having established suitable gating strategies, analysis of migrated leukocytes towards MSCs was carried out using the same approaches. UC MSCs were not tested.

The total cell number of WBCs migrating towards MSCs and control wells are graphed in **Figure 5-11 A**. Details on how the total cell numbers were enumerated are outlined in Section 2.4.3.5. Is MSCs attracted the most white blood cells out of Va, Ad and BM MSCs under homeostatic and inflammatory conditions. During homeostatic conditions, interestingly only Is MSCs attracted significantly more WBCs (P=0.00366) above background controls, whereas Va MSCs attracted substantially more. In contrast, Ad and BM MSCs attracted fewer white blood cells than homeostatic background migration controls. Under inflammatory stimulation, all MSC populations induced the migration of immune cells above background inflammatory controls, where Is MSCs and Va MSCs attracted significantly more (Is P=0.00138, Va P=0.0285) WBCs, and Ad and BM MSCs attracted substantially more WBCs than inflammatory background controls.

Perhaps not surprisingly due to the extensive secretion of neutrophil chemoattractants by MSCs, the predominant WBC present in the migrated immune cell population was the neutrophil (CD 66b+, Siglec 8 –ve cells) (**Figure 5-11 B**). Although not significant, Is MSCs attracted the most neutrophils out of all MSC populations under homeostatic and inflammatory conditions, highlighting that MSCs isolated from different tissues display differential behaviour *in vitro*. Under homeostatic conditions, Is and (arguably) Va MSCs were the only MSC populations to attract neutrophils at substantial levels above background, whereas Ad and BM MSCs attracted slightly less than background controls. Under inflammatory stimulation, neutrophil migration towards all MSCs was enhanced compared to homeostatic conditions, with all MSCs attracting more neutrophils than inflammatory background controls.

Similarly, fitting with the observation that Is MSCs were one of the top CCL2 secretors, they also attracted the largest numbers of monocytes (**Figure 5-11 H, I and J**). Classical monocytes ($CD16^{low}$ CD14++) play important roles in

scavenging dead cells and debris during inflammation. Under homeostatic conditions, classical monocytes specifically migrated towards Is MSCs only (**Figure 5-11 H**). Is MSCs attracted significantly more classical monocytes than Va MSCs ($P<0.05$), Ad MSCs ($P<0.05$) and BM MSCs ($P<0.05$). This specific classical monocyte chemoattraction by Is MSCs was maintained under inflammatory conditions where Is MSCs attracted significantly more MSCs than Va ($P<0.05$) and Ad MSCs ($P<0.05$). BM MSCs also attracted a large proportion of classical monocytes under inflammatory conditions. Conversely, all MSCs tested did not induce substantial levels of intermediate (CD16+ CD14++) or non-classical (CD16++ CD14+) monocyte chemotaxis above background migration controls (**Figure 5-11 I & J**). Arguably, it could be suggested that Va MSCs showed a preferential attraction of intermediate and non-classical monocytes in comparison to other MSC populations. This preferential attraction of cells towards Va MSCs remained true for eosinophils when MSCs were homeostatically maintained, however all other populations of MSCs did not attract eosinophils under homeostatic or inflammatory conditions **(Figure 5-11 G)**. Consistently, small numbers of NK cells **(Figure 5-11 C)**, T cells **(Figure 5-11 D),** DCs **(Figure 5-11 E)**, and B cells **(Figure 5-11 F)** specifically migrated towards Va MSCs only under both homeostatic and inflammatory conditions, whereas all other MSC populations did not induce migration of these cells above background control levels.

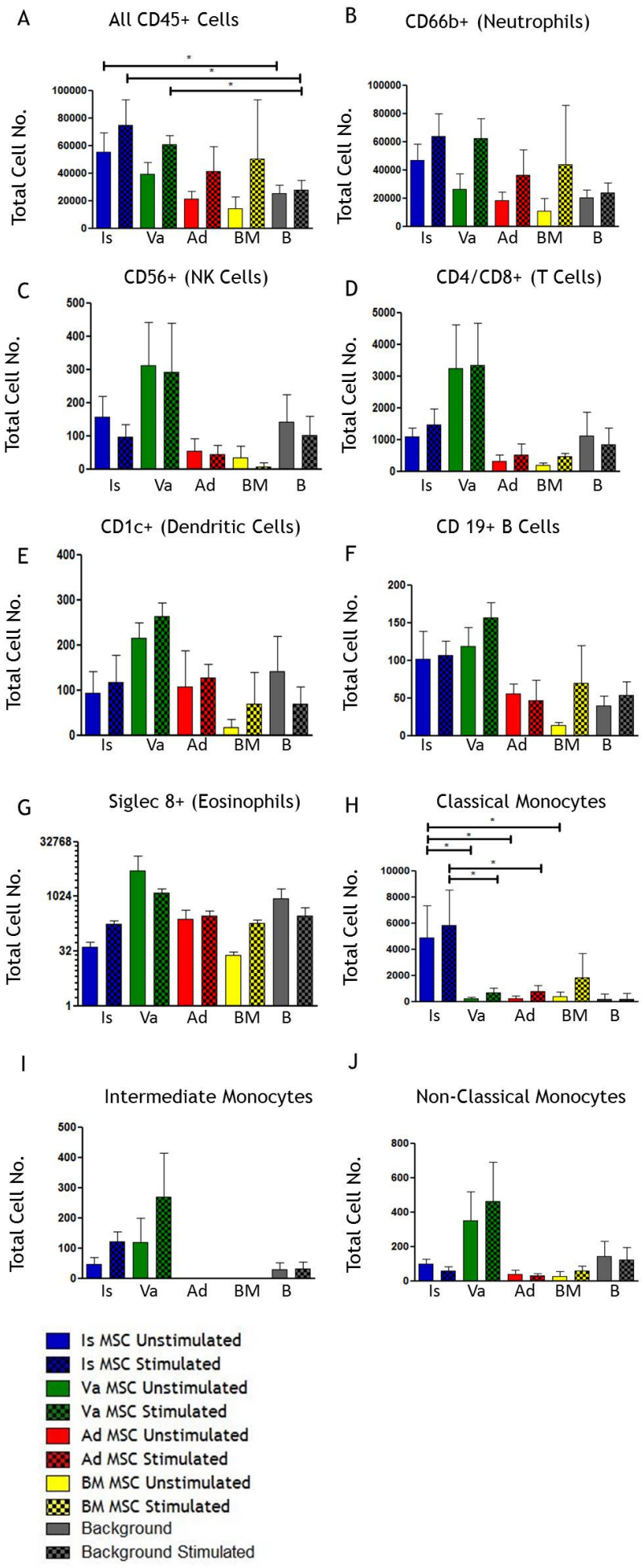

A
All CD45+ Cells
B
CD66b+ (Neutrophils)
C
CD56+ (NK Cells)
D
CD4/CD8+ (T Cells)
E
CD1c+ (Dendritic Cells)
F
CD 19+ B Cells
G
Siglec 8+ (Eosinophils)
H
Classical Monocytes
I
Intermediate Monocytes
J
Non-Classical Monocytes
Total Cell No.
Is
Va
Ad
BM
B
Is MSC Unstimulated
Is MSC Stimulated
Va MSC Unstimulated
Va MSC Stimulated
Ad MSC Unstimulated
Ad MSC Stimulated
BM MSC Unstimulated
BM MSC Stimulated
Background
Background Stimulated

Figure 5-11 Flow cytometry analysis of the number of immune cells migrating towards Is, Va, Ad, BM or plastic under homeostatic or inflammatory conditions.
WBCs were allowed to migrate in a transwell assay, through 5µm pores, for 3 hours towards Is MSCs (blue), Va MSCs (Green), Ad MSCs (red), BM MSCs (yellow) and plastic (Grey - background). MSCs and background controls had either been left under homeostatic conditions (coloured bars) or pre-inflamed with 10ng/mL of IFN-ϒ, TNF-α and IL-1β (Coloured, checked bars). Using the gating strategies in Figure 5-8, the total number of CD45+ (**A**), neutrophils (**B**), NK cells (**C**), T cells (**D**), DCs(**E**), B cells (**F**), eosinophils (**G**), classical monocytes (**H**), intermediate monosytes (**I**) non-classical monocytes (**J**) migrating towards MSCs or plastic were counted and graphed as above. Is MSCs: n=3, Va MSCs: n=3, Ad MSCs: n=3, BM MSCs: n=3. Background controls show the background migration of all transwell assays combined - total number of blood donors: n=6. Statistical analysis: Students T test was used when comparing MSCs from one source (unstim vs stim) and when comparing one source of MSC to background migration in unstimulated or stimulated conditions (P values stated throughout text). ONE WAY ANOVA with Tukey's post-test was used to assess statistical differences in immune cell migration towards MSCs from different sources (*=P<0.05, **=P<0.001, ***=P<0.0001).

Due to the adherence of WBCs to MSCs after transwell migration (Figure 5-7), cells were lifted from the plastic using TrypLE and analysed for the presence of monocytes and T cells. To account for background adherence to the plastic, cells that had adhered to the plastic in background controls were also included in the dataset (Figure 5-12).

During homeostatic conditions, the number of CD45+ve cells adhering to all populations of MSCs did not exceed the number of CD45+ve cells adhering to plastic in control wells (**Figure 5-12 A**). Inflammatory stimulation resulted in an upregulation of CD45+ cells adhering to MSCs from all tissue sources, which was above inflammatory background adherence controls in all MSC samples. This could suggest that under inflammatory conditions, MSCs were maintaining a specific interaction with immune cells. Through an unknown mechanism, it appears that Va MSCs are more efficient at mediating interactions with CD45+ve immune cells than other MSC populations as they showed substantially higher CD45+ve adherence than other MSC populations (4 fold higher than Is MSCs, 11 fold higher than Ad MSCs, 14 fold higher than BM MSCs and 33 fold higher than background controls) (**Figure 5-12 A**).

Under homeostatic conditions, classical monocytes ($CD16^{low}$ CD14++), specifically adhered to BM MSCs which was 40 fold higher than classical monocyte adherence to background controls (**Figure 5-12 B**). Inflammatory stimulation resulted in an increase of classical monocytes sticking to MSCs from all sources above background controls, with the exception of Ad MSCs. Similar to homeostatic

conditions, BM MSCs had the highest number of classical monocytes adhered to them compared to Is, Va and Ad MSCs.

Intermediate monocytes did not consistently adhere to any population of MSC above background levels under either homeostatic or inflammatory conditions **(Figure 5-12 C).** Similarly, under homeostatic conditions, non-classical MSCs did not adhere to any MSC populations above background controls **(Figure 5-12 D).** Inflammatory stimulation resulted in a significant upregulation of non-classical monocytes adhering to Is (P=0.0342) and Ad MSCs (P=0.0117), however, Is derived MSCs were the only tissue source to consistently facilitate significantly more adhesion of non-classical monocytes to their surface above background controls (P=0.0288).

CD8 T cells **(Figure 5-12 E)** and CD 4 T cells **(Figure 5-12 F)** did not adhere to MSC populations in great numbers above background controls, suggesting that MSCs might not specifically interact with these cells.

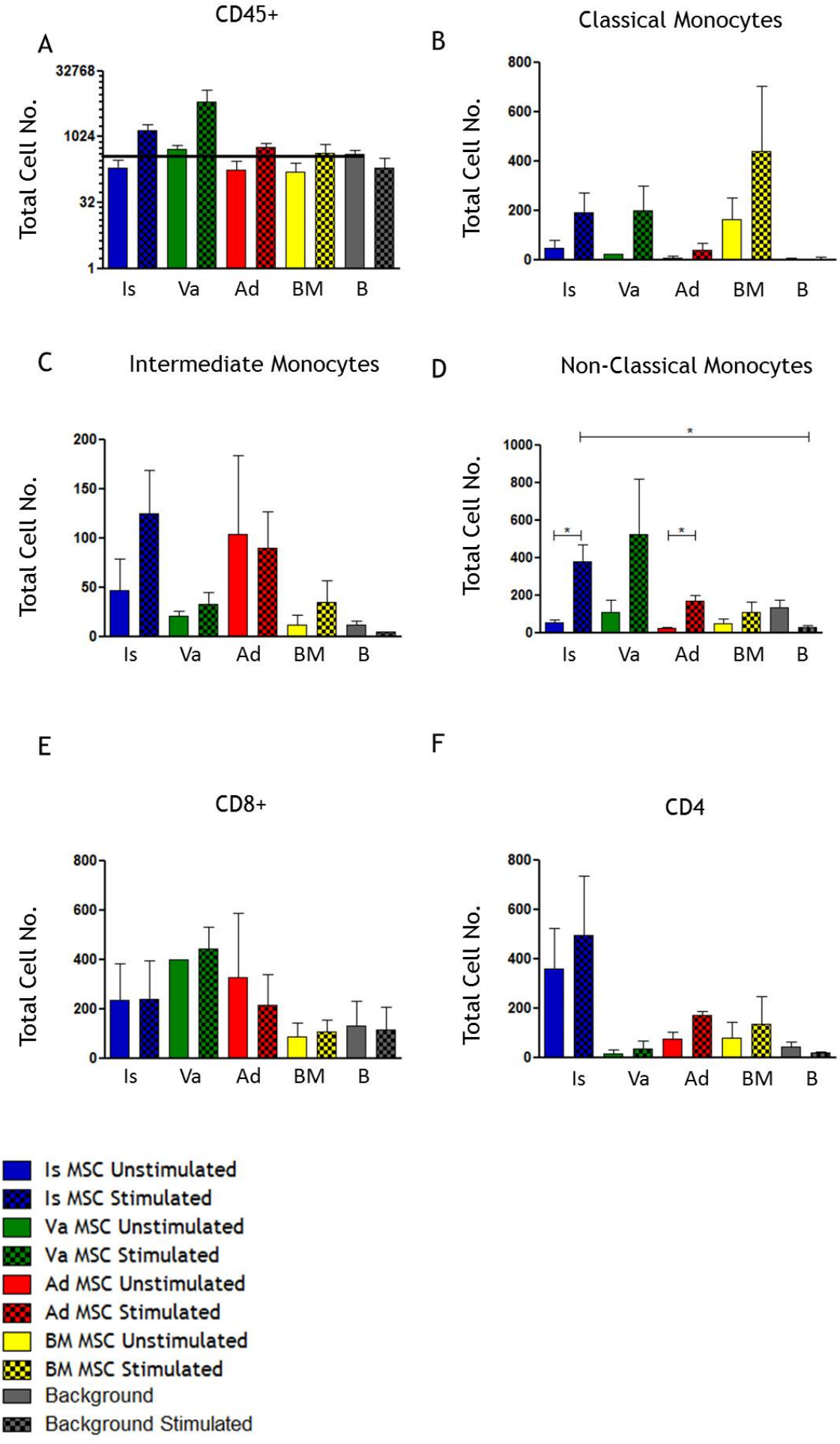

A
CD45+
Total Cell No.
32768
1024
32
1
Is
Va
Ad
BM
B
B
Classical Monocytes
Total Cell No.
800
600
400
200
0
Is
Va
Ad
BM
B
C
Intermediate Monocytes
Total Cell No.
200
150
100
50
0
Is
Va
Ad
BM
B
D
Non-Classical Monocytes
Total Cell No.
1000
800
600
400
200
0
*
*
*
Is
Va
Ad
BM
B
E
CD8+
Total Cell No.
800
600
400
200
0
Is
Va
Ad
BM
B
F
CD4
Total Cell No.
800
600
400
200
0
Is
Va
Ad
BM
B
Is MSC Unstimulated
Is MSC Stimulated
Va MSC Unstimulated
Va MSC Stimulated
Ad MSC Unstimulated
Ad MSC Stimulated
BM MSC Unstimulated
BM MSC Stimulated
Background
Background Stimulated

Figure 5-12 Flow cytometry analysis of the number of immune cells migrating towards, and adhering to, Is, Va, Ad, and BM MSCs or plastic under homeostatic or inflammatory conditions.
WBCs were allowed to migrate in a transwell assay, through 5µm pores, for 3 hours towards Is MSCs (blue), Va MSCs (Green), Ad MSCs (red), BM MSCs (yellow) and plastic (Grey - background). MSCs and background controls had either been left in homeostatic conditions (coloured bars) or pre-inflamed with 10ng/mL of IFN-ϒ, TNF-α and IL-1β (coloured, checked bars). MSCs were washed and leftover adherent WBCs and MSCs were detached and analysed via flow cytometry (**A-F**)

Using the gating strategies in Figure 5-9, the total number of CD45+ (**A**), Classical monocytes (**B**), intermediate monocytes (**C**), non-Classical monocytes (D), CD8 (E) and CD4 (F) adhering to MSCs or plastic were counted and graphed as above. Is MSCs: n=3, Va MSCs: n=3, Ad MSCs: n=3, BM MSCs: n=2. Background controls show the background adherence to plastic of all transwell assays combined - total number of blood donors: n=6. Students T test was used when comparing MSCs from one source (unstim vs stim) and when comparing one source of MSC to background migration in unstimulated or stimulated conditions (P values stated throughout text). ONE WAY ANOVA with Tukey's post-test was used to assess statistical differences in immune cell migration towards MSCs from different sources (*=P<0.05, **=P<0.001, ***=P<0.0001).

To summarise, Is MSCs attracted the most WBCs cells under both homeostatic and inflammatory conditions. The majority of cells migrating towards MSCs and plastic (background controls) were neutrophils. The total number of each WBC attracted to each MSC population and background is plotted in 'stack bars' to visualise if a particular tissue source of MSC preferentially attracted a specific immune cell **Figure 5-13**. The graph illustrates preferential attraction of classical monocytes by Is MSCs under homeostatic and inflammatory conditions, and eosinophils by Va MSCs under homeostatic conditions. Ad and BM MSCs show a similar composition of migrated WBCs to background controls.

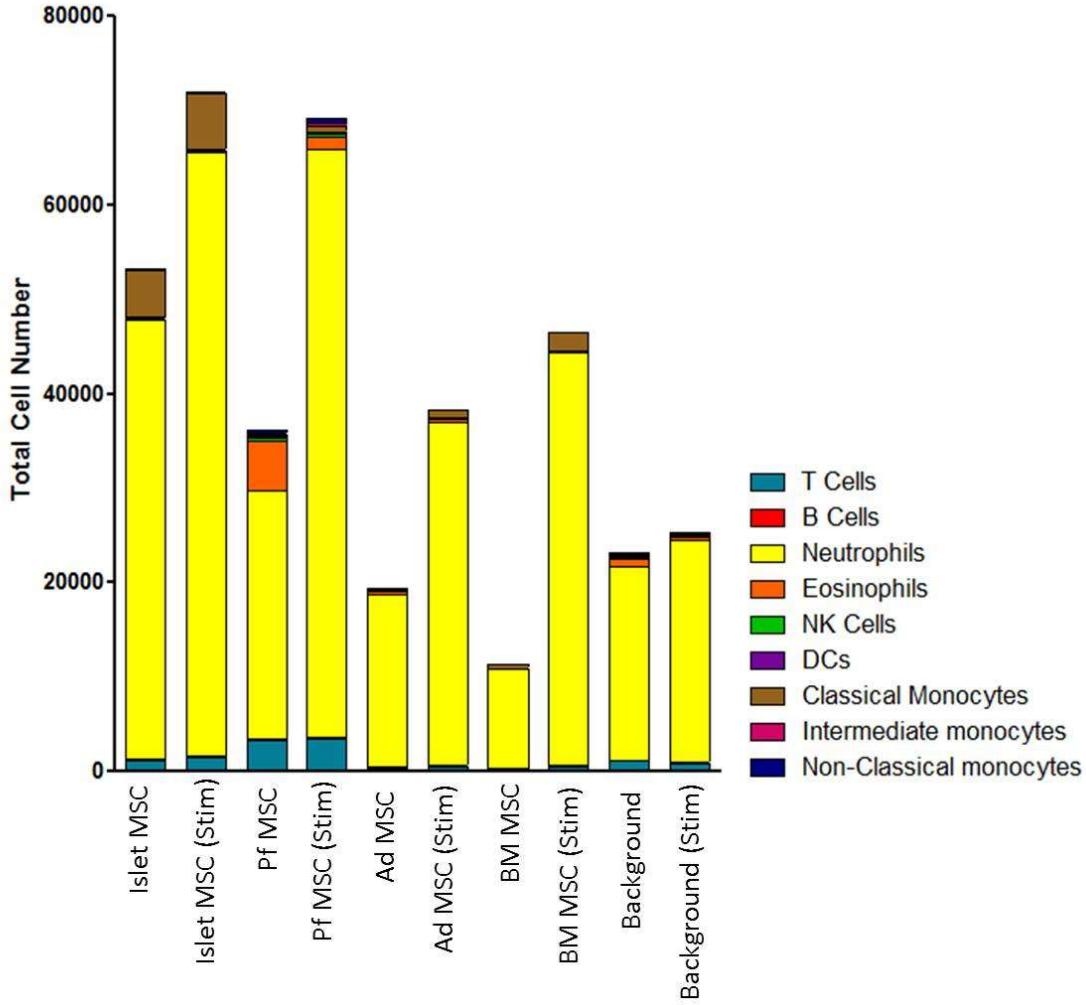


Figure 5-13 Summary of transwell Data
The same data graphed in Figure 5-11 is represented in a stack bar graph to summarise transwell assays and allow for easier visualisation of the immune cell attraction profile of Is, Va, Ad and BM MSCs.

5.5 Discussion

The aim of this chapter was to examine if genes of interest identified in the previous chapter were transcribed into protein. Additionally, through the use of standardised isolation procedures and experiments, this chapter aimed to objectively compare the chemokine and chemokine receptor expression at a protein level by MSCs isolated from the Is, Va, Ad, BM and UC under homeostatic and inflammatory conditions. Lastly, this chapter aimed to assess and compare the functionality of Is, Va, Ad and BM MSC secreted chemokines under homeostatic and inflammatory conditions. This discussion will combine the findings in the previous chapter (transcript expression of chemokines and their receptors by Is, Va, Ad, BM and UC under homeostatic and inflammatory conditions), and the current chapter. The discussion will focus predominantly on

the differences of chemokine and chemokine receptor expression between MSC sources and what this might mean in a clinical setting. To keep this discussion focussed, differences observed between MSC chemokine and receptor transcriptional expression vs. protein expression will not be addressed as the reasons for these variabilities could be manifold.

5.5.1 **Chemokine receptor expression by MSCs**

The use of MSCs for cell therapy somewhat relies on these cells homing and engrafting/persisting within the target tissue(s). Despite the exact mechanisms behind MSC homing and engraftment being poorly understood, they are presumed to involve chemokine receptors, along with other molecules such as integrins (244, 256, 257).

No significant differences were observed in the transcription of CC and CXC receptors by MSCs isolated from Is, Va, Ad, BM and UC MSCs under homeostatic or inflammatory conditions Section 4.3.1 and Section 4.3.2). This could be due to low transcription levels of the receptors by all populations of MSCs, therefore resulting in higher variability of the results. This observation fits with the literature as low chemokine receptor transcripts have been reported for MSCs (258), however , it has been shown that this does not impact surface chemokine receptor expression (259). Here we found low to moderate transcription of CCR7, CCR10, CXCR2, CXCR3, CXCR4, CXCR6, ACKR3 and ACKR4 on MSCs isolated from all sources under homeostatic and inflammatory conditions, and tested CCR7, CCR10, CXCR4, CXCR6, ACKR3 and ACKR4 at a protein level. Surface expression of these molecules on MSCs could result in MSCs migrating towards the lymph node (CCR7), skin (CCR10), bone marrow / lung (CXCR4) and potentially the liver (CXCR6). ACKR3 and ACKR4 are atypical chemokine receptors which play a regulatory role by scavenging the chemokines CXCL11, CXCL12 (ACKR3) and CCL19, CCL21 and CCL25 (ACKR4) from the surrounding environment. The current study found very little to no surface expression of CCR10 and CXCR6 on the surface of all tested MSCs which largely contradicts a large body of literature describing the presence of CCR10 and CXCR6 on Ad and BM MSCs (151, 242, 247, 260, 261) (262). The discrepancies between these observations and the current study could range from different culture conditions, to varied methods of chemokine receptor detection and importantly,

the notoriously poor quality of chemokine receptor antibody staining. It is also worth noting that, although little to no surface staining was observed for chemokine receptors, this is a common phenomenon reported in the MSC literature, however chemokine receptors still appear to be present and functional when tested in migration assays (263).

Due to the way this study was conducted, what can be concluded is that when Is, Va, Ad, BM and UC MSCS are objectively compared using standardised techniques, CCR7, CXCR4 and ACKR3 expression was specific to Va and BM derived MSCs. This suggests that chemokine receptor expression by MSCs relates to MSC tissue origin. To add another layer of complexity, inflammatory stimulation only appeared to significantly affect CXCR4 expression on BM MSCs, again suggesting a tissue specific response to inflammatory stimulation, which affects chemokine receptor expression. This has implications when one considers using MSCs in the clinic, where high levels of MSC CXCR4 expression would be desirable for hematopoietic stem cell transplantation for the enhancement of hematopoietic engraftment or treatment/prevention of graft versus host disease and therefore Va and BM MSCs might prove to be the preferential tissue for MSC isolation for use within this setting (264-266). Moreover, the highest levels of CXCR4 were observed on BM MSCs under inflammatory stimulation, perhaps providing a premise to pre-treat MSCs before infusion into patients to improve BM MSC homing with hope of ultimately improving clinical outcome. Similarly, enhanced expression of CCR7 by BM and Va MSCs could increase the lymph node homing of these MSCs in comparison with other tissue sources of MSCs which would intensify their *in vivo* immunomodulatory effects through their superior migration to SLO and interaction with T cells (267, 268), potentially having a beneficial impact on the prevention of GVHD.

In contrast to conventional signalling receptors, literature on MSC ACKR3 and ACKR4 expression are minimal, making it hard to evaluate the clinical relevance of MSC expression of these molecules, however it suggests that MSCs play a role in regulating the availability of CXCL11, CXCL12 (ACKR3) and CCL19, CCL21 and CCL25 (ACKR4).

Overall, chemokine receptor transcripts in all MSC populations were low and no significant differences between MSC populations were observed. No surface

expression of CCR10 and CXCR6 by any population of MSC tested were observed, implying that these populations of MSCs maintained in the specified conditions would not be desirable in clinical situations where skin (CCR10) and (potentially) liver (CXCR6) MSC homing/engraftment were important for clinical outcome. Importantly, moderate CCR7, CXCR4 and ACKR3 expression were specific to Va and BM MSCs, indicating that the tissue origin of MSCs affects the chemokine receptor expression and therefore potentially their *in vivo* migratory capacity. These data provide evidence to support further study into the expression and function of chemokine receptors by MSCs – in a standardised manner – as the tissue source of MSCs could impact MSC *in vivo* migratory capacity and thereby influence clinical outcome.

5.5.2 **Chemokine secretion by MSCs**

In vitro, constitutive secretion of a multitude of chemokines in the CC and CXC families by MSCs has been reported, including; CCL2, CCL3, CCL4, CCL5, CCL7, CCL20, CCL26, CXCL1, CXCL2, CXCL5, CXCL8, CXCL10, CXCL11, CXCL12 and CX3CL1, along with other non-chemokine related factors such as VEGF (269, 270). However, there is a lack of data surrounding the comparison of chemokines secreted by MSCs isolated from different tissues and what this might mean in a clinical setting- the majority of studies focus on chemokine secretion by BM MSCs. From the chemokines cited above, target immune cells that could be attracted towards MSCs include; neutrophils, monocytes, eosinophils, basophils, T Cells, B cells, DCs and NK cells (271-273). Chemokine secretion could be considered a vital immunomodulatory effect of MSCs *in vivo*, as chemokines mediate interactions between MSCs and surrounding immune cells, however, it is important to add that the chemoattraction of immune cells might not always lead to MSC immunomodulation of these cells. Moreover, the types, combination and the effects of chemokines secreted by MSCs may vary depending on the specific microenvironment and surrounding immune cells.

In the current study, chemokines that were transcribed and secreted at the highest levels by MSCs maintained under homeostatic conditions included; CCL2, CXCL1, CXCL5, CXCL6 and CXCL8. These chemokines are strong chemoattractants for monocytes and neutrophils, which were the predominant cell types to undergo chemotaxis towards MSCs in the transwell assays (68, 271,

274). Under inflammatory stimulation, MSCs from all tissue sources tested, upregulated the transcription and secretion the aforementioned chemokines, whilst also inducing the transcription and secretion of CCL20, CXCL9, CXCL10 and CXCL11. Although these data present many interesting findings, for the purpose of this discussion, I will focus on the chemokines that were secreted at high levels (CCL2, CCL20, CXCL1, CXCL5, CXCL6, CXCL8, CXCL9, CXCL10 & CXCL11) by MSCs and the specific immune cells they attracted.

CCL2 is produced by a variety of cell types including endothelial cells, fibroblasts, epithelial cells, smooth muscle cells, monocytes and macrophages (275-277). It regulates the migration and infiltration of monocytes, memory T cells, NK cells (278, 279) and is implicated in multiple excessive inflammatory disorders including atherosclerosis and multiple sclerosis whilst also being a mediator of angiogenesis and neovascularisation (274, 280). Several reports document the homeostatic secretion of CCL2 by BM and Ad derived MSCs and discuss MSC secreted CCL2 effects (281, 282). However, to my knowledge, differential CCL2 transcription, secretion or subsequent monocyte chemoattraction by MSC isolated from various tissues has not been documented. Here I show that Is, Va and Ad MSCs transcribed (Section 4.3.1 Figure 4-9 A) and secreted (Figure 5-4 A) substantial levels of CCL2 when maintained under homeostatic conditions. Transcription and secretion of CCL2 was markedly upregulated after inflammatory stimulation by MSCs isolated from all tissue sources. Is MSCs secreted the most CCL2 under inflammatory stimulation, which is consistent with the observation that Is MSCs attracted the most monocytes under inflammatory conditions, whereas Va, Ad and BM MSCs secreted less CCL2 and attracted fewer monocytes. Evidence suggests that MSCs secrete CCL2 to induce the migration of CCR2 positive inflammatory monocytes and drive monocyte differentiation towards a tumour associated macrophage phenotype, confirmed through inhibition of monocyte TNF secretion, and increased IL-10 secretion (283), driving an ‘M2-like’ phenotype. Differences in the monocyte populations (Classical, intermediate or non-classical) are ascribed to their surface molecule phenotype, cytokine production, antigen uptake and antigen presentation, however, there is little consensus in the literature about these attributes. It is largely accepted that classical monocytes are rapidly recruited to sites of inflammation and phagocytose microorganisms and dying cells (284).

Intermediate monocytes are recruited at a later stage of inflammation and are involved in high secretion of pro-inflammatory cytokines and chemokines and wound healing (285, 286). Non-classical monocytes display a patrolling behaviour and are also involved in inflammation via TNF-alpha production (286). CD16 expressing monocytes (intermediates and non-classical) have been implemented as proinflammatory cells, as they are rapidly expanded during inflammation (287). However, several reports contradict these findings and term classical monocytes as pro-inflammatory (288) and non-classical monocytes as pro-angiogenic (289). Phenotyping of the migrated monocytes towards MSCs showed that the majority of monocytes attracted to Is, Ad and BM MSCs were classical monocytes under homeostatic and inflammatory conditions, whereas the majority of monocytes attracted to Va MSCs were non-classical monocytes, further suggesting that the tissue origin of MSCs could impact their *in vivo* behaviours. As a result of the literature describing conflicting roles of the monocyte populations, which is likely due to their function being shaped by their surrounding environment, it is difficult to conclude whether the attraction of specific subsets of monocytes by a particular tissue source of MSC would be beneficial or detrimental in a clinical environment. Importantly, the attraction of monocytes is probably not only CCL2 dependent. Other chemokines identified in this study that could play a role in the differential MSC chemoattraction of monocytes include; CCL3 and CCL15 (290-292).

With the exception of CCL2, the CXC chemokines were secreted at higher levels by all MSCs populations than other CC chemokines, this mirrors the transcript data, where the CXC chemokines were the highest transcribed chemokines by all tissue sources of MSCs (Figure 5-5). MSC secretion of CXCL1, 5, 6 and 8 has been reported for mouse and human MSCs (293) and differential secretion of CXCL1, CXCL2, and CXCL5 has been observed between human Ad and BM MSCs. Here I report differential transcription and secretion of the neutrophil chemoattractants CXCL1, CXCL5, CXCL6 and CXCL8 by Is, Va, Ad, BM and UC MSCs. Under homeostatic conditions, CXCL1- involved in angiogenesis, arteriogenesis, tumorigenesis, wound healing and inflammation (7, 294)- was secreted in large amounts by UC MSCs only. Conversely, CXCL8 was secreted by all tissue sources of MSCs under homeostatic conditions, namely Is and Ad derived MSCs (295, 296). Interestingly the high levels of CXCL1, 5, 6 and 8

secretion by Is MSCs was mirrored by the specific chemoattraction of large numbers of neutrophils towards Is MSCs. Moreover, the MSC secretion of the aforementioned chemokines was upregulated following inflammatory stimulation, which was also associated with an influx in migrating neutrophils towards MSCs isolated from all tissue sources assayed – Is, Va, Ad and BM MSCs. The differences observed in MSC neutrophil chemoattraction abilities, where Is MSCs attracted substantially more than any other tissue source of MSC under homeostatic and inflammatory conditions, are difficult to interpret. Firstly, the chaotic redundancy within the chemokine family makes it impossible to easily understand the favoured/key neutrophil chemoattractancts involved in this process. Put simply, neutrophils bear CXCR1 and CXCR2 which together, bind a combination of all of the neutrophil chemoattractants listed above, ultimately resulting in neutrophil migration, thus making it nearly impossible to identify a single chemokine that is specific for the induction of neutrophil migration towards Is MSCs specifically. Secondly, given that Ad and BM MSCs secreted high levels of CXCL5 and CXCL8 under homeostatic conditions, which was not mirrored by neutrophil migration towards these populations of MSCs, it is also likely that, Is MSCs could specifically be secreting other un-assayed factors that enhance neutrophil migration. Alternatively, Ad and BM MSCs could secrete mediators such as Tumor necrosis factor inducible gene 6 (TGS-6) which has been shown to inhibit neutrophil migration in a similar transwell system by binding to CXCL8 and inhibiting its interaction with CXCR2 (297).

CXCL9, 10 and 11 are structurally related chemokines that bind to CXCR3 and promote chemotaxis of T cells and NK cells. Generally, they are not secreted under physiological conditions but are strongly induced during injury or infection (298). CXCL9, 10 and 11 secretion by human and mouse BM MSCs has been documented (299, 300) where mouse BM MSCs CXCL9, 10 and 11 secretion was important in the attraction, and subsequent immunomodulation of T cells (301). Data surrounding CXCL9, 10 and 11 secretion by MSCs isolated from alternative tissue sources are minimal. In the current study, the results show differential transcription and secretion of CXCL9, 10 and 11. In accordance with the literature, these chemokines were not transcribed or secreted by MSCs under homeostatic conditions, however upon inflammatory stimulation, transcription and secretion of CXCL9, 10 and 11 were upregulated by all MSCs, where Va MSCs

secreted substantially more CXCL9 and 11 than Is, Ad, BM and UC MSCs. These data are somewhat confirmed by the enhanced chemoattraction of small numbers of NK, CD4 and CD8 T cells towards Va MSCs only. However it does not explain the absence of T cell migration towards Is, Ad and BM MSCs despite their secretion of CXCL9, 10 and 11. The low number of T cells migrating towards MSCs could be explained by the absence of activated T cells in the peripheral blood. In the transwell system, the majority of T cells would have been naïve T cells which bear the receptor CCR7 and not CXCR3 (302). These T cells would respond to CCL19 and CCL21. CCL19 was transcribed at very low levels and likely not secreted by MSCs (Section 4.3.1). Similarly, CCL21 was transcribed at very low levels by MSCs and was not secreted in large amounts (Figure 5-4). In fact, it appeared that Is MSCs were degrading CCL21, perhaps accounting for the lower number of T cells migrating towards Is MSCs than background controls.

5.6 **Conclusions**

To summarise and conclude, this chapter aimed to determine if MSCs expressed genes –identified in the previous chapter- that were differentially/highly expressed at a protein level. More specifically, MSCs were assessed for their surface expression of CCR7, CCR10, CXCR4, CXCR6, ACKR3 and ACKR4 under homeostatic and inflammatory conditions. Low surface expression of all receptors assessed by flow cytometry was observed -this could be due to poor antibody staining of receptors. Low surface expression of chemokine receptors could mean that MSCs are unlikely to migrate away from a graft site if they are directly delivered there. Importantly, stimulation with cytokines resulted in an upregulation of CXCR4 which could give reason to pre-treat MSCs with cytokines to upregulate receptors and improve MSC homing to target tissues. To truly understand differential receptor expression and functionality of receptors expressed by Is, Va, Ad, BM and UC MSCs, migration assays towards chemokines would have to be performed.

Additionally this chapter aimed to assay chemokine secretion by Is, Va, Ad, BM and UC MSCs and to understand if tissue source of MSC resulted in differential chemokine secretion under homeostatic and inflammatory conditions. Similar to transcript data, MSCs were observed to produce large quantities of CCL2, CCL11, CCL20 and CXCL1, CXCL5, CXCL6, CXCL8, CXCL9, CXCL10 and CXCL11 at

differential levels depending on MSC tissue origin. Importantly, the secretion of these chemokines appeared to induce migration of target immune cells such as monocytes (CCL2) and neutrophils (CXCLs1,5,6 & 8) towards all MSCs, whereas migration of T cells and NK cells (CCL20, CXCL9,10 &11) was observed to a lesser extent. Interestingly, the substantial attraction of immune cells by Is MSCs under homeostatic and inflammatory conditions compared to Va, Ad and BM MSCs highlighted them as MSCs with potent and differential chemoattraction ability. This chapter clearly highlighted differential chamoattraction abilities of MSCs depending on their tissue origin and suggests that MSCs isolated from various tissues would act differentially *in vivo*. The chemoattraction of immune cells could be beneficial or detrimental in a clinical setting depending on the phenotype of the migrated immune cells and the interactions that MSCs have with them. Determining if MSCs exhibit the same immune cell attraction *in vivo* would be an important next step in understanding the potential behaviour of MSCs *in vivo*.

Chapter 6

Defining the immune cell attraction profile of islet derived MSCs *in vivo*

Chapter 6 Defining the immune cell attraction profile of islet derived MSCs *in vivo*

6.1 Introduction and aims

Results presented in the previous chapter showed that MSCs isolated from the islet (Is), visceral adipose (Va), adipose (Ad), bone marrow (BM) and umbilical cord (UC) expressed low levels of chemokine receptors on their surface. Additionally, Is, Va, Ad, BM and UC MSCs secreted CCL2, CCL20, CXCL1, CXCL5, CXCL6 and CXCL8 during homeostasis which induced chemotaxis of target cells (monocytes and neutrophils) in an *in vitro* transwell system (with the exception of UC MSCs which were not assessed in the transwell system). Inflammatory stimulation resulted in a substantial upregulation in the secretion of the aforementioned chemokines with additional secretion of CCL11, CXCL9, CXCL10 and CXCL11 by Is, Va, Ad, BM and UC MSCs. This upregulation of chemokine secretion under inflammatory conditions was mirrored by an increase in monocyte (classical) and neutrophil migration towards all MSCs. Migration of NK cells, DCs, eosinophils, T cells and B cells towards MSCs was barely above background controls, despite MSC secretion of CCL11 (eosinophils), CXCL9, 10 and 11 (NK cells, T cells). As inflammatory stimulation is considered a mechanism of MSC licensing whereby MSCs have to be stimulated with IFN-ϒ, TNF-α and IL-ß to exert their anti-inflammatory effects (133, 303-305), the current study's findings of mass immune cell migration towards MSCs post-inflammatory stimulation, seem contradictory to the belief that MSCs are anti-inflammatory after licensing. Thus, this has wider implications when one considers the infusion of MSCs into a patient with an existing inflammatory condition, where immune cells, recruited by MSC derived products could ultimately exacerbate and prolong inflammation. Conversely, the relatively new body of literature surrounding the concept of pro-inflammatory and anti-inflammatory/pro-angiogenic neutrophils in mice and humans could suggest that MSCs might attract a particular type of neutrophil, or immunomodulatory neutrophils (306). Moreover, the attraction of specific subsets of monocytes has also been reported to have inflammatory (307, 308) or pro-regenerative and anti-viral effects in mice and humans (309, 310). Thus, as Is and Va MSCs attracted immune cells without inflammatory stimulation, they have the potential to exaggerate inflammation or, promote an anti-inflammatory/pro-

regenerative outcome without being pre-licensed. Therefore, it is possible that these MSCs could be beneficial or detrimental to tissue regeneration in a clinical setting, depending on the phenotype of the immune cells they attract.

The overall aim of this thesis was to gain insight into the *in vivo* behaviour of MSCs by assessing which immune cells MSCs attract and therefore potentially interact with. Having observed the immune cell attraction profile of MSCs *in vitro*, understanding if MSC chemokine secretion resulted in a similar immune cell attraction profile *in vivo* was the next logical step in this study. To assess this, a murine air pouch model was used. The air pouch model is well-established to study immune cell infiltration - often promoted by the injection of an inflammatory agent (LPS/Carrageenan) - into an artificially created air pouch on the back of mice/rats (model discussed further in Section 6.2.1) (311, 312). Thus, the air pouch served as an ideal, relevant *in vivo* environment to assess immune cell infiltration in response to pre-inflamed Is MSC introduction into the air pouch of mice. Pre-inflamed Is MSCs were used in the *in vivo* system as they attracted the largest number of immune cells *in vitro*. Similar to the *in vitro* system, MSCs were stimulated with 10ng/mL of IFN-ϒ, IL-1ß and TNF-α for 24 hours and thoroughly washed prior to infusion into the air pouch to ensure immune cell attraction was MSC specific and not a result of the inflammatory cytokines used.

Therefore, this chapter aimed to assess; i) the *in vivo* immune cell attraction profile of pre-inflamed Is MSCs, ii) the phenotype of infiltrated immune cells and iii) the potential anti-inflammatory and immunomodulatory mechanisms of MSCs on chemoattracted immune cells by measuring the transcription of several genes including Tumour necrosis factor inducible gene 6 (TSG6), indoleamine 2,3-dioxygenase (IDO), complement factor H (CFH), CD 274, hepatocyte growth factor (HGF), TGF-beta (TGF-ß) and Granulocyte macrophage colony stimulating factor (GMCSF) under homeostatic and inflammatory conditions.

Results

6.2 Assessing the *in vivo* immune cell attraction profile of stimulated Is MSCs by using a murine air pouch model

6.2.1 Overview of air pouch model, timeline and set up

To prevent human MSC rejection, the air pouch model studies were initially performed in immunocompromised NOD/SCID-GAMMA (NSG) mice (Details of genotype in Section 2.6.2.1). NSGs are considered immunocompromised due to multiple defects in the innate and adaptive immune systems including lack of; complement, T cells, B cells and NK cells coupled with defective macrophages and DCs (313, 314). To assess the immune cell attraction profile of stimulated Is MSCs *in vivo*, an air pouch was created under the dorsal skin of NSG mice.

After concluding that NSG mice could tolerate the air pouch procedure and injection of human MSCs, OT-1 mice were used to assess the effect of human MSCs in mice with a fully-functional immune system. OT-1 mice became available to use throughout the experimental timeline and therefore served as a comparison to the NSG mouse strain. OT-1 are transgenic mice, where the CD8+ T cells express a TCR specific for the SIINFEKEL peptide of ovalbumin, however all other immune cells are present and fully functional (315).

The comparison of results between NSG and OT-1 mice assessed the validity of the NSG results. In other words, using OT-1 mice made it possible to determine if the results from NSG mice may relate to their incomplete immune system, or not. Additionally, OT-1 mice allowed an assessment of the infiltration of adaptive immune cells into the air pouch. Therefore, the following sections include air pouch data from both NSG and OT-1 mice.

The timeline of how the air pouch was created and maintained is shown in **Figure 6-1 A.** Due to the availability of mice, 10 female NSG mice between 14 and 16 weeks and 5, 14 week old OT-1 males were used. On Day 7 post pouch creation, mice were injected with 1 mL of sterile PBS (control mice) or $5.5x10^5$ pre-stimulated Is MSCs (10ng/mL of TNF-α, IL-1β and IFN-Ɣ for 24 hours) in 1mL of PBS (treated mice) and left for 24 hours. Mice were then sacrificed and the air

pouch was dissected and assessed (For full detail, see Section 2.6). The immune cell infiltration into the air pouch fluid, upper and lower membranes of each mouse **(Figure 6-1 B)** was assessed using flow cytometry and histology.

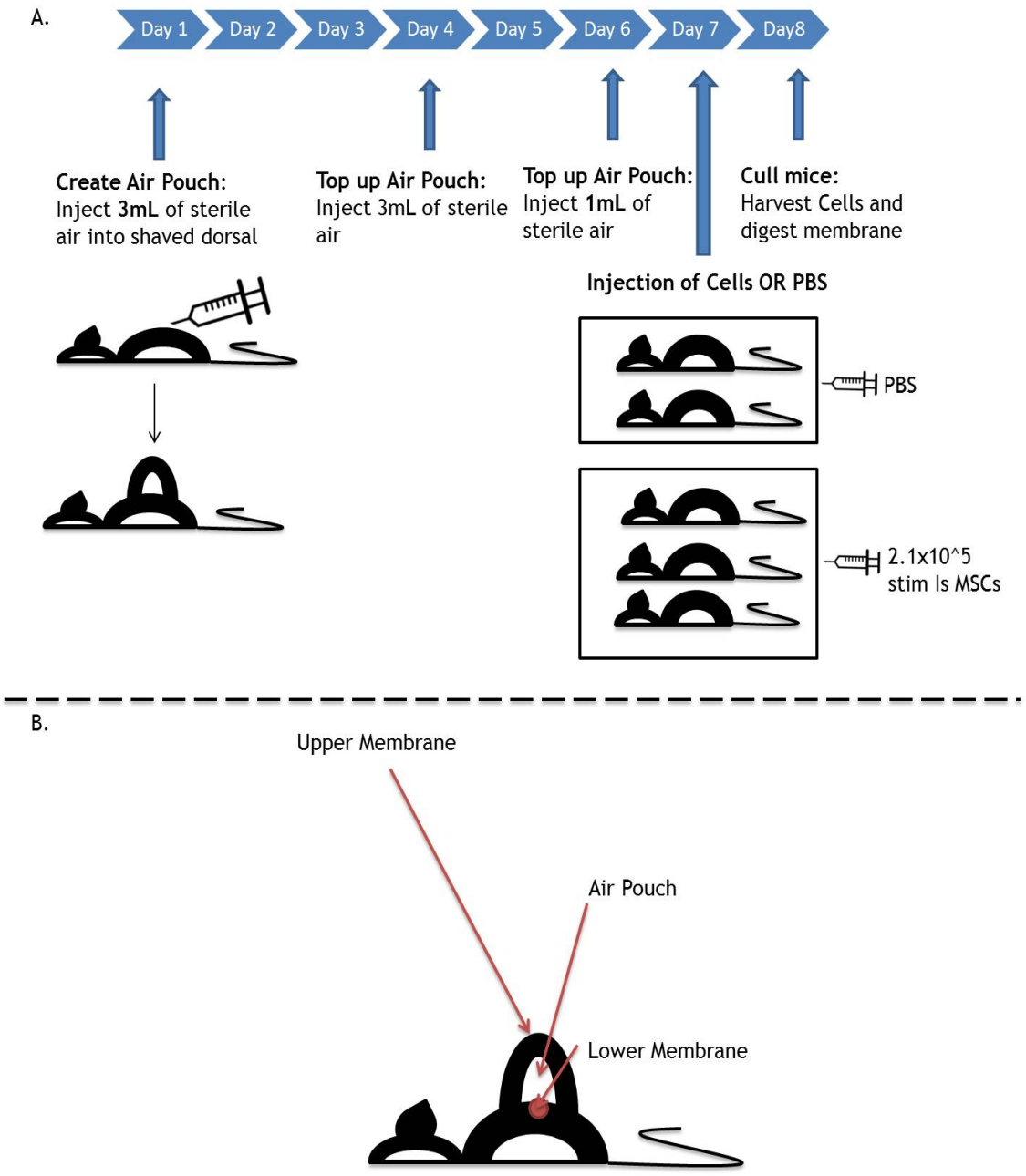


Figure 6-1 Diagrammatic illustration of the time course and anatomy of the air pouch model on NSG and OT-1 mice.
The timeline of the air pouch model is illustrated in **A**. On day 0, the dorsal skins of mice were shaved. Day1, 3 mL of sterile air was injected subcutaneously into the dorsal of each mouse. Mice were left for two days prior to a 3mL sterile air top up into the air pouch (Day 4). Mice were left for one day before topping up the air pouch again with 1mL of sterile air (Day 6). 24 hours post air injection, mice were injected with 1mL of sterile PBS (control mice) or $5.5x10^5$ pre-inflamed Is

MSCs in 1mL of sterile PBS (Day7) and left for 24 hours before mice were sacrificed (Day 8). The air pouches of all mice were dissected by injecting 1mL of PBS into the air pouch and carefully removing all liquid from the air pouch – all cells within this sample were termed “air pouch” samples (**B**). The upper membrane of the pouch was dissected and digested – all cells within this sample were termed “upper membrane”. The lower membrane was dissected and digested in a similar manner to the upper membrane – all cells isolated from the lower membrane were termed “lower membrane”.

6.2.2 **Flow cytometry gating strategies for the NSG and OT-1 air pouch models**

Flow cytometry was used to assess the immune cell infiltration into the air pouch fluid, upper and lower membranes of NSG and OT-1 mice after 24 hours. An array of mouse cell markers including; CD11c, F480, CD11b, Ly6c, Ly6g, CD45, Siglec F and human CD105, coupled with a specific gating strategy **(**details in **Figure 6-2)** was used to identify neutrophils (**Figure 6-2 B**), macrophages **(Figure 6-2 C)**, eosinophils **(Figure 6-2 D)** monocytes **(Figure 6-2 E)**, DCs **(Figure 6-2 F)**, and human Is MSCs**.**

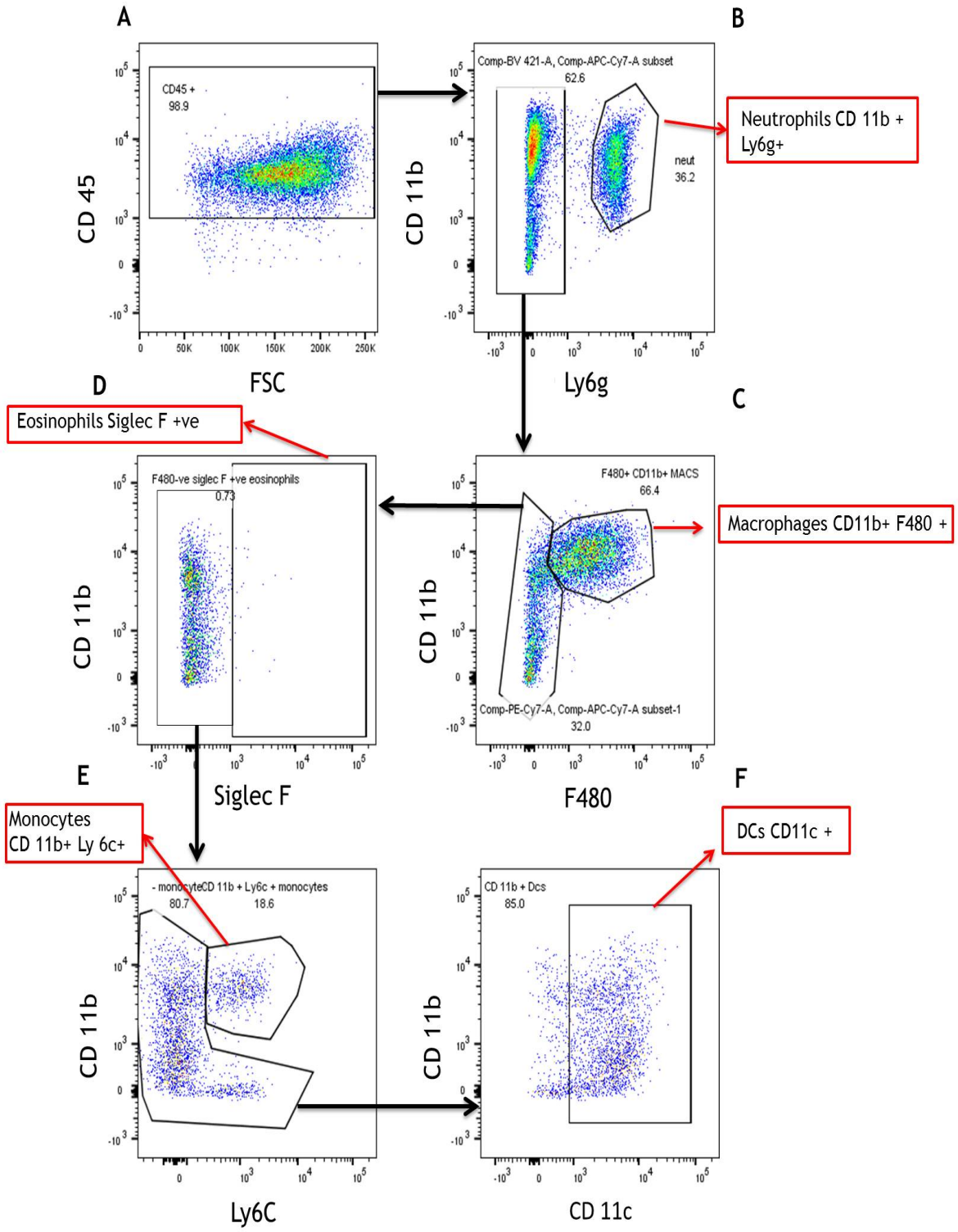


Figure 6-2 Flow cytometry gating strategy to identify various mouse innate immune cells in the NSG and OT-1 air pouch Model

Flow cytometry was used to assess and identify the immune cell infiltration into the air pouches of NSG and OT-1 mice. Doublets were excluded and live cells were selected as described in previous chapters (plots not shown). Black arrows highlight the gating pathway and red arrows highlight gated cell populations. CD45 +ve cells were selected **(A)** and CD11b +ve, Ly6g +ve cells were considered neutrophils **(B).** The remaining population were assessed for their expression of F480. F480 +ve, CD11b +ve cells were considered macrophages **(C).** The remaining population of cells were assessed for expression of Siglec F. Siglec F +ve cells were considered eosinophils **(D).** CD11b +ve, Ly6c +ve cells were considered monocytes **(E).** The remaining cells were assessed for the expression of CD11c. Any cells expressing CD11c were classified as dendritic cells **(F).** Positive staining was measured through the use of 'Fluorescence minus one' controls.

As OT-1 mice have an adaptive immune system, an additional flow cytometry panel (details in **Figure 6-3**) was used in conjunction with the aforementioned panel (Figure 6-2), to assess the infiltration of adaptive immune cells including NK cells (**Figure 6-3 B**), CD4 and CD 8 T cells **(Figure 6-3 C)** and B cells **(Figure 6-3 D).**

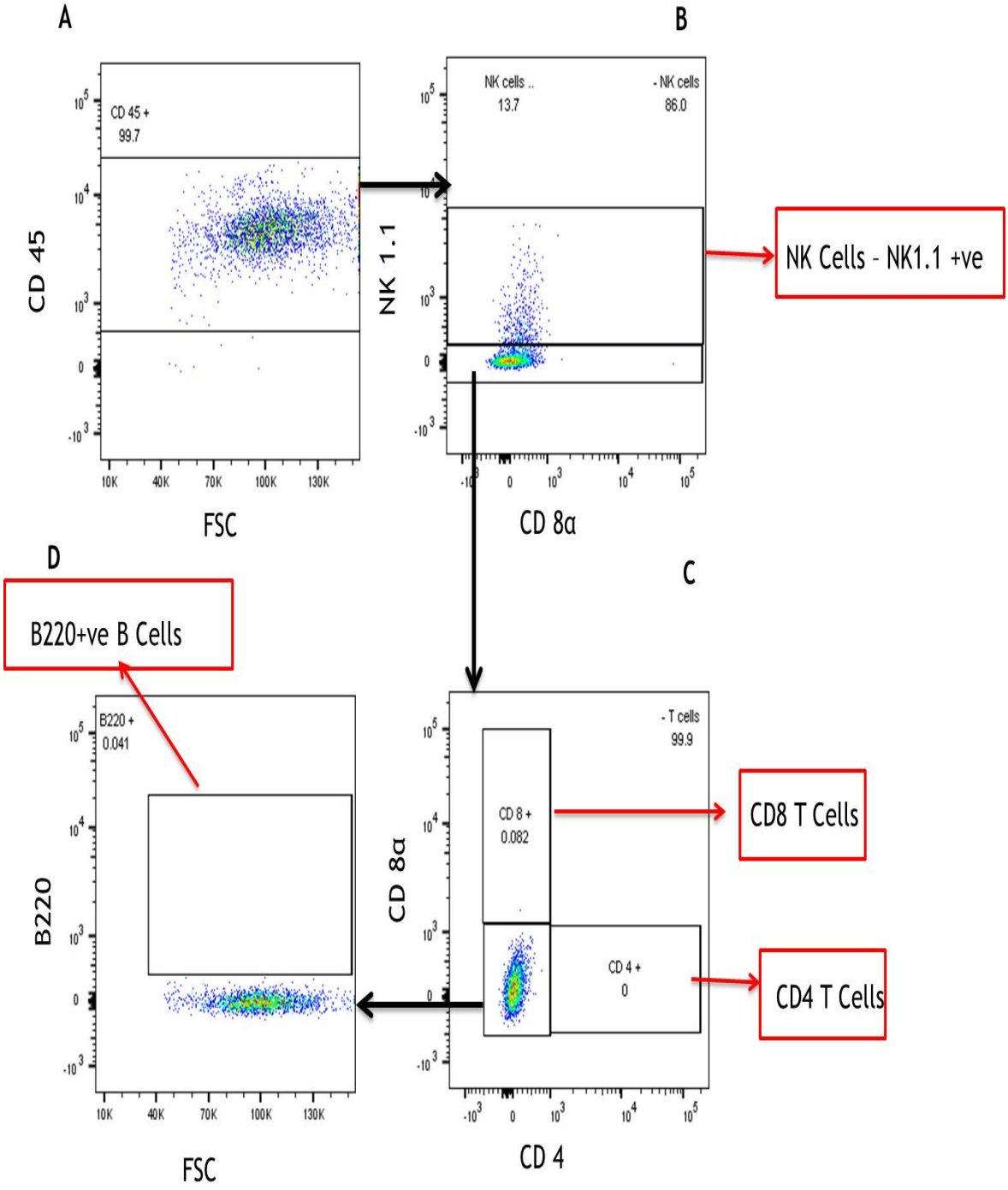


Figure 6-3 Flow cytometry gating strategy to identify cells of the adaptive immune system in the OT-1 air pouch model.
Flow cytometry was used to assess the presence of adaptive immune cells in the OT-1 air pouch model. Doublets were excluded and live cells were selected as described in previous chapters

(plots not shown). Black arrows highlight the gating pathway. Red arrows highlight the gated cell population. CD45 +ve cells were selected **(A)** and assessed for their expression of NK1.1, CD8alpha, CD4 and B220. NK1.1 positive cells were considered NK cells **(B)** and NK1.1 –ve cells were assessed for the expression of CD4 and CD8 **(C)** and considered CD4 T cells or CD 8 T cells, respectively. Cells negative for CD4 and CD8 were assessed for their expression of B220 and positive cells were considered B cells **(D).** Positive staining was measured through the use of fluorescence minus one controls.

Although the fine details of specific marker expression by immune cell subsets is beyond the scope of this chapter, immune cells broadly characterized using the gating strategies mentioned above will be referred to as neutrophils, macrophages, monocytes, eosinophils, DCs, NK cells, T cells and B cells.

6.2.3 Analysis of the cellular infiltration into the air pouches of NSG and OT-1 Mice.

6.2.3.1 Immune cell infiltration into the air pouches of NSG mice

Using flow cytometry, the total numbers of CD45+ cells that had infiltrated into the air pouches of NSG and OT-1 mice were analysed and are graphed in **Figure 6-4** (details on how the total numbers were calculated are described in Section 2.4.3.5). Minimal migration of CD45 +ve cells into control NSG air pouches was observed. This demonstrated that the creation of the air pouch or PBS injection itself, does not result in substantial immune cell infiltration/inflammation at the 24 hour time point. Macrophages were the predominant immune cell present in the air pouches of control mice, followed by smaller numbers of neutrophils, DCs, monocytes and eosinophils **(Figure 6-4 A)**. Pre-stimulated Is MSCs in the NSG mice air pouches (treated) resulted in a 30 fold increase in CD45 +ve cells compared to control mice. In contrast to control animals which predominantly attracted macrophages into the air pouch, neutrophils were the predominant infiltrating cell type in treated animals, attracting of 91 fold more neutrophils compared to control mice. This highlights that Is MSCs preferentially attracted neutrophils in NSG mice and that the immune cell attraction was not just an amplification of the inflammatory reaction/immune cell infiltrate, observed in control mice. Macrophage infiltration increased by 14 fold compared to control mice and made up the second largest population of cells within the air pouch. DCs, monocytes and eosinophils made up smaller proportions of the infiltrating

CD45 +ve immune cells in treated NSG air pouches and their infiltration increased 5, 18 and 8 fold, respectively, when compared to control mice.

6.2.3.2 Immune Cell Infiltration into the Air Pouches of OT-1 mice

Similar to NSG control mice, OT-1 control mice did not recruit large numbers of CD45 +ve cells into the air pouch and macrophages were the predominant CD45 +ve cell (**Figure 6-4 B**). Pre-stimulated Is MSCs in OT-1 air pouches upregulated CD45 +ve cell infiltration 153 fold compared to control mice. Again, the predominant infiltrating cell type was neutrophils in treated OT-1 mice, which infiltrated 1610 fold higher than in control OT-1 mice. The switch in the predominant cell type in the air pouches of control vs treated mice, again, suggested a selective attraction of neutrophils towards Is MSCs in OT-1 mice. An increase in macrophage infiltration by 53 fold was observed when comparing control to treated mice and a significant upregulation of DC infiltration by 30 fold (P=0.0090), monocyte infiltration by 71 fold and eosinophil infiltration by 46 fold was also seen.

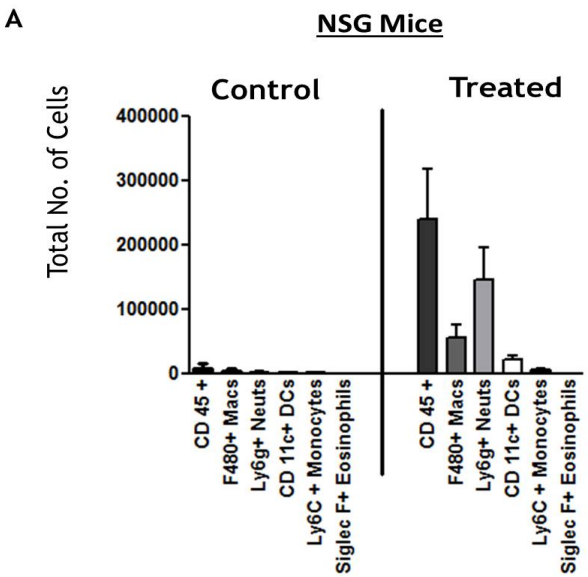
The presence of adaptive immune cells in the air pouch of OT-1 mice was low in control mice **(Figure 6-4 C).** The presence of pre-stimulated MSCs resulted in a substantial increase in NK cells and a small increase in CD8 and CD 4 T cells and B cells. However, adaptive immune cell infiltration into MSC containing pouches remained relatively low. This complements the *in vitro* data observed in the previous chapter.

6.2.3.3 Comparing the immune cell Infiltration into NSG and OT-1 air pouches.

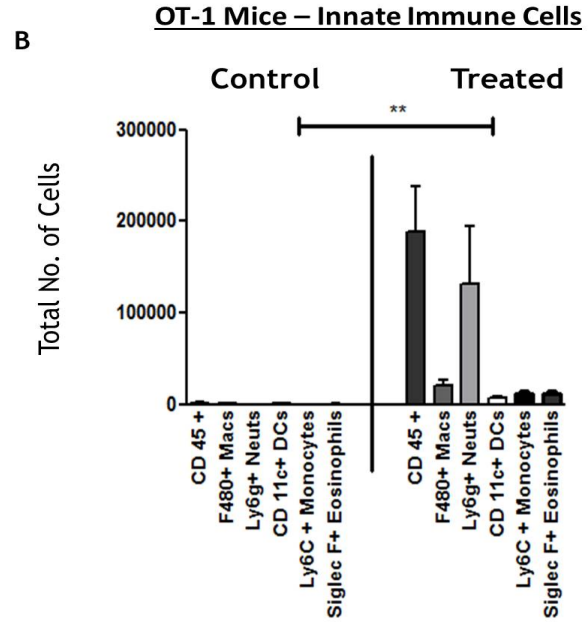
To assess if the immune cell infiltration in treated NSG and OT-1 mice was specific to the presence of Is MSCs and not a result of the mouse genotype used, a direct comparison between control NSG vs. OT 1 mice and treated NSG vs. OT-1 mice was made. No significant differences were observed in the presence of immune cells in the air pouches of control NSG and OT-1 mice, suggesting that similar numbers of immune cells infiltrating into the air pouches of control mice are equally capable of migrating in both mouse strains (**Figure 6-4 D**).

Eosinophil migration into the air pouches of treated mice was significantly reduced in NSG mice, compared to OT-1 mice (P=0.0053). This could be due to a defect in NSG eosinophils which is not widely reported within the literature, and/or, a result of defects in NSG cytokine secretion capabilities. All other immune cells were fully capable of migrating in similar numbers towards Is MSCs. This suggests that the majority of immune cells observed infiltrating into the air pouches of NSG and OT-1 mice were as a result of Is MSCs and not due to the mouse genotype (**Figure 6-4 E**).

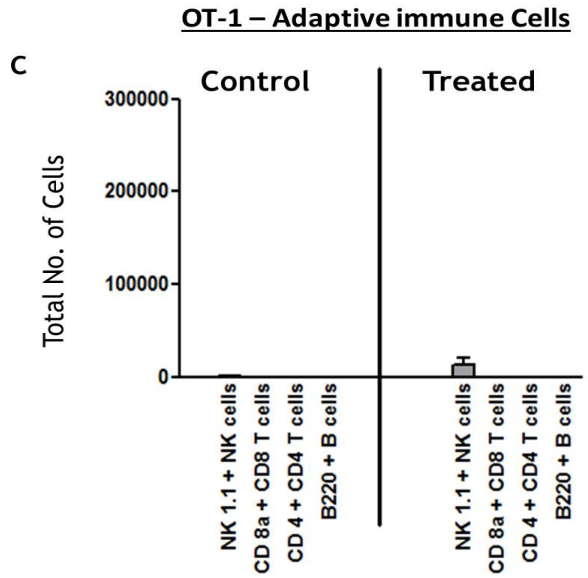
A


NSG Mice
Control
Treated
Total No. of Cells
400000
300000
200000
100000
0
CD 45 +
F480+ Macs
Ly6g+ Neuts
CD 11c+ DCs
Ly6C + Monocytes
Siglec F+ Eosinophils

B


OT-1 Mice – Innate Immune Cells
Control
Treated
**
Total No. of Cells
300000
200000
100000
0
CD 45 +
F480+ Macs
Ly6g+ Neuts
CD 11c+ DCs
Ly6C + Monocytes
Siglec F+ Eosinophils

C


OT-1 – Adaptive immune Cells
Control
Treated
Total No. of Cells
300000
200000
100000
0
NK 1.1 + NK cells
CD 8a + CD8 T cells
CD 4 + CD4 T cells
B220 + B cells

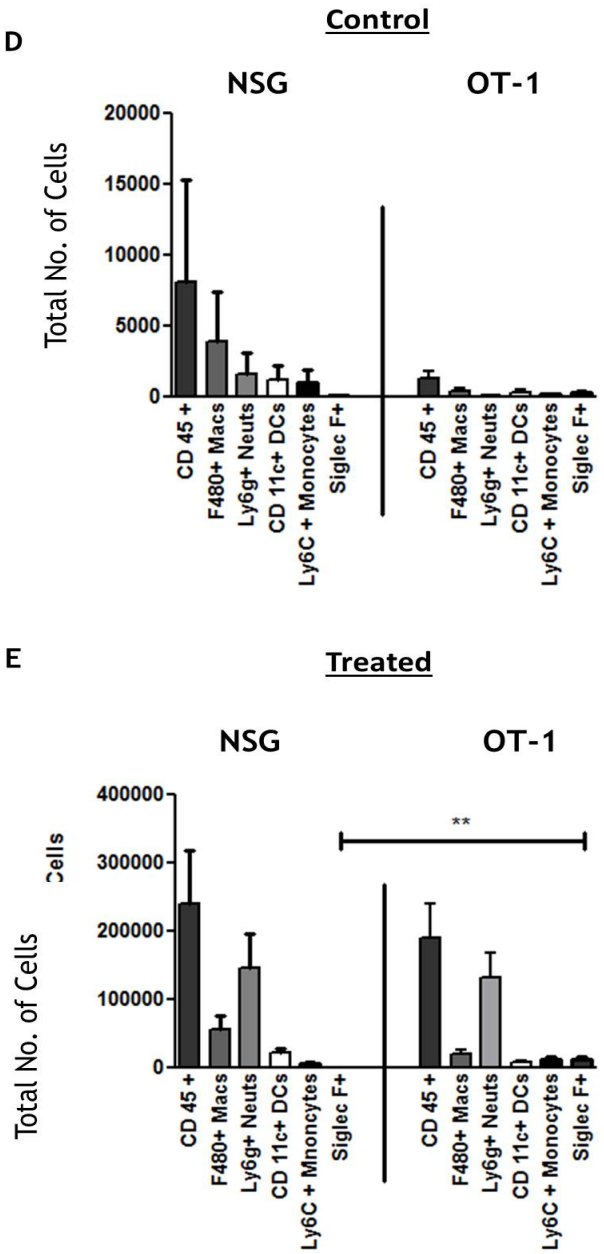


Figure 6-4 Immune cell infiltration into the air pouches of NSG and OT-1 mice.
An air pouch was created on the dorsal of 10 female mice between 14-16 weeks old (NSG) and 5, 14 week old male mice (OT-1). After 6 days, mice received either 1 mL of PBS (control mice n=4 NSG, n=2 OT-1) or 5.5x10^5 pre-stimulated Is MSCs in 1mL of PBS (treated mice n=6 NSG, n=3 OT 1) for 24 hours before they were sacrificed. Flow cytometry was used to assess the immune cell infiltration into the air pouches of NSG and OT-1 mice. The total number of CD45 +ve, F480 +ve macrophages, Ly6g +ve neutrophils, CD11c +ve DCs, Ly6c +ve monocytes and Siglec F+ve eosinophils that had infiltrated into air pouches of control and treated NSG and OT 1 mice are graphed in **A** and **B**, respectively. The total number of NK1.1 +ve (NK cells), CD8α +ve (CD8 T cells), CD4 +ve (CD4 T cells) and B220 +ve (B cells) that had infiltrated into the air pouches of OT-1 mice are graphed in **C**. To assess if mouse strain had any effect on the immune cell infiltrate into the air pouches of NSG and OT-1 mice, a direct comparison between control NSG vs OT-1 (**D**) and treated NSG vs. OT-1 mice was made (**E**). 2 Is MSC donors were used for the NSG air pouch experiment (3 mice per MSC donor) and 1 Is MSC donor was used for the OT-1 air pouch experiment.

Students T test (unpaired) was used to assess control vs. treated immune cell infiltration. Significance is marked where appropriate and P values are stated throughout the text.

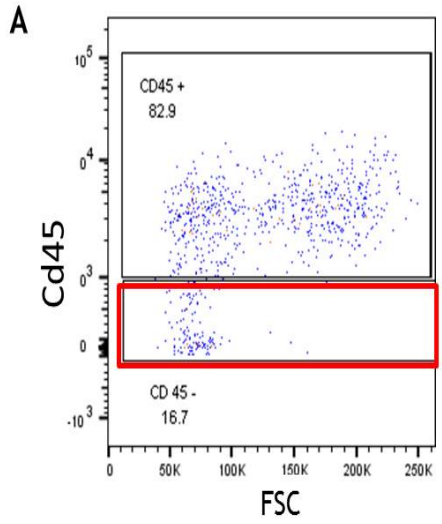
6.2.3.4 **CD45 –ve cell infiltration into the air pouches of NSG and OT-1 mice**

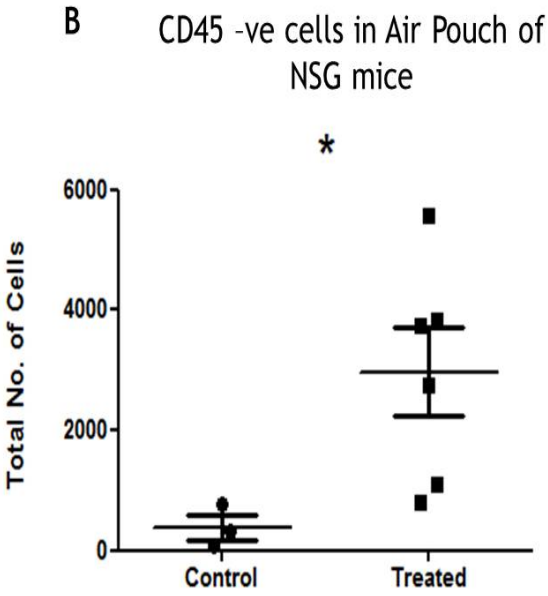
To assess if CD45 –ve cells infiltrated into the air pouches of NSG and OT-1 mice, CD45 –ve cells were gated (**Figure 6-5 A**) and results are graphed in **Figure 6-5 B & C.** In NSG control mice, small numbers of CD45-ve cells were present in the air pouches compared to treated animals which had significantly more (P=0.0491) CD45-ve cells **(Figure 6-5 B).**

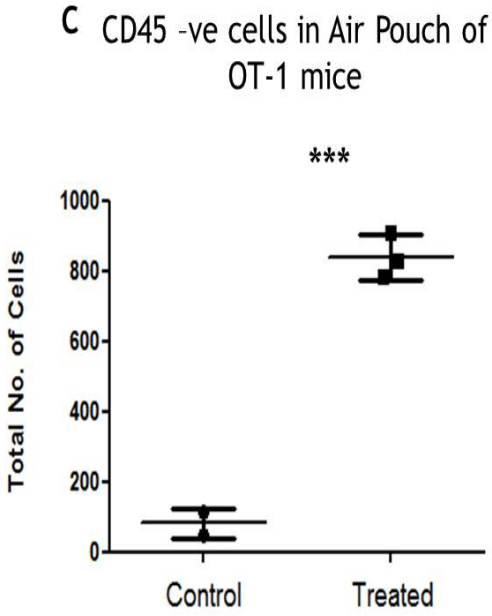
Similarly, CD45-ve cells were present in significantly higher (P=0.0008) numbers in treated OT-1 mice air pouches compared to OT-1 controls **(Figure 6-5 C).**

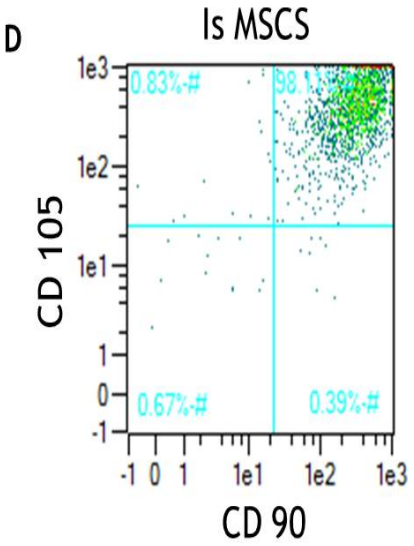
To ensure that the significant increase in CD45-ve cells observed in treated NSG and OT-1 mice was not a result of the presence of CD45-ve human Is MSCs, and to assess if human MSCs resided within the air pouch, NSG and OT-1 air pouches were analysed for the presence of CD105+ve Is MSCs. Previous data (Section 3.3, Figure 3-3) showed that human Is MSCs highly expressed CD105 **(Figure 6-5 D).** Moreover, the human CD105 antibody was not cross reactive with mouse cells **(Figure 6-5 E**), therefore, human CD105 was included in the panel to identify Is MSCs.

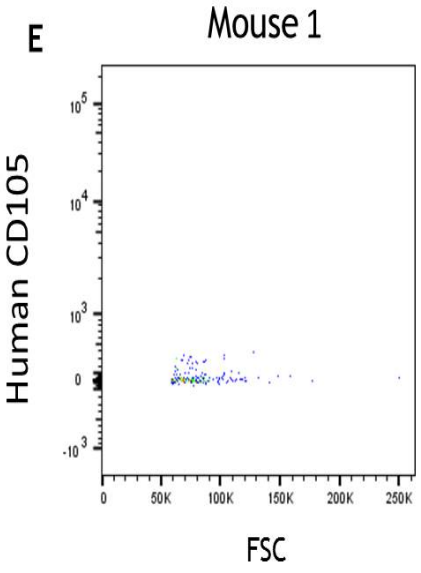
As expected, CD105+ve Is MSCs were not observed in the air pouches of NSG and OT-1 control mice (**Figure 6-5 F.i, F.ii).** Surprisingly, Human CD105+ve Is MSCs were also not observed in the air pouches of NSG and OT-1 treated mice **(Figure 6-5 G.i, G.ii)**. To rule out that human Is MSCs might be dead and residing in the air pouches of NSG and OT-1 mice, the dead cell gate was removed and the air pouches were assessed for the presence of human CD105+ve Is MSCs **(Figure 6-5 H.i, H.ii)**. Live or dead human CD105+ve cells were not detected in the air pouches of NSG or OT-1 mice.


A
CD45 +
82.9
CD 45 -
16.7
Cd45
FSC


B
CD45 -ve cells in Air Pouch of NSG mice
*
Total No. of Cells
Control
Treated


C
CD45 -ve cells in Air Pouch of OT-1 mice
***
Total No. of Cells
Control
Treated


D
Is MSCS
0.83%-#
0.67%-#
0.39%-#
CD 105
CD 90


E
Mouse 1
Human CD105
FSC

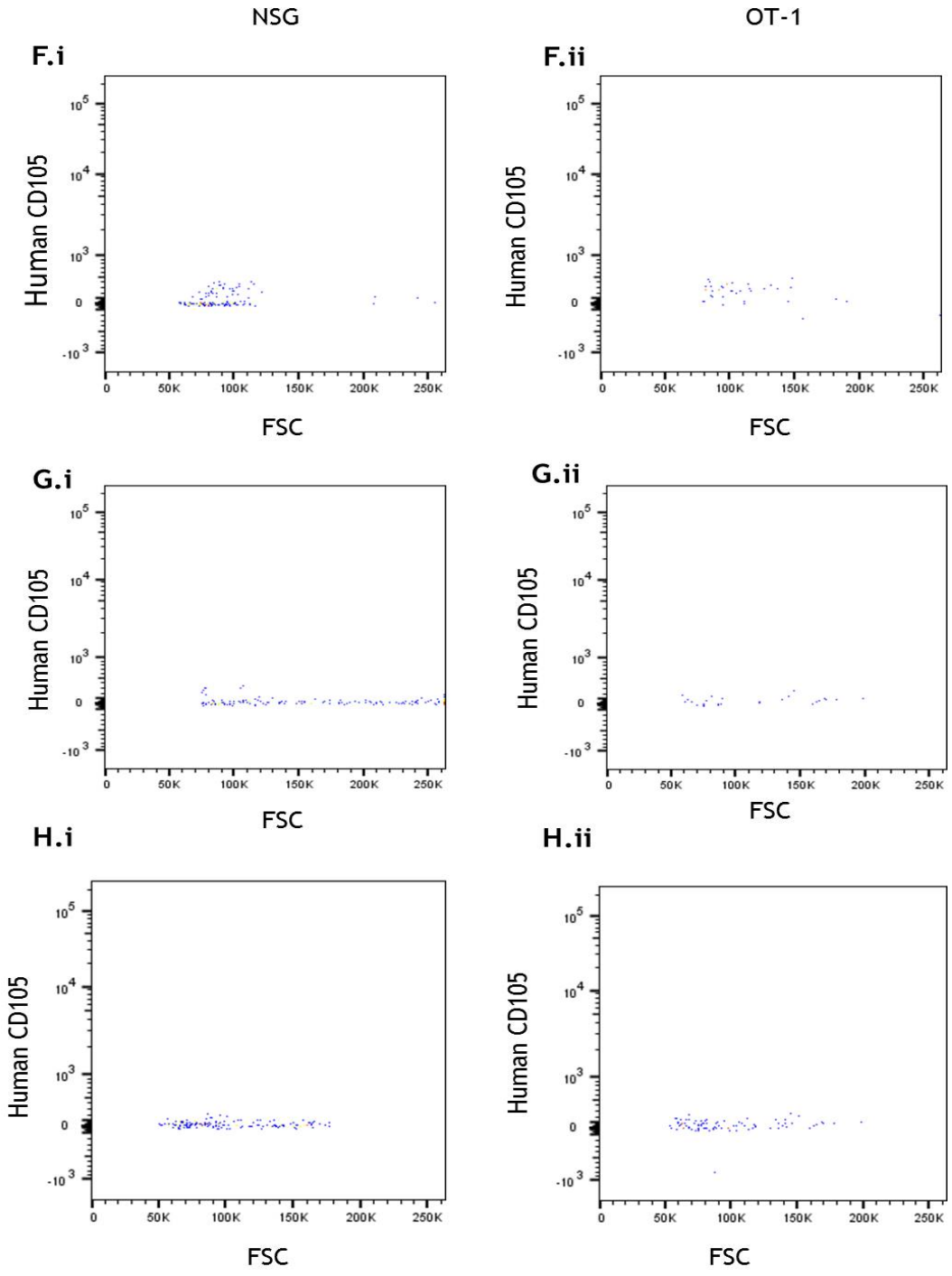


Figure 6-5 Assessment of the CD45-ve cell infiltration into the air pouches of NSG and OT-1 mice

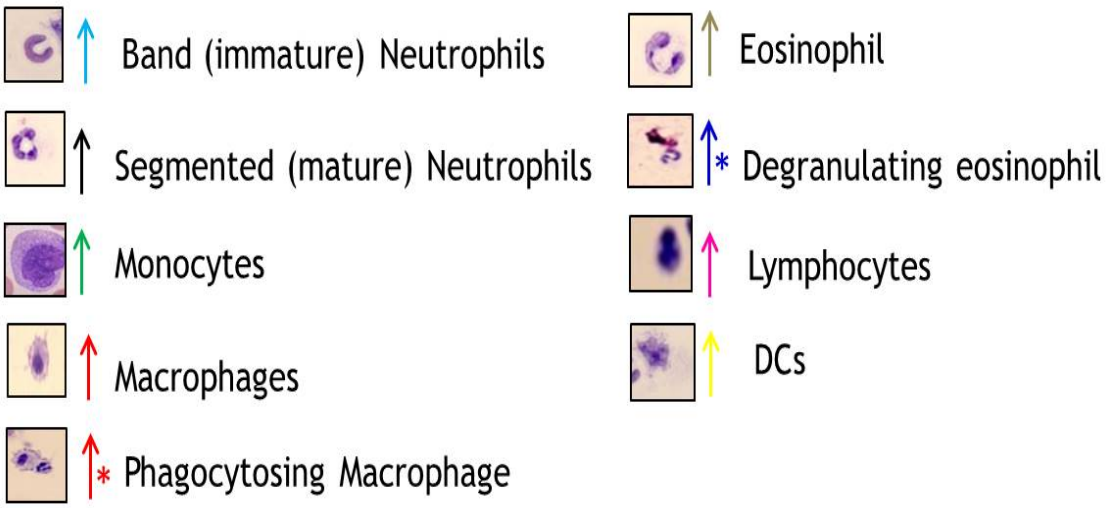
To assess the infiltration of CD45-ve cells into the air pouches of NSG and OT-1 mice, CD45 cells were gated on **(A)** and the total numbers of CD45-ve cells that had infiltrated into the air pouches of NSG and OT-1 mice were counted and graphed in **B** and **C** respectively. To ensure that the increased number of CD45-ve cells observed in the air pouches of treated mice was not a result of the presence of Is MSCs, NSG and OT-1 air pouches were assessed for positive staining of human CD105. Previous data highlighted strong positive staining of human CD105 on Is MSCs **(D)** and this marker did not cross react with mouse cells **(E).** CD105 expression was assessed in control NSG **(F.i)** and OT-1 **(F.ii)** air pouches**.** CD105 expression was also assessed in treated NSG **(G.i)** and OT-1 **(G.ii)** air pouches. To ensure the MSCs were not dead within the air pouches, the dead cell gate was removed and CD45–ve cells were assessed for their expression of CD105 in NSG **(H.i)** and OT-1 **(H.ii)** mice.

To summarise, Is MSCs specifically attracted significantly more CD45-ve cells into the air pouches of NSG and OT-1 mice compared to control mice, which could consist of an array of mouse cells including; fibroblasts, pericytes and MSCs, along with others. Moreover, live or dead CD105+ve Is MSCs were not detected in the air pouches of NSG and OT-1 mice, suggesting that MSCs might have migrated out of the air pouch into the upper and/or lower membranes of the pouch.

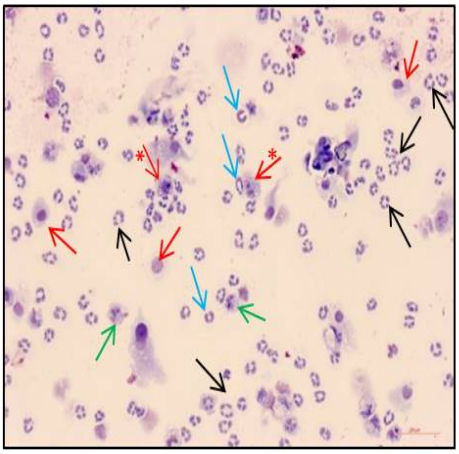
6.2.3.5 **Morphology of immune cells within the air pouch of NSG and OT-1 mice**

To analyse the morphology of immune cells within the air pouch, a cell sample from each mouse was examined using Giemsa stained cytospin **(Figure 6-6).** The immune composition of NSG (**Figure 6-6 A-F**), OT-1 (**Figure 6-6 G-J**) and control (Figure 6-6 **K-N**) mouse cytospins complimented the flow cytometry data, where the predominant cell type in NSG and OT-1 cytospins were mature neutrophils (black arrow). Mature neutrophils were characterised by segmented nuclei, whereas, immature neutrophils were characterised by band shaped nuclei (blue arrow). Macrophages (red arrow) were also present in NSG and OT-1 air pouch cytospins. Macrophages were characterised by their light purple stained cytoplasm and ruffled edges, and can be seen phagocytosing (red arrow with an asterisk) neutrophils and other cellular debris. Fewer monocytes (green arrow) and dendritic cells (yellow arrow) were observed in NSG and OT-1 air pouches. Consistent with the flow cytometry data, small numbers of eosinophils were observed. Eosinophils can be characterised by their 'S' shaped nuclei and, in some cases, appeared to be degranulating – highlighted by dark pink granules emerging from the cell (blue arrow with asterisk **(Figure 6-6 F)).** Lymphocytes (pink arrow) were present in the cytospins of OT-1 mice and are identified by their small round appearance with a large nucleus (**Figure 6-6 G&H**).

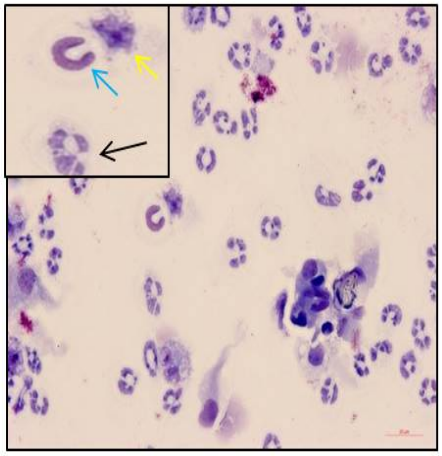
No immune cells were observed on the cytospins from the air pouches of control mice (**Figure 6-6 K-N).**


Band (immature) Neutrophils
Segmented (mature) Neutrophils
Monocytes
Macrophages
* Phagocytosing Macrophage
Eosinophil
* Degranulating eosinophil
Lymphocytes
DCs

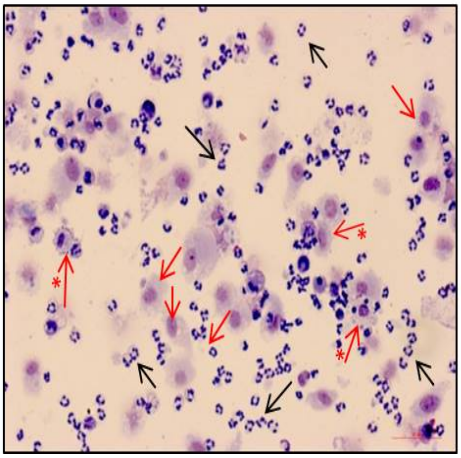
A



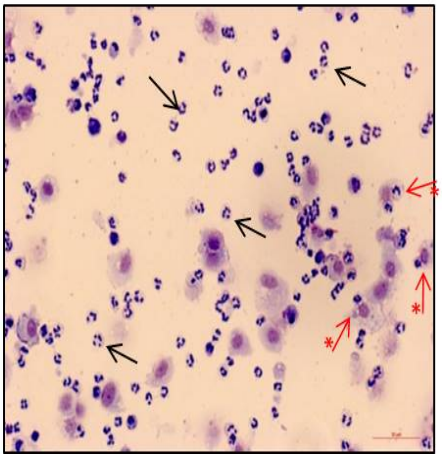
B



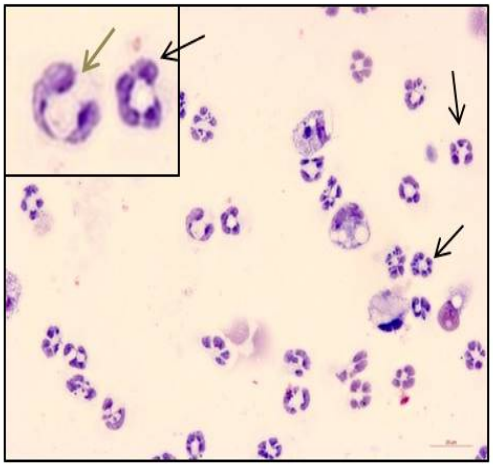
C



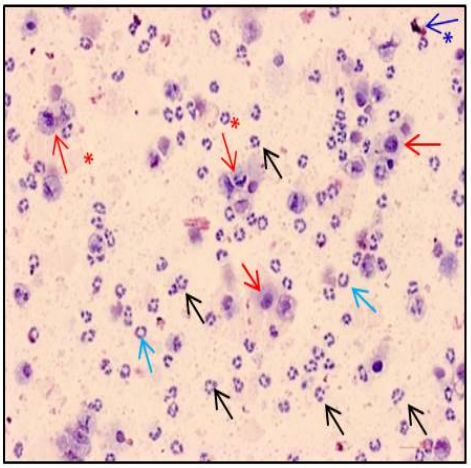
D



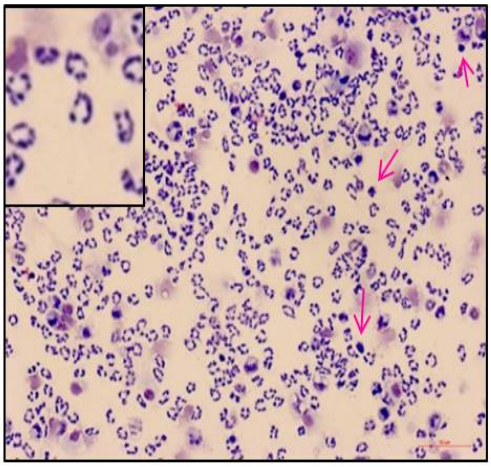
E



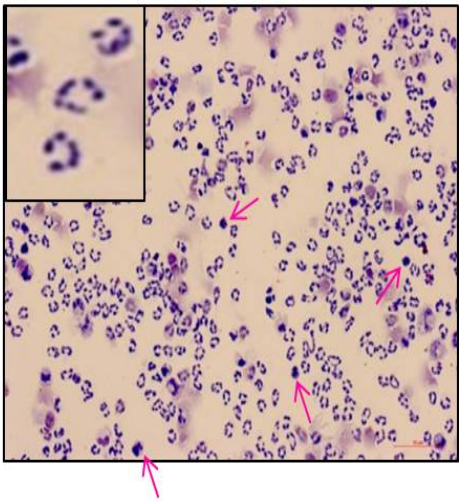
F



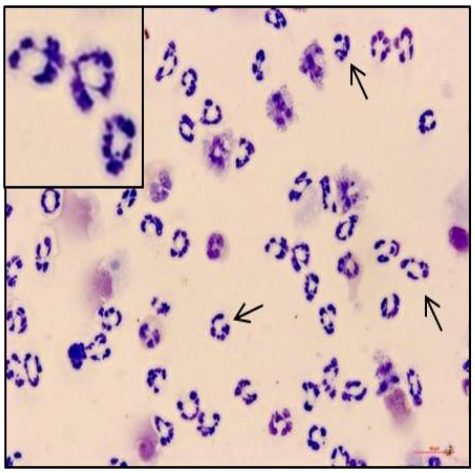
G



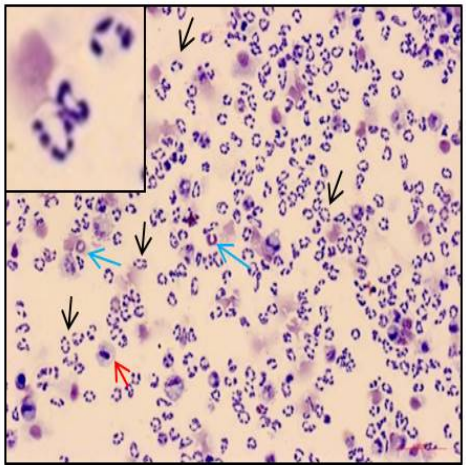
H



I



J



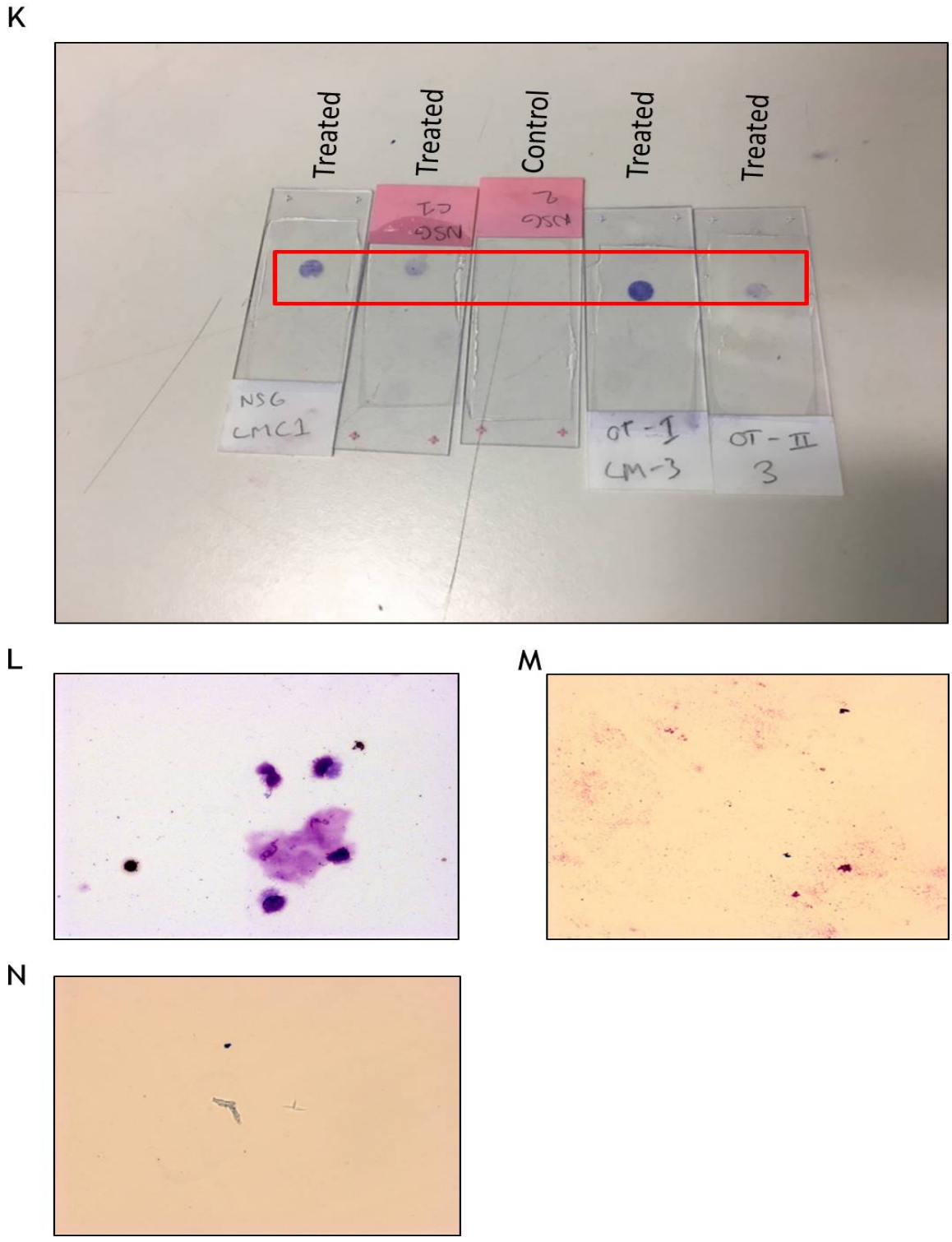


Figure 6-6 Histology of the cellular infiltration into the air pouches of control and treated NSG and OT-1 mice.
$2x10^4$ cells isolated from the air pouches of treated NSG (**A-F**) and OT-1 (**G-J**) and control (**K-N**) mice were prepared for cytospins. All slides were stained with Giemsa and imaged using an epifluorescent microscope - brightfield channel. Light blue arrows point to immature neutrophils (band/ringed shaped nuclei), black arrows point to mature neutrophils (segmented/hyper-segmented nuclei), red arrows point to macrophages, red arrows with an asterisk (*) point to phagocytosing macrophages, green arrows point to monocytes, dark green arrows point to eosinophils, dark blue arrows with an asterisk (*) point to degranulating eosinophils (**F**), pink arrows point to lymphocytes, yellow arrows point to DCs. Magnified inserts on **B,E,G,H,I and J** allow for clearer visualisation of nuclear morphology. Control slides had a noticeable reduction of cells from cytospin analysis (**K-N**).

6.2.4 Summary of the cells present in the air pouch of control vs treated mice

To summarise, the presence of pre-inflamed Is MSCs in the air pouches of NSG and OT-1 mice substantially increased the number of CD45+ve and CD45 -ve cells recruited into the air pouches compared to control pouches. The composition of immune cells in the air pouch of NSG and OT-1 control mice (predominantly macrophages) differed from treated NSG and OT-1 mice (predominantly neutrophils), suggesting that Is MSCs preferentially attracted neutrophils and that they did not just exaggerate the immune cell infiltration caused by the generation of the air pouch -observed in control animals. Neutrophils in treated NSG and OT-1 air pouches had segmented/hyper-segmented nuclei, indicative of mature neutrophils. Macrophages were also common in the air pouches of NSG and OT-1 mice and were often phagocytosing neutrophils and cellular debris. An increase in DCs, monocytes and eosinophils was observed in the air pouches of treated NSG and OT-1 mice compared to control mice. However they appeared in substantially lower numbers than macrophages and neutrophils. Extremely low numbers of T cells and B cells were observed in the air pouches of control and treated OT-1 mice, this could be due to the 24 hour time point used, which might not be long enough to trigger an adaptive response.

With the exception of eosinophils, no significant differences were observed in immune cell infiltration between mouse strains in control or treated mice. This highlights that the observed results were specific to the presence of Is MSCs and not an artefact of the mouse genotype used.

6.2.5 Analysis of CD45 +ve and CD45 -ve cells present in the upper membrane of the air pouch

6.2.5.1 Assessment of CD 45+ve cells present in the upper membrane of NSG mice

As the results of the air pouch fluid were consistent between the two mouse strains, assessment of the CD45 +ve and CD45 -ve cells present in the upper and lower membranes of the air pouch was only carried out on NSG mice.

The presence and composition of immune cells in the upper membrane of the air pouch were assessed using the same gating strategies as previously performed in

NSG mice. The numbers of macrophages, neutrophils, DCs, monocytes and eosinophils are expressed as a percentage of the total CD45 +ve cells in the upper membrane and are graphed in **Figure 6-7.** Total numbers of immune cells in the membrane were not graphed as the amount of membrane dissected from each mouse varied. Due to the thin membrane, standardising the amount of membrane extracted was extremely difficult and therefore not carried out.

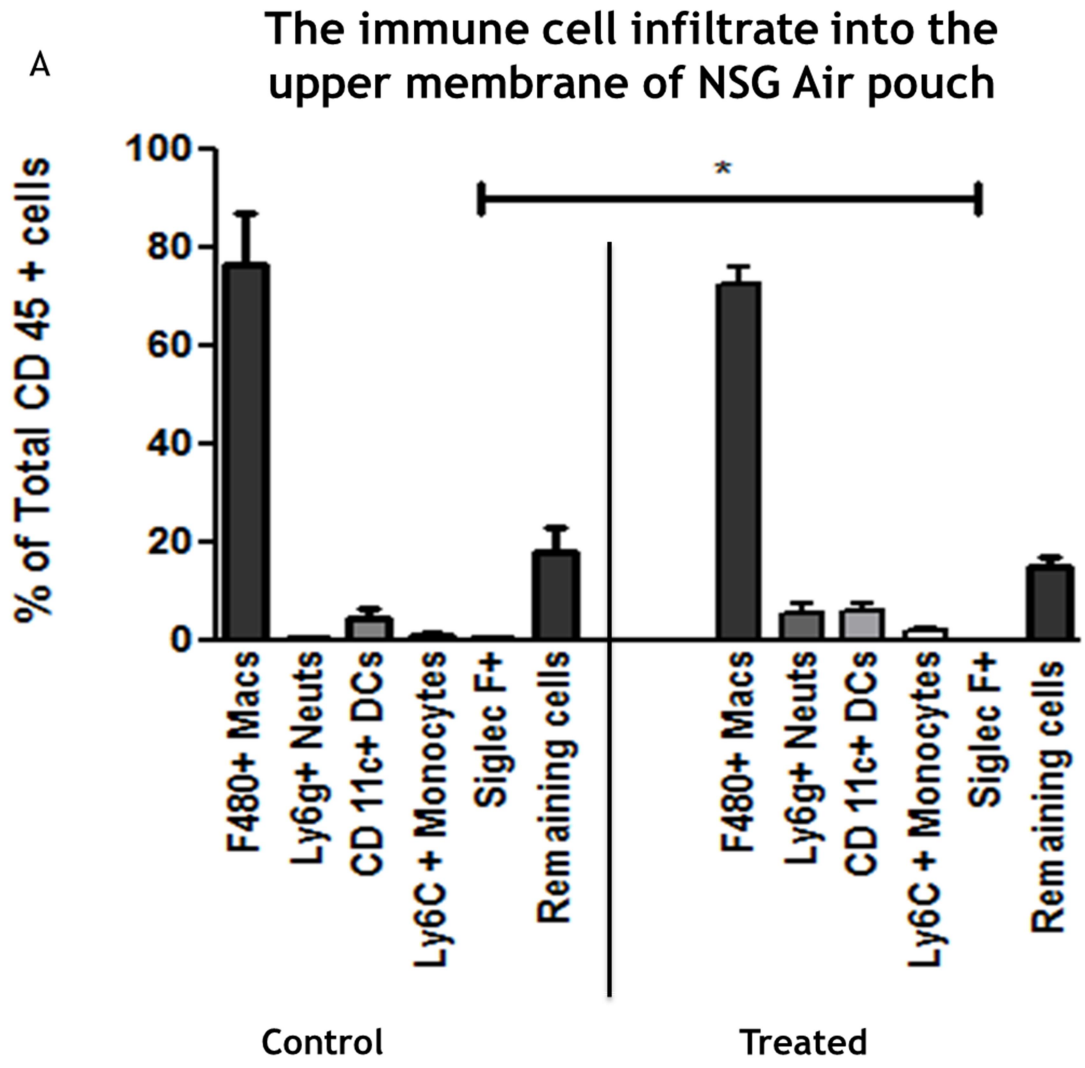
In control NSG mice, macrophages were the predominant CD45 +ve immune cells in the upper membrane. DCs, monocytes and neutrophils made up a small portion of the upper membrane in NSG control mice. The remaining portion of cells which could not be identified with the flow cytometry panel used could consist of basophils and mast cells and are represented as 'remaining cells'.

Macrophages made up the largest proportion of immune cells in the upper membrane of the air pouch in control mice, followed by the undefined proportion of CD45+ve 'remaining cells'. Substantially smaller percentages of DCs, neutrophils, monocytes and eosinophils were present.

The presence of pre-inflamed Is MSCs resulted in a slight decrease in the percentage of macrophages and "remaining cells" present in the upper membrane, whereas the percentage of eosinophils significantly decreased (P=0.0267). The reduction in the percentages of these cells in the upper membrane of treated could be a result of them migrating out of the membrane, into the air pouches in treated mice. A small increase in the percentage of DCs, monocytes and neutrophils in the upper membranes of treated NSG mice compared to control mice was observed.

Cytospins from the upper membrane of NSG mice, further confirmed that the majority of immune cells present were macrophages (red arrow) (**Figure 6-7 B**). Moreover, the immune cell composition of the cytospins from control and treated mice looked very similar, therefore complementing the observations from the flow cytometry data, where no major differences were observed between control and treated mice.

A


The immune cell infiltrate into the upper membrane of NSG Air pouch
% of Total CD 45 + cells
100
80
60
40
20
0
*
F480+ Macs
Ly6g+ Neuts
CD 11c+ DCs
Ly6C + Monocytes
Siglec F+
Remaining cells
F480+ Macs
Ly6g+ Neuts
CD 11c+ DCs
Ly6C + Monocytes
Siglec F+
Remaining cells
Control
Treated

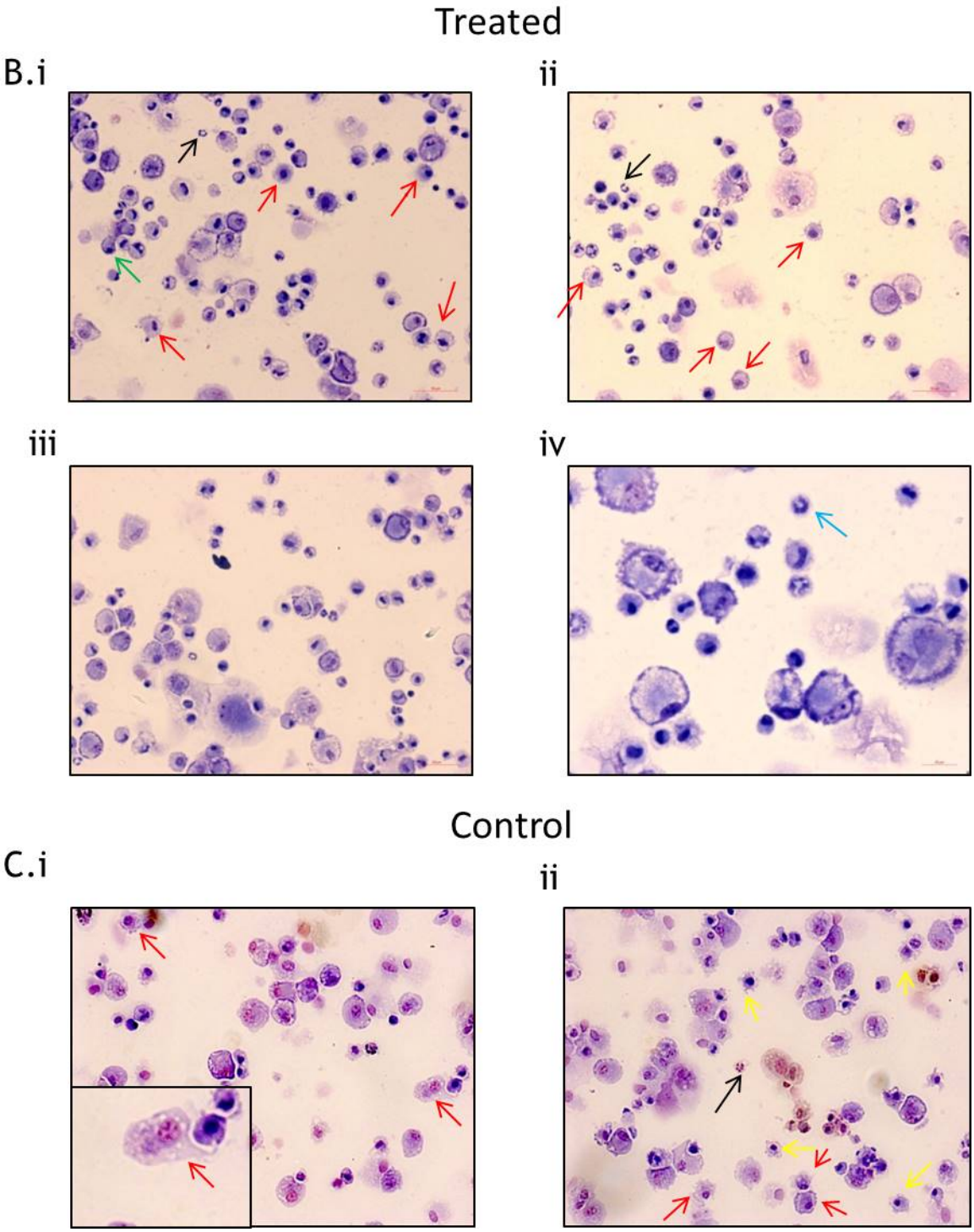


Figure 6-7 Assessing the immune cell composition of the upper membrane in NSG air pouches using flow cytometry and histology
Flow cytometry was used to identify the immune cell composition of the upper membrane of NSG control and treated mice **(A)**. Each bar in the control group represents 4 mice ± SEM. Each bar in the treated group represents 6 mice and 2 Is MSC donors ± SEM. Students (unpaired T test was used to assess statistical differences between control and treated mice. Significance is marked where appropriate and P value(s) are stated throughout the text. Histology (cytospins and Giemsa staining) was used to further assess the phenotype of cells in the upper membrane of treated (**B)** and control (**C**) NSG mice. As before, red arrows point to macrophages, yellow arrows point to DCs, black arrows point to mature neutrophils, blue arrows point to immature neutrophils and green arrows point to monocytes.

6.2.5.2 Assessment of the upper membrane of the air pouch in NSG mice for CD105+ve human Is MSCs

To assess whether human MSCs resided in the upper membrane of the air pouch in NSG mice, the CD45 -ve portion of the upper membrane was analysed for positive expression of human CD105 (**Figure 6-8**). No live or dead human CD105 +ve Is MSCs were observed in the upper membrane of treated NSG air pouches.

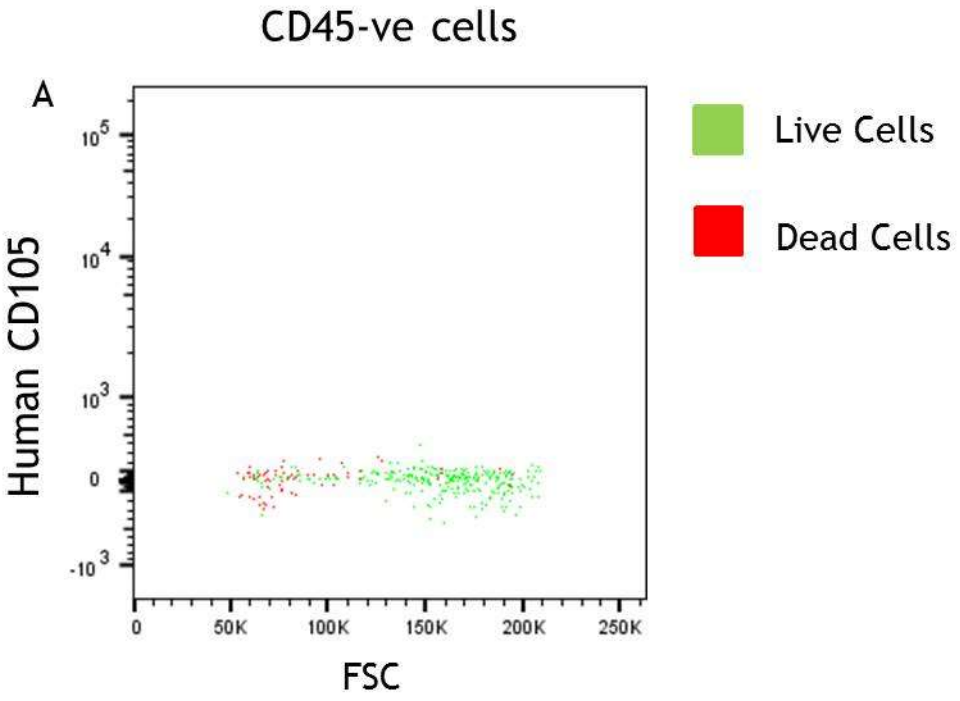


Figure 6-8 Assessment of the upper membrane for live and dead CD105 +ve Is MSCs in NSG mice
Using flow cytometry, the upper membrane of treated NSG air pouches were assessed for CD105+ve Is MSCs. As in Figure 6-5, CD45–ve cells were gated on and assessed for positive CD105 staining. Neither live (green) nor dead (red) CD105+ve Is MSCs were present in the upper membrane of NSG mice air pouches.

To summarise, with the exception of eosinophils, no significant differences were observed in the immune cell composition of the upper membrane between control and treated mice. The significant decrease in eosinophils and slight decrease in macrophages observed in the upper membrane of treated animals might be due to eosinophil and macrophage egress into the air pouch from the upper membrane. Equally, the substantial increase in DC and neutrophil percentages could have altered the immune cell composition of the upper membrane in such a way that it appears as if macrophages and eosinophils have decreased in percentage. Neither live, nor dead, Is MSCs resided in the upper membrane of NSG air pouches.

6.2.6 Analysis of CD45 +ve and CD45 –ve cells present in the lower membrane of the air pouch

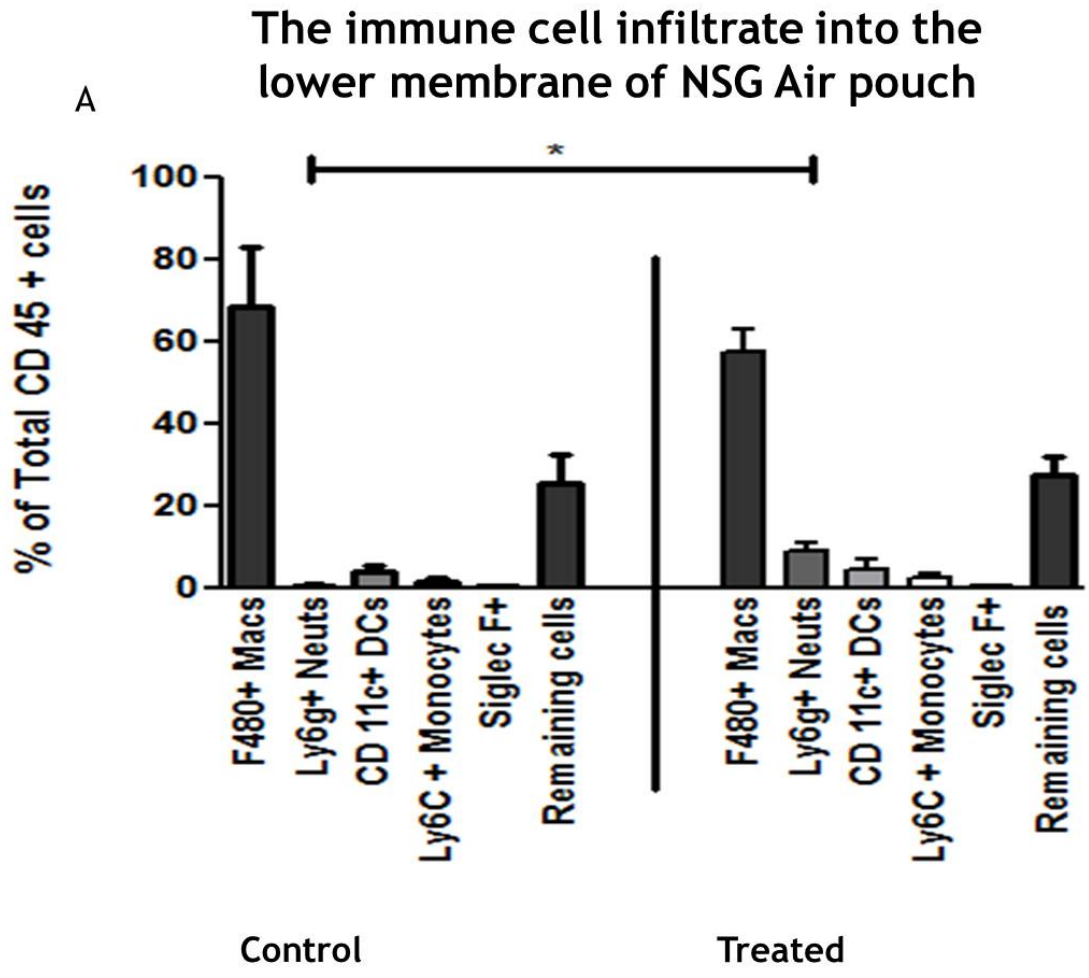
6.2.6.1 Assessment of the CD45 +ve cells present in the lower membrane of NSG mice

The number of macrophages, neutrophils, DCs, monocytes and "remaining cells" as a percentage of the total CD45 +ve cells in the lower membrane of control NSG mice were similar to the percentages observed in the upper membrane of control NSG mice. Macrophages were the predominant immune cell type and the remaining CD45 +ve cells were made up of small fractions of neutrophils, DCs, monocytes and "remaining cells" **(Figure 6-9 A).**

Is MSCs in the NSG air pouch resulted in a change in the lower membrane immune cell composition. Similar to the upper membrane, the percentage of macrophages decreased along with a very marginal degrease in eosinophil percentages when comparing control to treated mice. A slight increase in DCs, monocytes and "remaining cells" was observed, coupled with a significant increase in the percentage of neutrophils (P=0.0314) **(Figure 6-9 A)**.

Cytospins from the lower membrane of treated NSG mice (**Figure 6-9 B**) showed that the majority of immune cells were macrophages and that neutrophils (black arrows = mature, blue arrows = immature) are present throughout the lower membrane of treated mice. Conversely, in control mice (**Figure 6-9 C**), the lower membrane lacks neutrophil infiltration, whilst maintaining high numbers of macrophages.

A


The immune cell infiltrate into the lower membrane of NSG Air pouch
% of Total CD 45 + cells
100
80
60
40
20
0
*
F480+ Macs
Ly6g+ Neuts
CD 11c+ DCs
Ly6C + Monocytes
Siglec F+
Remaining cells
F480+ Macs
Ly6g+ Neuts
CD 11c+ DCs
Ly6C + Monocytes
Siglec F+
Remaining cells
Control
Treated

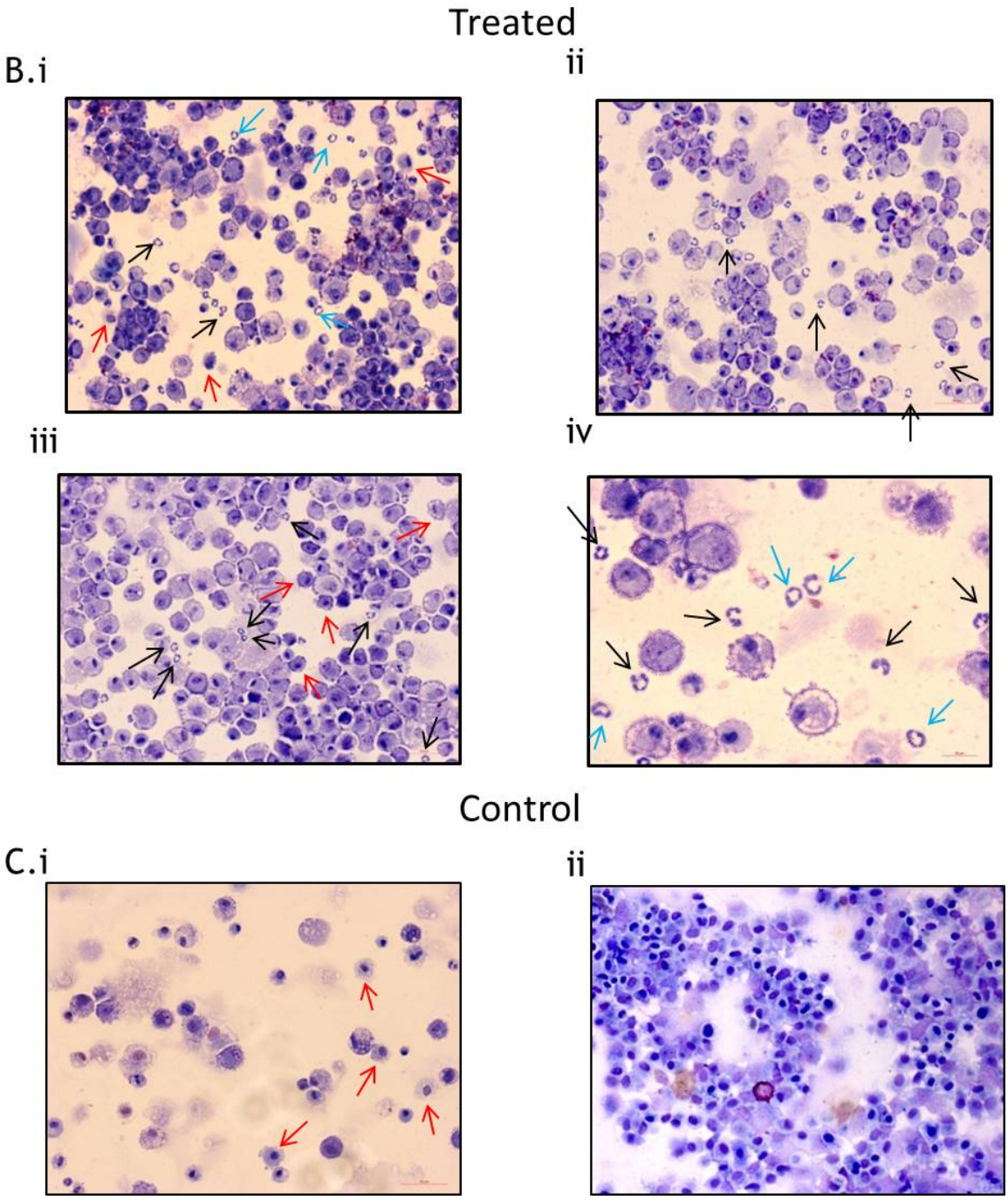


Figure 6-9 Assessing the immune cell composition of the lower membrane in NSG Air pouches using flow cytometry and histology
Flow cytometry was used to identify the immune cell composition of the lower membrane in NSG control and treated mice (**A**). Each bar in the control group represents 4 mice ± SEM. Each bar in the treated group represents 6 mice and 2 Is MSC donors ± SEM. Students unpaired T test was used to assess statistical differences between control and treated mice. Significance is marked where appropriate and P value(s) are stated throughout the text. Histology (cytospins and Giemsa staining) were used to further assess the phenotype of cells in the upper membrane of treated (**B**) and control (**C**) NSG mice. As before, red arrows point to macrophages, yellow arrows point to DCs, black arrows point to mature neutrophils, blue arrows point to immature neutrophils and green arrows point to monocytes.

6.2.6.2 Assessment of the lower membrane of the air pouch in NSG mice for CD105+ve human Is MSCs in

The presence of CD105 +ve Is MSCs in the lower membrane of the air pouch in NSG mice was assessed in the same way as for the upper membrane. Similar to the upper membrane of the air pouch, no live or dead (**Figure 6-10**) human CD105+ve MSCs were detected in the lower membrane of the air pouch in NSG mice.

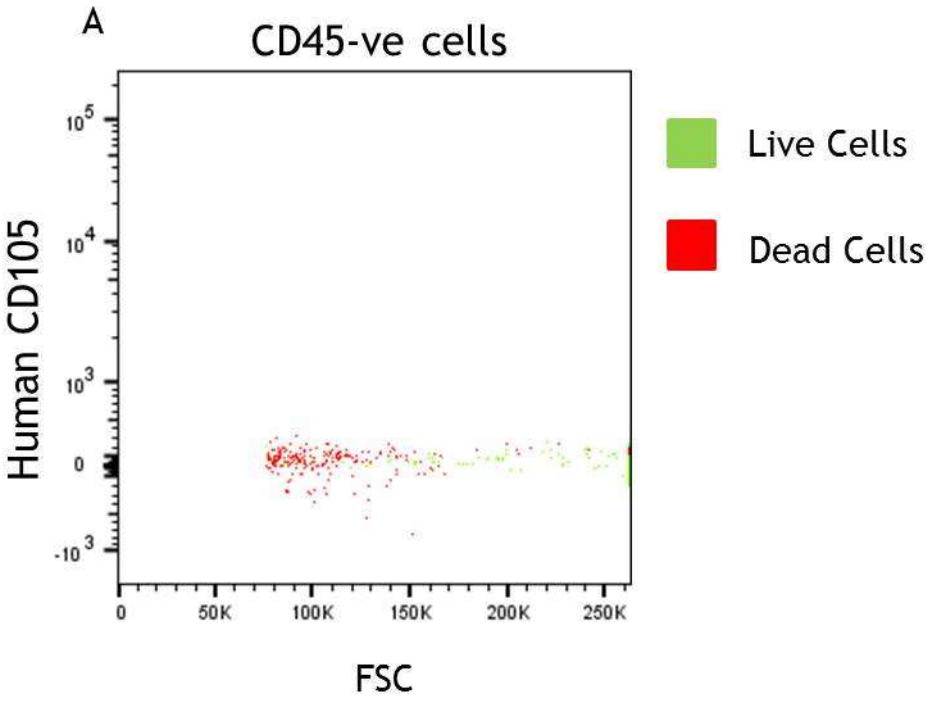


Figure 6-10 Assessment of the lower membrane for the presence of live or dead CD105 +ve Is MSCs in NSG mice.
Using flow cytometry, the lower membrane of treated NSG air pouches were assessed for their presence of CD105 +ve Is MSCs. As in Figure 6-5, CD45 –ve cells were gated on and assessed for positive CD105 staining. Neither live (green) nor dead (**red**) CD105 +ve Is MSCs were present in the lower membrane of NSG mice air pouches.

In summary, the lower membrane of control and treated NSG mice showed similar immune cell composition with the exception of neutrophils which made up a significantly higher percentage of the immune cells in treated animals compared to control animals, likely due to a large influx of neutrophils migrating through the membrane into the air pouch. A mixture of mature (segmented nuclei) and immature neutrophils (band neutrophils) was observed in the lower membrane of treated mice. Live or dead MSCs were not detected in the lower

membrane of the air pouch, suggesting that they might have migrated through the membrane and away from the air pouch.

6.3 Transcriptional analysis of immunomodulatory and anti-inflammatory genes in Is, Va, Ad, BM and UC MSCs.

To assess if MSCs isolated from various sources could have differential immunomodulatory and anti-inflammatory capacity on immune cells in close proximity under homeostatic and inflammatory conditions, Is, Va, Ad, BM and UC MSCs were assessed for their transcriptional expression of several non-chemokine related genes including; Tumour necrosis factor inducible gene 6 (TSG-6), indoleamine 2,3-dioxygenase (IDO), complement factor H (CFH), CD274, hepatocyte growth factor (HGF), TGF-beta (TGF-ß) and Granulocyte macrophage colony stimulating factor (GMCSF).

TSG-6 is known as a multi-functional protein which mediates anti-inflammatory and protective effects in disease models by reducing neutrophil infiltration and inhibiting the presentation of chemokines on the surface of glycosaminoglycans (316). Under homeostatic conditions, Is, Va, Ad, BM and UC MSCs transcriptionally expressed TSG-6 at similar, moderate levels. Inflammatory stimulation resulted in Is, Va, Ad and UC MSCs substantially upregulating transcription of TSG-6 and BM MSCs significantly upregulating TSG-6 transcription ($P=0.0176$). BM MSCs transcribed the highest levels of TSG-6, significantly more than Is ($P=<0.05$), Va ($P=<0.05$) and Ad ($P=<0.05$) MSCs and 2 fold higher than UC MSCs **(Figure 6-11 A**).

As previously discussed in this thesis, MSC secretion of IDO, is a well-documented mechanism of MSC immunomodulation within the literature and causes a downregulation of T cell proliferation (317). Under homeostatic conditions, IDO was not transcribed by Is, Va, Ad, BM and UC MSCs. Inflammatory stimulation resulted in an upregulation of IDO transcription by Is, Va, Ad and UC MSCs and a significant upregulation by BM MSCs ($P=0.0360$). BM MSCs transcribed the most IDO, exhibiting a $2^{(-\Delta CT)}$ 6.3 fold higher than Is MSCs IDO expression (**Figure 6-11 B).**

CFH is an anti-inflammatory factor that inhibits the formation of complement and its secretion by MSCs is considered a mechanism of immunosuppression (109). CFH was transcribed at moderate and similar levels during homeostatic conditions by all MSC tissue sources. Inflammatory stimulation resulted in a moderate upregulation in CFH transcription by Is, Va and Ad MSCs to similar levels. Conversely, BM (P=0.0020) and UC (P=0.0243) MSCs significantly upregulated CFH transcription to similar levels. BM and UC MSCs transcribed significantly more CFH than Is MSCs which transcribed the least CFH under inflammatory conditions (BM: P=<0.01, UC: P=<0.05) **(Figure 6-11 C).**

CD274, otherwise known as programmed death-ligand 1 (PD-L1), is a transmembrane protein which plays an important role in the suppression of the immune system during autoimmune disease and tissue allografts via binding to its receptor PD-1 on the surface of T cells and ultimately inhibiting their proliferation (318). Its expression on MSCs has been documented and is considered another method of immunosuppression by MSCs (319). Under homeostatic conditions, Is, Va, Ad, BM and UC MSCs transcribed CD274 at very low levels. Inflammatory stimulation however, resulted in Is, Va and Ad MSCs substantially upregulating CD274 transcription and BM (P=0.0205) and UC (P=0.0349) MSCs significantly upregulating CD274 transcription. Inflammatory stimulation resulted in BM MSCs transcribing significantly more CD274 than Is (P=<0.001), Va (P=<0.05) and Ad (P=<0.05) MSCs. Similarly, UC MSCs also transcribed significantly more CD274 than Is (P=<0.01), Va (P=<0.05) and Ad MSCs (P=<0.01) (**Figure 6-11 D**).

HGF is a cellular growth and motility factor secreted by MSCs. It has been shown to have potent anti-inflammatory effects in multiple animal models of disease via NF-kappaB inhibition (320). Under homeostatic conditions, Is, Va and Ad MSCs HGF transcript levels were very low and transcribed at similar levels between these MSC tissue sources. Conversely, UC MSCs expressed moderately higher HGF transcripts than Is, Va and Ad MSCs and slightly less than BM MSCs. BM MSCs transcribed significantly higher levels of HGF than Is (P=<0.05), Va (P=<0.05) and Ad MSCs (P=<0.05) under homeostatic conditions. Inflammatory stimulation resulted in a slight upregulation in HGF transcript levels in Is, Va, Ad and UCs, whereas BM MSCs substantially upregulated HGF transcripts. BM MSCs had

significantly higher HGF transcript levels than Is (P=<0.001), Va (P=<0.001), Ad (P=<0.001) and UC MSCs (P=<0.001) (**Figure 6-11 E).**

TGF-β is a secreted protein that performs many functions in regulation of inflammation. The multi-faceted effects of TGF-β are cellular and environmental context dependent (321). MSCs from all tissue sources transcribed TGF-β at similar levels during homeostatic conditions. Inflammatory stimulation resulted in a slight upregulation or downregulation of TGF-β transcript depending on MSC tissue origin where Is MSCs, Ad MSCs and UC MSCs downregulated TGF-beta transcription and Va and BM MSCs upregulated TGF-β transcription **(Figure 6-11 F**).

GMCSF is a secreted protein that promotes neutrophil, monocyte and macrophage proliferation and maturation. Additionally, it can alter neutrophil receptor expression and inhibit their degranulation and migration (322). During homeostatic conditions, Is, Va, Ad, BM and UC MSCs expressed very low levels of GMCSF transcripts. Inflammatory stimulation resulted in a substantial upregulation of GMCSF transcription by Is MSCs only, with a slight upregulation by BM and UC MSCs and minimal upregulation by Ad MSCs (**Figure 6-11 G**).

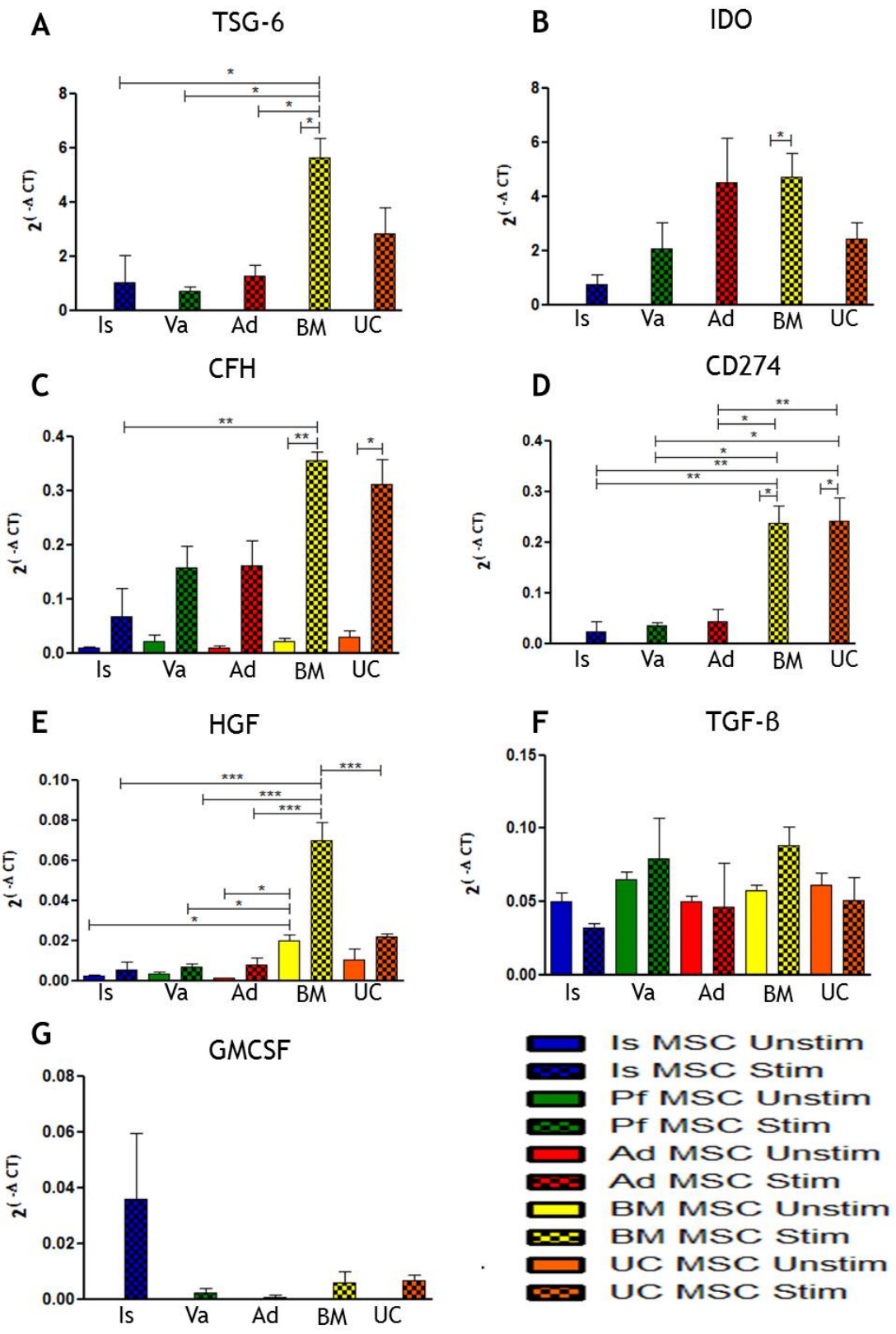


Figure 6-11 Assessing the transcriptional expression of anti-inflammatory and immunomodulatory genes under homeostatic and inflammatory conditions by Is, Va, Ad, BM and UC MSCs.

Is, Va, Ad, BM and UC MSCs were grown until 80% confluent at P3. MSCs were either stimulated or left in homeostatic conditions for 24 hours. After 24 hours, MSCs were detached, RNA was extracted and cDNA was synthesised prior to the assessment of the transcriptional expression of TSG-6 (**A**), IDO (**B**), CFH (**C**), CD274 (**D**), HGF (**E**), TGF-β (**F**) and GMCSF (**G**) using RT PCR. Each bar represents an n of 3 ± SEM. A students (paired) T test was used when measuring

statistical differences within one MSC tissue source (homeostatic vs. Inflammatory). A ONE WAY ANOVA with Tukey's multiple comparisons test was used to assess statistical differences between all MSC tissue sources. Significance is marked where appropriate and P values are stated throughout the text.

To summarise, the aforementioned anti-inflammatory and immunomodulatory genes were transcriptionally upregulated by MSCs isolated from all tissue sources after inflammatory stimulation, with the exception of TGF-β. Interestingly, the tissue origin of MSC had an effect on the transcriptional level of anti-inflammatory and immunomodulatory genes where BM and UC MSCs expressed significantly more CD274 and CFH than Is, Va and Ad MSCs. Moreover BM MSCs consistently expressed higher transcripts than other MSCs for several genes including TSG-6, IDO, CFH, CD271, HGF and TGF-β when maintained under inflammatory conditions, whereas Is MSCs expressed higher transcript levels of GMCSF than any other tissue source of MSC.

6.4 Discussion

Using the air pouch model on NSG and OT-1 mice, this chapter aimed to identify and examine the *in vivo* immune cell attraction profile of pre-stimulated Is MSCs via flow cytometry and histology.

Moreover, data from the previous chapter demonstrated that Is, Va, Ad, BM and UC MSCs secreted chemokines and attracted target immune cells *in vitro*, therefore, this chapter aimed to understand the potential immunomodulatory effects of Is, Va, Ad, BM and UC MSCs might have on immune cells within close proximity. Thus, the expression of several immunomodulatory and anti-inflammatory genes were assessed at a transcriptional level. These included; TSG-6, IDO, CFH, CD274, HGF, TGF-β and GMCSF.

6.4.1 The in vivo immune cell attraction profile of pre-stimulated Is MSCs

As discussed throughout this thesis, it is established that MSCs are anti-inflammatory and immunomodulatory cells as demonstrated in a variety of disease models (323, 324). Importantly, it is accepted that MSCs attract immune cells in close proximity in order to immunomodulate them and exert their anti-inflammatory properties on surrounding immune cells (325). The mechanisms by which MSCs apply their anti-inflammatory and immunomodulatory effects have

been thoroughly explored *in vitro* (133). However, the exact mechanisms of how this occurs *in vivo* are largely not understood.

The current study found that licensed Is MSCs (pre-stimulated with 10ng/mL of IFN-ϒ, TNF-α and IL-ß for 24 hours), recruited large numbers of leukocytes into the air pouches of NSG and OT-1 mice. The predominant recruited cell type in both mouse strains was neutrophils, followed by macrophages and smaller numbers of DCs, monocytes and eosinophils. Additionally, OT-1 mice also recruited NK cells. This is in contrast to control NSG and OT-1 mice which had markedly less CD45 +ve cells within the air pouch and the predominant infiltrating cell type was macrophages. This highlights that the creation of the air pouch does not result in a substantial immune cell reaction and that the immune cells recruited by Is MSCs was not influenced by the mouse genotype used, suggesting that Is MSCs specifically recruited neutrophils. Specific neutrophil chemoattraction by Is MSCs was further emphasised by the significant increase in the percentage of neutrophils in the lower membrane of treated NSG mice, compared to control. Literature surrounding MSC immune cell attraction in various disease models exists, however are somewhat contradictory to the current study. Georgiev-Hristov *et al* documented an Ad MSC dependant block in neutrophil recruitment and enhanced macrophage recruitment at the 24 hour time point within a tracheal anastomosis murine model (326). Moreover, Carceller *et al* showed that Ad MSCs alone in an air pouch model did not induce migration of neutrophils at a time point of 18 hours (327). The discrepancies between the studies is likely due to the different populations of MSCs used as the current study observed that unstimulated Ad MSCs did not attract neutrophils *in vitro* (Figure 5-11 Flow cytometry analysis of the number of immune cells migrating towards Is, Va, Ad, BM or plastic under homeostatic or inflammatory conditions.). Collectively, this suggests that the attraction of neutrophils is Is MSC specific and highlights that MSCs attract/block recruitment of specific, differential immune cells, depending on what tissue they are isolated from. Therefore, Is MSCs chemoattraction of neutrophils and the potential clinical implications is the focus of this discussion.

6.4.2 **Is MSCs and their *in vivo* attraction of neutrophils**

Neutrophils express high levels of CXCR1 and CXCR2 which bind to CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8 and result in neutrophil recruitment and migration into target tissues (328, 329). Out of these chemokines, Is MSCs were tested for their secretion of CXCL1, CXCL5, CXCL6 and CXCL8, which they secreted at high levels when maintained under homeostatic and inflammatory conditions. Therefore, although an array of molecules are involved in neutrophil migration (329), it is likely that Is MSC specific neutrophil attraction profile is due to the predominant secretion of CXC chemokines. In the first instance, the attraction of neutrophils towards Is MSCs could be regarded as detrimental within the clinic as large numbers of neutrophils are associated with inflammatory diseases such as rheumatoid arthritis (330) and transplant rejection, where neutrophil accumulation in the lungs after transplantation leads to acute graft failure (331).

Conversely, accumulating evidence proposes that the presence of neutrophils is not always related to inflammation and suggests that this view is a rather limited interpretation of neutrophil function.

Firstly, the engulfment of neutrophils by macrophages is an anti-inflammatory process in itself, which prevents the uncontrolled release of damaging proteolytic and oxidative mediators from neutrophils and also results in the production of anti-inflammatory cytokines such as IL-10 and TGF-β from phagocytosing phagocytes (332). Macrophages phagocytosing neutrophils and cellular debris was a common phenomenon visualised in the air pouches of NSG and OT-1 mice within this study **(**Figure 6-6 (red arrow with asterisk)).

Secondly, neutrophils may inhibit graft inflammation via promoting wound and tissue repair coupled with graft revascularisation. Lammermann *et al* described how neutrophils swarmed around sites of necrotic tissue *in vivo*, isolating it from surrounding viable tissue and limiting further damage (333). Additionally, Christoffersson *et al* discussed the importance of VEGF-A in attracting CD11b+/GR-1+CXCR4^{hi} neutrophils to the site of islet engraftment in mice. VEGF-A recruited neutrophils, expressed high levels of MMP9 and MMP9 deficient mice were unable to revascularise transplanted islets. Thus they found that a subset

of CD11b+/GR-1+CXCR4^{hi}, MMP9 secreting neutrophils were vital in islet graft revascularisation, likely due to increased vascular density and blood flow caused by MMP9 (334, 335). Similarly, the same group found that revascularisation of islets transplanted into striated muscles within mice was indispensably dependant on the presence of neutrophils (336).

The contradictory roles of neutrophils in inflammation and transplantation models could be cellular and environmental context dependent. However, recent observations suggest that different subsets of neutrophils exist in mice and humans – inflammatory and anti-inflammatory/pro-angiogenic. It is unclear whether these are truly distinct subsets of neutrophils or if they are a continuum of maturation -changing phenotype in response to extracellular queues.

Using mice infected with methicillin-resistant Staphylococcus aureus (MRSA), Tsuda *et al* observed different populations of neutrophils. Neutrophils isolated from mice resistant to MRSA and mice susceptible to MRSA showed distinct patterns in their cytokine production, TLRs and surface marker expression (337). The neutrophils isolated from MRSA resistant mice were considered inflammatory neutrophils and secreted IL-13 and CCL3, whereas neutrophils isolated from MRSA susceptible mice were considered anti-inflammatory and secreted IL-10 and CCL2. Moreover, inflammatory neutrophils were observed to have segmented nuclei (indicative of mature neutrophils) and anti-inflammatory neutrophils were observed to have ring-shaped nuclei (indicative of immature neutrophils). In the current study, the majority of neutrophils which had infiltrated into the lower membrane and air pouches of NSG and OT-1 mice had segmented/hyper-segmented nuclei, indicating that these might be inflammatory neutrophils, whereas small numbers of immature, ringed shaped nuclei neutrophils were observed on the cytospins of NSG and OT-1 mice.

As an added complication to establishing whether or not the attraction of neutrophils by Is MSCs *in vivo* would be detrimental or beneficial in a clinical setting, an ongoing debate exists on whether neutrophils and granulocytic myeloid derived suppressor cells (GMDSCs) are analogous or distinct cells. GMDSCs are well established as anti-inflammatory immune cells largely implicated in the negative regulation of immune responses in various cancers, ultimately promoting tumour angiogenesis, tumour cell invasion and metastasis

(338-340). They share identical surface markers to neutrophils and their nuclear morphology is debated within the literature. Greifenberg *et al* found that GMDSCs had ring shaped nuclei, whereas cells with segmented nuclei were not suppressive (341). Conversely others have extensively reviewed GMDSCs nuclear morphology and associated a segmented nucleus with immune suppression (342-346).

Given the current data, drawing conclusions on whether Is MSC *in vivo* chemoattraction of neutrophils would be beneficial or detrimental in a clinical setting is not possible. However, it has been proposed that MSCs attract immune cells in close proximity to immunomodulate them. Therefore, is it possible that MSCs attract inflammatory neutrophils and through undocumented interactions, steer them towards an anti-inflammatory neutrophil. Mediators potentially involved in this process are discussed below.

6.4.3 Immunomodulatory genes transcribed by Is MSCs and their potential effects on neutrophils

TSG-6 is a secreted glycoprotein expressed at sites of inflammation and injury. It is produced by endothelial cells, monocytes/macrophages and mast cells in response to inflammation or injury and interacts with glycosaminoglycans (GAGs) such as heparin sulphate as well as inhibiting the binding and presentation of CXCL8 on the surface of GAGs (347). TSG-6 is considered an anti-inflammatory protein, produced in episodes of inflammation to limit tissue damage during acute inflammatory episodes. The secretion of TSG-6 by MSCs is associated with the reduction in inflammation in rodent models of myocardial infarction and corneal transplantation (228, 348). In both models, this reduction of inflammation is associated with a reduction in infiltrating leukocytes – mainly neutrophils and macrophages. The anti-inflammatory effects of TSG-6 have been loosely associated with the inhibition of neutrophil extravasation into inflammatory sites by inhibiting the presentation of CXCL8 on GAGs. CXCL8 presentation on GAGs is a vital step in the rolling and tethering of immune cells prior to extravasation (297, 349). In the current study, high levels of TSG-6 transcription by inflamed Is MSCs was observed, coupled with high levels of neutrophil infiltration. This directly contradicts the findings discussed above

where TSG-6 inhibits neutrophil infiltration into sites of inflammation. Several proposals discussed below could account for this observation:

1. **Is MSCs do not transcribe TSG-6 into a protein/ produce TSG-6 in low quantities.** As data suggesting MSC inhibition of neutrophil migration via TSG-6 secretion were collected using BM isolated MSCs, it is possible that Is MSCs do not secrete functional TSG-6 or that they secrete low levels of TSG-6 (348, 350). In the current study, significantly higher TSG-6 transcription by BM MSCs compared to Is MSCs could suggest that BM MSCs secrete significantly more TSG-6 *in vivo*, therefore impairing neutrophil migration more effectively than Is MSCs. This would explain why BM MSCs have been demonstrated to inhibit leukocyte migration, whereas Is MSCs failed to.

2. **Neutrophils in the air-pouch are anti-inflammatory/pro-angiogenic and do not use the CXCL8/CXCR1, CXCR2 axis to gain entry into the air pouch, therefore the effects of TSG-6 are overridden**. Christoffersson *et al* described that VEGF secreting islets attracted MMP9-secreting neutrophils which were vital in islet transplant revascularisation in mice. $VEGFR^{-/-}$ mice resulted in a reduction of more than two fold in the recruited neutrophils towards the islets and this impaired islet revascularisation (334). Therefore, it could be possible that the neutrophils observed in the air pouch were recruited via VEGF secretion – which Is MSCs have been shown to secrete under homeostatic and inflammatory conditions (Figure 5-4).

3. **The neutrophils observed in the air pouch are GMDSCs and therefore use alternative methods of extravasation, thus the effects of MSC secreted TSG-6 on neutrophil recruitment are irrelevant.** As discussed, neutrophils and GMDSCs are often indistinguishable (351), however, despite the mechanisms of GMDSCs trafficking not being fully understood, it is reported that their means of trafficking involve molecules such as GMCSF, IL-1β, IL-6, VEGF and IL-10 as well as chemokines (351). Serafini et al demonstrated that GMCSF attracted GMDSCs *in vitro* which resulted in substantial immunosuppression of anti-tumour T cell responses (352). Interestingly, in the current study, GMCSF transcription was specific to Is

MSCs. Importantly, GMCSF transcription was upregulated after inflammatory stimulation. This could account for the specific neutrophil/GMDSC attraction by Is MSCs observed *in vitro* Figure 5-11 and *in vivo,* which was upregulated after inflammatory stimulation.

Other molecules involved in the migration and immunomodulation of neutrophils are discussed in the context of cancer, where the existence of tumour associated neutrophils are either N1 (inflammatory, anti-tumorigenic neutrophils) or N2 (anti-inflammatory, pro-tumour and pro-angiogenic neutrophils) (353). As mesenchymal stromal cells are widely implicated in cancer progression and its metastasis as a result of their immunosuppression of the immune system, it is reasonable to assume that the molecules they secrete could play a role in the N1/N2 paradigm (354-356). It is also reasonable to assume that MSCs could act similarly in other diseases other than cancer.

Here I report the transcription of TGF-ß1 by all MSCs at similar levels during homeostatic and inflammatory conditions. TGF-ß is a secreted multi-functional protein involved in many processes including immune suppression, inflammation and disease progression (357). It plays a role in neutrophil chemoattraction (358) and inhibition of neutrophil degranulation (359). In the context of cancer, TBF-ß is involved in tumour progression which has been associated with neutrophil polarisation (353). Through the use of a variety of injected tumour cell lines, Fridlender *et al* demonstrated the effects of TGB-ß blockade on an *in vivo* model of tumour progression. Blocking TGF-ß resulted in an accumulation of neutrophils with an N1 “inflammatory” phenotype, characterised by their expression of TNF-α and CCL3. Moreover, they demonstrated that neutrophil depletion in mice bearing tumours with no TGF-ß blocker (i.e. depletion of N2 neutrophils), increased CD8 T cell activation, whereas the depletion of neutrophils in mice bearing tumours with a TGF-ß blocker (i.e. depletion of mice with N1 neutrophils) resulted in decreased activation of intra-tumoral CD8 T cells (353).Thus, it is possible that TGF-ß secretion by Is MSCs in the air pouch could promote the recruitment of anti-inflammatory neutrophils, whilst also inhibiting their cytotoxic effects, resulting in downstream effects on other immune cells such

as CD8 T cells. As Is MSC TGF-β transcription is not necessarily transcribed into a protein, assumptions on the actions of MSC TGF-β secretion are not conclusive. It is important to note that the only true means of comparison between Fridlender *et al* and the current study is the nuclear morphology of the neutrophils, where Fridlender et al observed hyper-segmented nuclei in N1 inflammatory neutrophils, similar to the nuclear morphology of the neutrophils within the air pouch of NSG and OT-1 mice.

6.5 **Conclusions**

The initial aim of this work was to better understand the *in vivo* behaviours of MSCs isolated from various tissues through the assessment of MSC chemokine and chemokine receptor expression. Having extensively reviewed chemokine secretion by MSCs, it was obvious that the chemoattraction of specific immune cells towards MSCs - Is and Va MSC in particular - was a mechanism employed by MSCs *in vitro*. Moreover, the specific neutrophil chemoattraction observed *in vitro* was consistent *in vivo*. Given the evidence, it is appropriate to suggest that neutrophils were recruited into the air pouch of NSG and OT-1 mice under the influence of Is MSC secreted chemokines such as CXCL1, 5, 6 and 8. Other mediators which were transcribed by MSCs and therefore could be secreted such as TGF-β, TSG-6, IDO and GMCSF, could play a role in which type of neutrophil is recruited and influence overall neutrophil behaviour.

Overall, due to a newly emerging N1 and N2 field coupled with an inability to distinguish neutrophils from GMDSCs, makes it difficult to understand if neutrophil attraction by Is MSCs would be detrimental or beneficial in a clinical setting. This study highlights the specific *in vivo* attraction of neutrophils by licensed Is MSCs. Additionally it demonstrates that anti-inflammatory and immunomodulatory genes are upregulated after licensing which mirrors the upregulation of chemokine secretion and immune cell attraction. Through the secretion of TGF-β, IDO and GMCSF, Is MSCs could be attracting a specific type of neutrophil which could be beneficial in a clinical setting. However, it is important to address that it is probable that neutrophil phenotype is shaped by the surrounding microenvironment and therefore they exist in a spectrum of states, rather than the existence of two

distinct neutrophil populations. Thus, this work would greatly benefit from an investigation into the phenotype of infiltrating neutrophils towards MSCs.

Chapter 7

General Discussion

Chapter 7 General Discussion

7.1 Introduction

Bone Marrow (BM) MSCs are considered to be the "gold-standard" MSC and are widely studied as a cellular therapeutic in several clinical settings, including transplant and inflammatory diseases to act as anti-inflammatory and immunomodulatory mediators. Recently, other more easily accessible, less invasive and more cost-efficient tissues such as the ones utilised throughout this study – Islet (Is), visceral adipose (Va), adipose (Ad) and umbilical cord (UC) - have been identified as an attractive, alternative source of MSCs. This has resulted in a plethora of studies, using MSCs isolated from various alternative tissues to act as anti-inflammatory and immunomodulatory mediators for inflammatory diseases and in transplant settings. However, a full, standardised comparison of the phenotype and function of these MSCs does not exist, resulting in a lack of understanding of their true potential clinical applications. Additionally, more often than not, MSCs are frequently phenotyped when maintained under homeostatic conditions, which often disregards the ultimate fate of many MSCs - infusion into an inflammatory environment. Therefore, through a stringent set of standardised techniques, the aim of this study was to begin to understand and compare the potential *in vivo* function of Is, Va, Ad, BM and UC MSCs under homeostatic or inflammatory conditions. This would help us assess and possibly identify a "preferred" tissue source for the isolation of MSCs, for use within a broad range of clinical settings, with particular focus on the co-infusion of MSCs with pancreatic islets to act as anti-inflammatory mediators and treat individuals with Diabetes mellitus type 1 (DMT1). To address this aim, three key questions were considered:

1) Do Is, Va, Ad, BM and UC MSCs have the same phenotype?

2) Where could MSCs potentially migrate to when infused into a patient and does the tissue origin of MSCs impact this?

3) How do MSCs interact with surrounding target (or off-target) tissue(s) and does the tissue origin stimulation of MSC impact their behaviour?

Chapter 3 of this thesis addressed question number one and ensured that the MSCs studied met the minimum criteria outlined by the International Society of Cellular Therapy (ISCT) to be defined as a MSC (95). MSCs were assessed for basic phenotypical properties through passage by monitoring their spindle-shaped morphology and ability to adhere to plastic. Through a set of standardised techniques, the surface molecule expression, size and granularity of MSCs were assessed in homeostatic conditions and after inflammatory stimulation. Moreover, basic MSC function was assayed by the assessment of MSC differentiation potential into adipocytes, chondrocytes and osteocytes.

Chapters 4 and 5 addressed question number 2. Chemokine receptor expression by MSCs at a transcript (Chapter 4) and protein (Chapter 5) level, were assessed in homeostatic conditions and under inflammatory stimulation to understand if MSCs possessed migratory capacity and if MSC tissue origin could affect their migratory potential. An extensive assessment of all the chemokine receptors expressed by MSCs at a transcript level highlighted genes of interest to be assessed at a protein level and outlined specific anatomical locations that MSCs might be more inclined to migrate to when infused into a patient.

Chapters 4, 5 and 6 addressed question number 3. Chemokine expression by MSCs at a transcript level (Chapter 4) and protein level (Chapter 5) were assessed under homeostatic and inflammatory conditions to begin to understand how MSCs might behave when they reach target (or off-target) tissue(s). After highlighting that MSCs extensively transcribed and secreted a particular set of chemokines under homeostatic and inflammatory conditions, chemokine function was tested *in vitro* (Chapter 5) and *in vivo* (Chapter 6) by assessing the immune cell attraction profile of MSCs. Moreover, transcriptional analysis of anti-inflammatory and immunomodulatory genes (Chapter 6) provided a better insight into how MSCs might be interacting with the immune cells they attracted. The following sections will discuss these findings and how they relate to specific areas of clinical relevance.

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

Clinical performance of MSCs is often monitored by the temporary or prolonged resolution of symptoms and/or (in the case of transplants) the promotion of graft survival (216, 360). Depending on the specific clinical setting, the efficacy of MSCs is somewhat dependent upon the successful migration/delivery of MSCs to target tissues, their differentiation capacity and how MSCs interact with their surrounding allogeneic environment (tissues and immune cells) (226). Thus, as MSCs isolated from the Is, Va, Ad, BM and UC differentially express chemokine receptors, (which could impact their migratory potential) and chemokines, (which was shown to have impact on their immune cell attraction profiles), the data generated in this thesis provides evidence to support that MSCs isolated from particular tissues might be more beneficial than other tissue sources of MSCs in specific clinical settings. For the purpose of this discussion, I will focus on two clinical settings – transplantation and cancer- and highlight which tissue source of MSC might be more beneficial as a cellular therapy in these settings, whilst also discussing which tissue source of MSC might be less beneficial or detrimental.

7.2.1 The optimal tissue for MSC isolation to co-transplant with pancreatic islets

Islet transplantation is an attractive alternative to pancreas solid organ transplantation (SOT) to treat diabetes mellitus type 1 (DMT1) as it is minimally invasive and reduces the intensity of the immunosuppressive regime post-transplant because the islets present as a smaller immunogenic tissue mass (176). However, islet survival and function after transplantation is known to diminish over time due to graft rejection and failure of the islets to re-vascularise (361-363). Graft rejection is a multifactorial process, consisting of multiple immune mediated reactions including activation of complement, antibody mediated rejection, alloantigen specific induction of T-cell proliferation and the activation of T-cell effector functions (364). Thus, due to MSC anti-inflammatory, immunomodulatory and pro-regenerative properties, the co-infusion of islets with MSCs could reduce graft rejection, whilst prolonging graft survival.

Islets are typically infused into the hepatic portal vein and engraft within the liver. Infusion of cells into this vein increases the blood pressure, subsequently activating the endothelium and ultimately increasing immune cell transendothelial migration into the liver, which could promote unwanted inflammation (365, 366). Thus, considering the size of MSCs being co-infused with islets is important in minimising potential unwarranted immune cell trafficking into the liver parenchyma, as the infusion of smaller cells would result in decreased pressure (367). In the current study, MSCs isolated from the Is, Va, Ad, BM and UC were of identical shape (Figure 3-2) and size (Figure 3-7) under homeostatic and inflammatory conditions. Moreover, CXCR6 – a receptor thought to be involved in immune cell trafficking and retention within the liver – was expressed at slightly higher levels by BM MSCs than any other population of MSCs under both homeostatic and inflammatory conditions (Figure 5-1). Therefore, based on this observation, it could be proposed that BM MSCs possess greater migratory and retention capacity within the liver and thus could deliver enhanced therapeutic effects for the islets longer than other tissue sources of MSCs.

Once MSCs and islets are engrafted within the liver, MSCs must function to **i)** deter graft rejection whilst simultaneously **ii)** promoting graft survival and longevity.

i) To deter graft rejection, MSCs must control the activation and proliferation of immune cells, namely allo-specific T cells (176). Firstly, mismatched MSCs themselves should not provoke alloreactivity. It is widely accepted that under homeostatic or inflammatory conditions, MSCs do not express the key antigens involved in immediate rejection - ABO blood group antigens (368). Additionally, the expression of HLA-DR (another key molecule involved in graft rejection) on MSCs is documented only after inflammatory stimulation, an observation which the current study agrees with (Figure 3-6) (369). Conversely, Is, Va and Ad (BM and UC not tested) MSCs expressed HLA-ABC when maintained in homeostatic conditions which was substantially upregulated after inflammatory stimulation. Here we found that Ad derived MSCs expressed significantly more HLA-DR and substantially more HLA-ABC on their surface than Is derived MSCs in both homeostatic conditions and after inflammatory stimulation. Therefore the Islet

might be the more suitable MSC tissue source for use within transplantation settings to avoid allogeneic rejection of MSCs. Notably however, MSCs do not express co-stimulatory molecules and therefore they are unlikely to provoke extensive activation of T cells based on mismatched HLA-ABC expression and thus the expression levels of these molecules might not play a huge role in their rejection.

Secondly, MSCs must also induce tolerance and immunomodulate the surrounding cells to prevent the primary cause of early islet damage – inflammation (370). Infusion of islets into the hepatic portal vein can trigger complement activation which leads to the lysis of islets (364, 371). Complement factor H (CFH) is a complement regulatory protein which binds to GAGs and protects cells from complement-mediated destruction via the cleavage of C3b and accelerating the decay of C3-convertases. CFH deficiency promotes graft rejection and therefore CFH secretion by MSCs could serve as a mechanism of protection for transplanted islets (372, 373). MSC constitutive secretion of CFH has been documented which is upregulated after inflammatory stimulation. Similarly, the current study showed constitutive, moderate levels of transcription of CFH by all tissue sources of MSCs in homeostatic conditions, which was significantly upregulated by BM and UC MSCs and substantially upregulated by Is, Va and Ad MSCs after inflammatory stimulation (Figure 6-11). BM MSCs transcribed the most CFH, significantly more than Is MSCs. Assuming that the MSC transcription levels of CFH are proportionately transcribed into protein and that higher levels of CFH are optimal in controlling aberrant complement activation, the BM would be the desired tissue source for MSCs to avoid islet rejection via complement activation, whereas the islet would be the least desired tissue source due to low CFH transcription.

Akin to complement activation, allogeneic T cell proliferation and activation are prerequisites for islet allograft rejection, along with the concurrent activation of existing autoimmune T cells (374). A large body of literature shows that MSCs can inhibit T cell proliferation whilst also promoting a T regulatory cell phenotype through the secretion of several mediators including IDO, TGF-β and expression of CD 274 (133, 375, 376). Induction of tolerance is often associated with T regulatory cells and secretion of these mediators by MSCs has been

associated with islet allograft tolerance, function and regeneration (376-380). In chapter 6, substantial transcription of the above mediators was observed by all tissue sources of MSCs after inflammatory stimulation, where BM MSCs transcribed the most IDO, CD274 and marginally more TGF-β than Is, Va, Ad and UC MSCs (Figure 6-11). Collectively, based on the transcription of CFH, IDO, CD274 and TGF- β, BM MSCs might possess more potent immunosuppressive capabilities than other tissue sources of MSCs, which would promote islet survival, whilst also supporting graft tolerance. Importantly however, Chapter 5 and Chapter 6 demonstrated that despite MSC secretion of T cell chemoattractants (CCL20, CXCL9, 10 and 11) under inflammatory stimulation, MSCs do not attract any T cells *in vitro* or *in vivo*. This could suggest that MSCs do not need to be in close proximity to T cells to elicit their anti-inflammatory effects, or indeed that MSC and T cell interactions do not play a major role in *in vivo* MSC immunomodulatory/anti-inflammatory actions.

ii) In addition to immune cell modulation, MSCs must promote islet survival. As well as inflammation causing a significant amount of islet loss, a lack of blood supply also plays an important role. The process of isolating islets destroys the external vasculature whilst potentially damaging the internal islet vascular network (381). Ito *et al* demonstrated that the co-infusion of rat BM MSCs and rat islets into the liver of streptozotocin-diabeted synergetic recipients or transplanted under the renal capsule of NOD SCID mice resulted in an improvement of islet graph morphology and function, which was in part due to the revascularisation of the graft, mediated by MSCs (381). Factors such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) are widely recognised as pro-angiogenic mediators which are involved in the revascularisation of islets (382, 383). Importantly, in the current study MSCs were observed to secrete VEGF (Figure 5-4) and transcribe HGF (Figure 6-2). In homeostatic conditions MSCs secreted ~ 300pg/mL of VEGF, however, UC MSCs did not secrete VEGF above background levels. Is, Va, Ad, BM and UC MSCs upregulated VEGF secretion under inflammatory stimulation and BM MSCs secreted double the amount of VEGF than any other tissue source of MSC. Importantly, Walker *et al*. demonstrated that a dose response blockade of VEGF using the monoclonal antibody bevacizumab showed decreased vascular density in mouse brains with increased concentrations of bevacizumab (384). This

suggests that increased concentrations of VEGF could increase the vascular density of islet grafts and therefore higher volumes of VEGF may be optimal over lower volumes and thus BM MSCs would be the preferential tissue source of MSC to promote angiogenesis and islet re-vascularisation, whereas Ad derived MSCs would promote little angiogenesis. Conversely however, Carlsson *et al*. described that the amount of VEGF available to the islets does not correlate to the degree of re-vasularization of islets (385). This could be due to the presence of other angiogenic mediators secreted by MSCs such as HGF which is known to play a role in angiogenesis and islet survival. HGF is secreted by hepatocytes in the liver which support islet graft function and longevity. In Chapter 6, HGF was shown to be transcribed by all tissue sources of MSCs, with BM MSCs transcribing significantly more than Is, Va and Ad in homeostatic conditions, and significantly more than Is, Va, Ad and UC MSCs under inflammatory conditions, again suggesting that BM MSCs might possess greater angiogenic potential than other tissue sources of MSCs. Despite BM MSCs secreting and transcribing the most VEGF and HGF, respectively, it is important to recognise that these are not the only angiogenic mediators that Is, Va, Ad, BM and UC MSCs were found to secrete in this study. In Chapter 4 and 5, MSCs were shown to transcribe (Figure 4-10) and secrete (Figure 5-5) substantial levels of the pro-angiogenic chemokines CXCL1, 5, 6 and 8 (386-389). CXC chemokine secretion by MSCs was differentially affected depending on MSC tissue source and inflammatory stimulation. During homeostatic conditions CXCL8 was the highest secreted CXC chemokine by Is, Va, Ad and BM MSCs, whereas UC MSCs secreted more CXCL1 than any other CXC chemokine assayed. The pro-angiogenic role of CXCL8 has been described in various models and in some cases, is associated with aberrant blood vessel formation (390). The angiogeneic contribution of CXC chemokines in islet transplantation is – to my knowledge not documented. However, their detrimental role in islet graft survival has been reported, where CXCR1/2 (receptors for CXCL1, 5, 6 and 8) blockade with an allosteric inhibitor - reparixin - enhanced islet survival after transplantation. This could be due to a decrease in neutrophil infiltration towards the graft site as neutrophils are considered to have detrimental effects on lung and cardiac allografts, due to their inflammatory secretory profiles and interaction with surrounding immune cells (391-394). Challenging the observation that neutrophils are detrimental in a transplant setting is work carried out by Christoffersson et al, which describes

that MMP9 secreting neutrophils were pivotal in the revascularisation of islets that had been transplanted into the striated muscle of mice (334). In the current study, neutrophils were the predominant CD45 +ve cell attracted towards MSCs *in vitro* (Chapter 5) and *in vivo (*Chapter 6*)*. Is and Va MSCs were the only tissue sources of MSCs to attract substantial numbers of neutrophils above background levels in homeostatic conditions, whereas Ad and BM MSCs did not. Under inflammatory stimulation, neutrophil attraction towards all MSCs was upregulated above background levels *in vitro*, mirroring the upregulation in neutrophil chemoattractant chemokine secretion by all tissue sources of MSCs. Is derived MSCs attracted the most neutrophils under homeostatic and inflammatory conditions, whereas BM MSCs attracted the least. The immune cell attraction profile of Is MSCs was mirrored exactly when moving from *in vitro* analysis into the *in vivo* air pouch models in two individual mouse strains, where neutrophils were the predominant CD45 +ve cell in the air pouch. Additionally, Is MSCs attracted significantly more CD45 -ve cells than in control mice. It therefore could be proposed that MSCs enhance graft revascularisation not only through substantial VEGF and HGF secretion but by the extensive secretion of pro-angiogenic chemokines that chemoattract neutrophils, ultimately resulting in prolonged graft survival. Moreover, the observation that Is MSCs attracted CD45 –ve cells *in vivo* suggests that MSCs could attract endogenous MSCs to the graft site and perpetuate MSC anti-inflammatory and immunomodulatory effects. Transplanted MSCs attracting endogenous MSCs has been documented (395).

The limitations of this hypothesis are that MSC neutrophil chemoattraction was observed in an air pouch model and not in an islet transplant model. Moreover, Christoffersson *et al*. described that the neutrophils required for islet revascularisation were attracted via VEGF and that these were of a pro-angiogenic phenotype. Despite nuclear morphology analysis of the neutrophils – which were hypersegmented – a conclusive phenotype of the infiltrating neutrophils could not be made.

To summarise, MSCs isolated from various tissues express different levels of CXCR6 which could impact MSC migration and retention within the liver. Additionally, BM MSCs secreted the largest quantities of VEGF and transcribed HGF at the highest levels in homeostatic conditions and after inflammatory.

MSCs isolated from all sources secreted substantial amounts of pro-angiogenic chemokines under homeostatic and inflammatory conditions, which could impact the vascularisation of islets via the angiogenic nature of chemokines themselves or through the attraction of pro-angiogenic neutrophils. The current study highlights that MSCs differentially transcribed and secreted pro-angiogenic mediators, depending on their tissue origin. This could have profound impact, not only in islet transplantation, but in any transplant setting. Additionally, the tissue source of MSCs also dictates 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. The differential immune cell attraction profile of MSCs could also be harboured in other transplant settings. For example, the presence of macrophages positively impacts the regenerative potential of hepatocytes in the liver (396, 397). Thus infusing MSCs that attract monocytes and macrophages (Is MSCs) with hepatocytes could promote the attraction of endogenous MSCs to the liver and aid in liver regeneration.

7.2.2 The optimal tissue for MSC isolation for potential therapeutic use in Cancer

MSCs have been described as a double edged sword in cancer, providing pro-and anti-tumorigenic signals. Despite florid documentation on MSCs role in promoting tumour growth and metastasis (398), they are an attractive candidate for delivery of anti-tumour agents due to their ability to home to tumour sites and secrete cytokines/chemokines (399).

It has been suggested that BM and Ad derived MSCs can migrate to sites of tumour development via the expression of several different receptors such as VEGFR, CCR2 and CXCR4. Additionally, MSC localisation to the inflammatory tumour environment might also involve other receptors (CXCR6 and ACKR3) that respond to the chemokines that the tumour environment produces (400-402). Thus, the expression levels of these receptors by MSCs might influence their migratory potential to the tumour. The current study highlighted that BM MSCs expressed the highest surface expression of CXCR4, CXCR6 and ACKR3, whereas Is and Ad MSCs expressed low surface levels of these receptors. Moreover, BM MSCs markedly upregulated their expression of ACKR3 and CXCR4 under inflammatory stimulation, suggesting that pre-treating MSCs with inflammatory

mediators prior to infusion into cancer patients could improve BM MSC homing capabilities to the tumour site.

Once within the tumour site, MSCs can work in two ways i) promoting tumour growth and metastasis or ii) inhibiting tumour growth. Their dual role within several clinical settings has prompted investigations into the possibility of two MSC phenotypes – anti-inflammatory and pro-inflammatory. It is not assumed that these phenotypes are distinct subsets of MSCs, but rather thought to be a result of MSCs being able to sense their environment (403). It is possible that MSCs isolated from different tissues might be more inflammatory than others due to the environment they were isolated from. Thus MSCs isolated from different tissue sources could promote tumour growth or inhibit it.

i) Through the secretion of immunomodulatory and pro-angiogenic mediators (along with an array of other cytokines and chemokines), it is believed that MSCs are involved in the regulation of immune surveillance, apoptosis and angiogenesis during tumour development (404-407). The expression of IDO by BM MSCs has been reported to inhibit T-cell-mediated immune responses against tumours (404). Moreover, BM MSC secretion of TGF beta was found to stimulate the proliferation, migration and invasion of a prostate cancer cell line *in vitro* and VEGF secretion by BM MSCs was observed to contribute to blood vessel density and tumour angiogenesis in pancreatic carcinoma (408, 409). As mentioned earlier, BM MSCs secreted twice as much VEGF in an inflammatory environment than other tissue sources of MSCs. Similarly, BM MSCs transcribed IDO and TGF-beta at higher levels than any other tissue source, suggesting that despite BM MSCs high surface expression of chemokine receptors that would promote delivery of BM MSCs to the tumour environment, the BM would not be the desired tissue origin of MSCs to use when MSCs reach the tumour environment, as they appear to be relatively anti-inflammatory cells. Conversely, Is and Ad derived MSCs would be preferential due to their low transcription of TGF-beta and IDO (Is MSCs) and low secretion of VEGF (Ad MSCS) under both homeostatic and inflammatory conditions. Their low expression of receptors however could result in poor migration towards the tumour.

ii) Despite the current study finding that BM MSCs appear to be largely anti-inflammatory, they have been described to have anti-tumour capabilities, where

BM MSCs inhibited the survival of non-Hodgkin's Lymphoma cells in a mouse xenograft model (410). Additionally, Ad and UC MSCs have also been described as anti-tumorigenic cells where Ad MSCs were documented to inhibit the proliferation of breast cancer cells (411) and UC MSCs significantly inhibited the proliferation of glioblastomas in a co-culture system, whilst simultaneously increasing apoptosis of glioma cells (412). As this is a relatively new emerging field, detailed mechanisms of how MSCs contribute to the prevention of tumour progression are somewhat unexplored. CCL2 is involved in the attraction of monocytes and macrophages to the tumour site and stimulates macrophage cytolic activity against tumour cells. Moreover, CCL2 injection into the tumour site results in mononuclear cell infiltration, leading to rapid tumour rejection (413, 414). CCL2 was transcribed and secreted by all tissue sources of MSCs when maintained in homeostatic and inflammatory conditions. During homeostatic conditions, Is and Ad MSCs secreted the largest volumes of CCL2 (~5000 pg/mL), whereas under inflammatory stimulation only Is MSCs, secreted the largest volumes of CCL2 (24410pg/mL) (Figure 5-4). Despite high levels of CCL2 secretion by all MSCs, Is MSCs were the only tissue source to attract large numbers of classical monocytes *in vitro*. The attraction of monocytes and macrophages towards pre-inflamed Is MSCs was also observed in the *in vivo* air pouch models. The *in vitro* immune cell attraction profile data in this study suggests that Is derived MSCs have a greater capacity to attract monocytes and macrophages than other tissue sources of MSCs, which could be very beneficial in a tumour environment. In addition to CCL2 secretion, MSC CXCL8 secretion could also prevent tumour metastasis, through the attraction of neutrophils (415). Large neutrophil chemoattraction towards Is and Va MSCs was observed in homeostatic conditions, whereas Ad and BM MSCs did not attract any neutrophils above background levels. Inflammatory stimulation resulted in the upregulation of neutrophil migration towards all tissue sources of MSCs, with Is MSCs inducing the largest neutrophil chemoattraction despite similar secretion of CXCL chemokines compared to other MSCs, this could be a result of Is MSC GM-CSF secretion as Is MSCs were the only tissue source of MSC to transcribe this neutrophil chemottractant. Neutrophils were also the predominant cell type attracted towards pre-stimulated Is MSCs *in vivo*. Based on these observations, the islet could serve as a very promising tissue source for MSC isolation to inhibit the progression of tumours.

As well as immune cell attraction, chemokines themselves play an important role in the tumour microenvironment, where the ELR +ve chemokines (CXCL1, 2, 3, 5, 6, 7 and 8) promote tumour angiogenesis and the ELR-ve chemokines (CXCL 9, 10 and 11) play an angiostatic role in the tumour microenvironment (416). Through the interaction with CXCR3, the ELR –ve chemokines have been observed to inhibit the angiogenic effects of ELR +ve chemokines (417). As Is, Va, Ad, BM and UC MSCs secreted high volumes of both ELR +ve and ELR –ve chemokines in the current study under inflammatory stimulation, it could be proposed that only in inflammatory environments (such as cancer) MSCs employ a safety mechanism to avoid aberrant angiogenesis via the secretion of ELR –ve angiostatic chemokines (416). It is therefore of no surprise that MSCs are termed 'the double-edged sword' in cancer. Their chemokine secretion leads to immune cell attraction which could either result in further immune cell immunomodulation to support the tumour microenvironment, or it could result in tumour destruction. Equally, the pro-angiogenic chemokines that MSCs secrete in an inflammatory environment could enhance tumour angiogenesis, whereas the simultaneous secretion of ELR –ve chemokines could inhibit this.

In summary, it is clear that MSCs isolated from various tissues have the potential to function differentially with respect to the mediators they secrete and immune cells they attract. BM MSCs could potentially home to tumour sites more efficiently than other MSC tissue sources due to their high receptor expression, however their large secretion of pro-angiogenic mediators and substantial transcription of immunomodulatory factors suggests that they might act as an anti-inflammatory MSC in the context of cancer. On the other hand, MSCs isolated from the Is and Va might home less efficiently in comparison to BM MSCs, but through the attraction of higher numbers of immune cells and lower transcription of immunomodulatory mediators than BM MSCs, Is and Va MSCs might function as a more pro-inflammatory MSC in the cancer setting. A schematic in Figure 7-1 highlights the differences observed between Is MSCs and BM MSCs throughout this study and summarises how these differences might influence the tumour microenvironment.

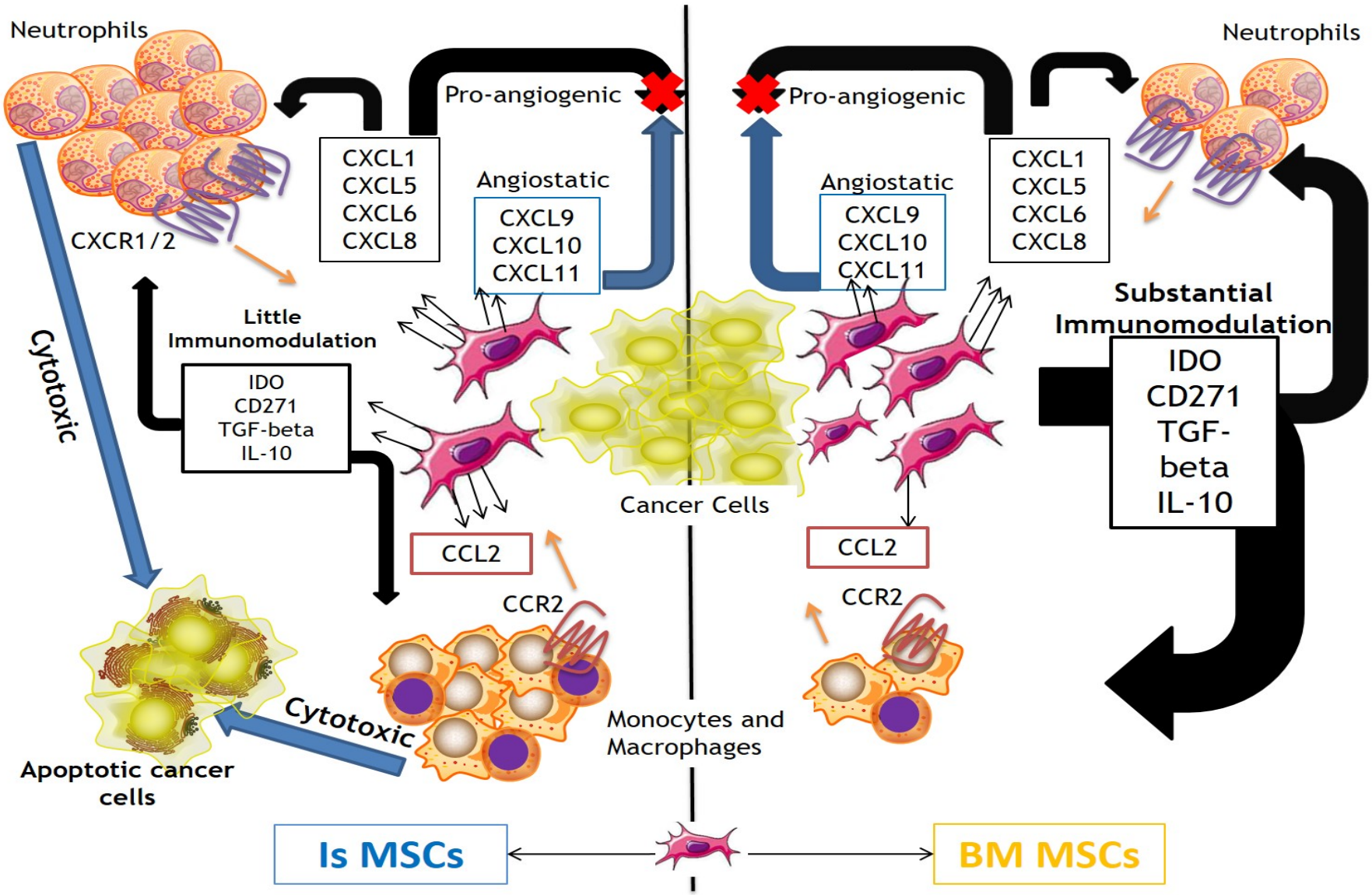

Neutrophils
CXCR1/2
Cytotoxic
Little Immunomodulation
IDO
CD271
TGF-beta
IL-10
Pro-angiogenic
CXCL1
CXCL5
CXCL6
CXCL8
Angiostatic
CXCL9
CXCL10
CXCL11
Cancer Cells
CCL2
CCR2
Cytotoxic
Apoptotic cancer cells
Monocytes and Macrophages
Is MSCs
BM MSCs
Neutrophils
Substantial Immunomodulation
IDO
CD271
TGF-beta
IL-10

Figure 7-1 Simplified schematic highlighting the potential actions of Is MSCs (left) vs. BM MSCs (right) in a tumour microenvironment
BM MSCs (right) are potentially capable of migrating to tumour microenvironments due to their higher surface expression of chemokine receptors which is depicted by the increased number of BM MSCs surrounding the cancer cells compared to Is MSCs (left). Once within the tumour microenvironment, Is MSCs secrete substantially more CCL2, which results in a large influx of CCR2 +ve monocytes and macrophages to the tumour, whereas BM MSCs reduced secretion of CCL2 attracts substantially less monocytes and macrophages. Is and BM MSCs secrete similar levels of ELR +ve neutrophil chemoattractants (CXCL1, 5, 6 and 8), however Is MSCs attract substantially more neutrophils, perhaps due to the secretion of GM-CSF (Chapter 6). Is and BM MSC secreted CXCL1, 5, 6 and 8 have pro-angiogenic effects with potential to promote tumour angiogenesis which are inhibited by the MSC secretion of ELR –ve angiostatic chemokines – CXCL9, 10 and 11. BM MSCs potentially secrete substantially more immunomodulatory factors than Is MSCs and therefore surrounding immune cells could be immunomodulated and unable to kill cancer cells, whereas Is MSCs low secretion of immunomodulators results in the apoptosis of cancer cells due to the large number of infiltrating immune cells having cytotoxic effects on cancer cells. Notably, it is also important to appreciate that the large attraction of immune cells towards Is MSCs could be detrimental if they are of an anti-inflammatory phenotype.

7.3 Overview

It is widely appreciated that MSCs are anti-inflammatory cells capable of immunomodulating surrounding immune cells and repairing damaged tissue and for that reason they are routinely used in clinical trials to act as anti-inflammatory mediators. As mentioned, BM MSCs are considered the gold standard MSC due to their routine use within the clinic, however their frequency within the bone marrow is low and therefore other tissues are being widely exploited for MSC isolation. The wide use of MSCs isolated from alternative tissue sources, cultured in different conditions and used throughout experiments at varied passage makes comparing MSC literature near impossible. The standardised experiments throughout this study comparatively explored the potential *in vivo* differences of MSCs isolated from the islet, visceral adipose, adipose, bone marrow and umbilical cord. Through the assessment of chemokine receptors at a transcript and protein level, this study highlighted that MSCs isolated from various tissue sources could harbour greater migratory potential. Moreover, pre-treating MSCs with inflammatory mediators could increase MSC migratory potential due to an upregulation in chemokine receptor expression. This finding has clinical implications when considering which tissue source of MSC to utilise when MSCs are being infused systemically, where high chemokine receptor surface expression could provide more efficient homing to target tissues. Based solely on chemokine receptor expression, the ideal tissue to isolate MSCs from in order to direct migration towards specific anatomical locations is outlined in Figure 7-2 Overview of chemokine receptor expression by MSCs at a transcript and protein level under homeostatic and inflammatory stimulation.

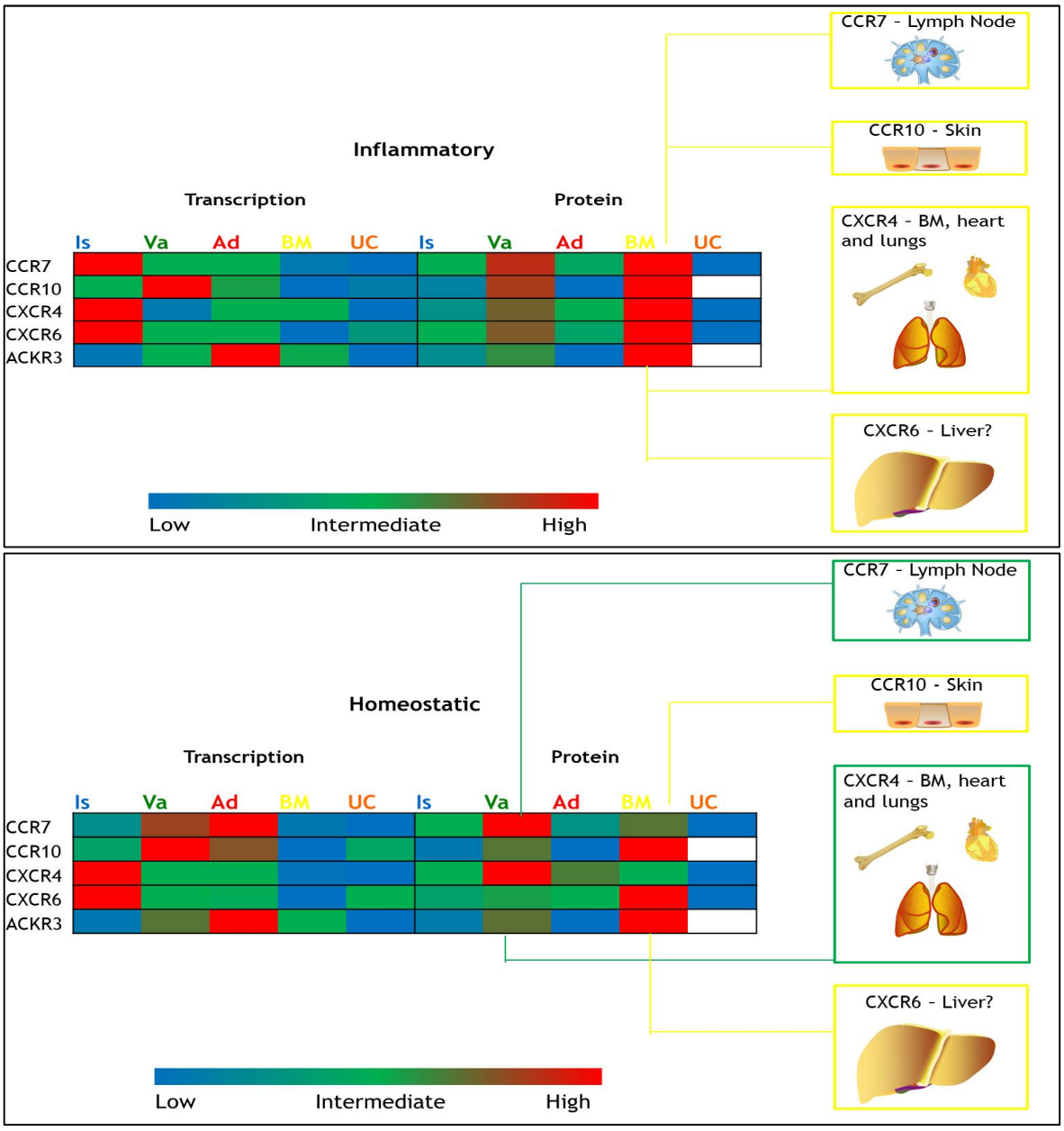
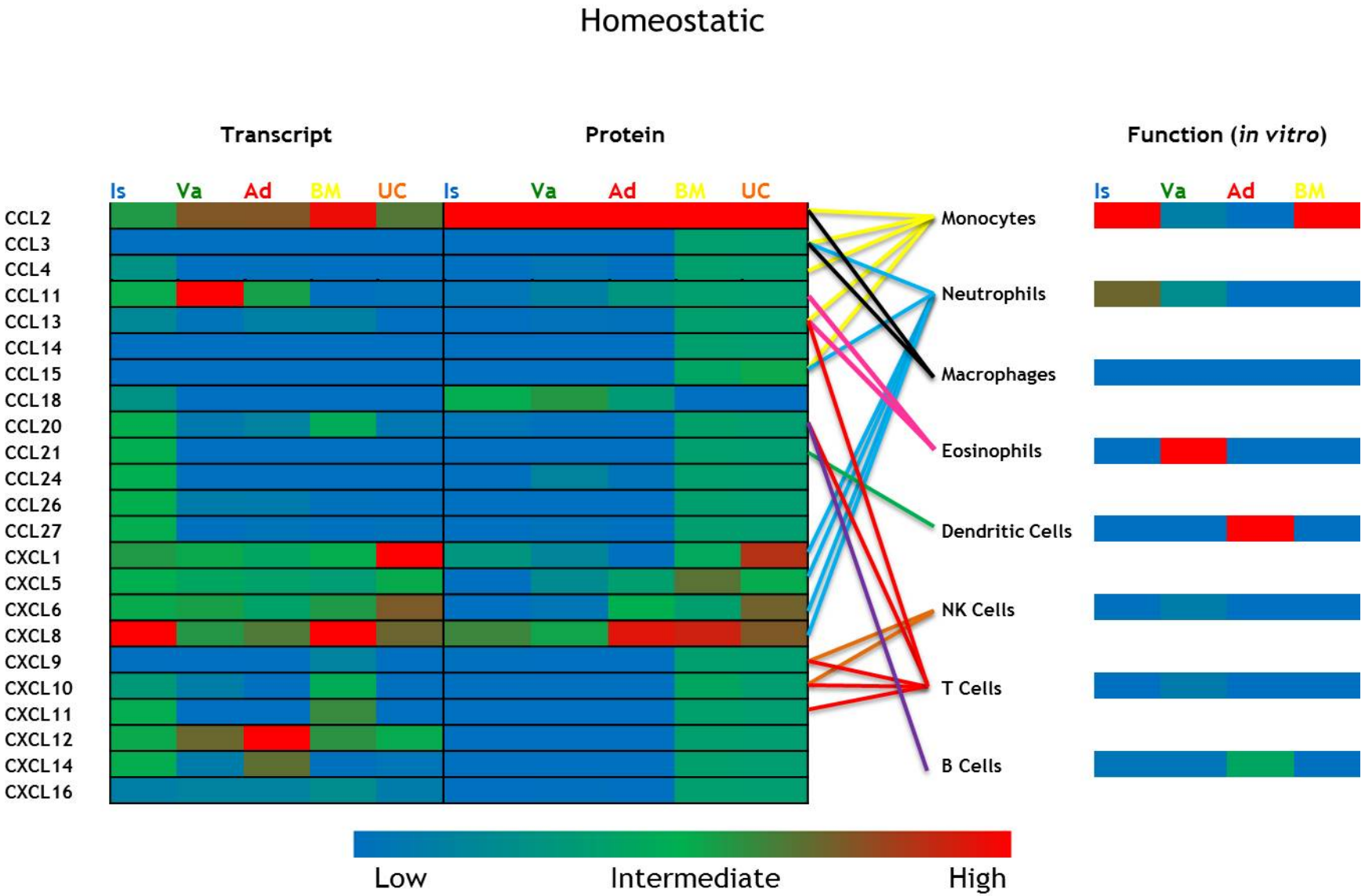


Figure 7-2 Overview of chemokine receptor expression by MSCs at a transcript and protein level under homeostatic and inflammatory stimulation.

Chemokine receptor expression by MSCs isolated from the Is Va, Ad, BM and UC were compared to each other in homeostatic (top) and inflammatory (bottom) conditions to understand which tissue source of MSC expressed the highest levels of CCR7, CCR10, CXCR4 and CXCR6 at a transcript (left) and protein (right) level. Colour coding highlights tissue sources that express receptors at the highest (red) intermediate (green) and lowest (blue) levels (blank boxes depict receptors that were not tested). In homeostatic conditions, Va MSCs expressed the highest protein levels (red boxes) of CCR7 and CXCR4, suggesting that visceral adipose might be the preferred tissue to isolate MSCs to enhance migration towards the lymph node (CCR7) and bone marrow, heart and lungs (CXCR4) (highlighted by green lines). Conversely, BM MSCs expressed the highest surface levels of CXCR6 and CCR10 (red boxes), suggesting that the BM might be the desired source of MSCs to enhance trafficking to the liver and skin, respectively. After inflammatory stimulation, the BM would be the optimal tissue for MSC isolation for enhanced migration to the listed tissues. ACKR3 is not involved in migration and therefore is not listed. Importantly, this summary diagram highlights that transcriptional expression of receptors by MSCs does not correspond to protein expression and highlights a need for rigorous testing of receptors at a protein level. Transcript data shown here is summarised from Chapter 4, Figure 4-7 and Figure 4-8 . Protein data shown here is summarised from Chapter 5, Figure 5-1.

The *in vitro* experiments in this study also highlighted the influence of MSC tissue origin in dictating how they might interact with their surrounding environment when infused into a patient. In homeostatic conditions, all MSCs had a similar chemokine secretion profile, where CCL2 was the top chemokine secreted by all MSC populations. However, the immune cell attraction profile of MSCs differed drastically where Is and Va MSCs attracted substantially more immune cells than BM and Ad, which did not attract any above background levels. Inflammatory stimulation resulted in an upregulation of CCL2 secretion as well as a huge shift towards the secretion of ELR +ve and ELR –ve CXC chemokines. The secretion of these CXC chemokines was met with the specific chemoattraction of neutrophils towards all tissue sources of MSCs, however, the number of cells attracted towards MSCs varied considerably depending on MSC tissue origin, where Is MSCs attracted substantially more than BM MSCs, which attracted the least. This has huge clinical implications as inflammatory chemokine secretion and monocyte, macrophage and neutrophil attraction are largely viewed as detrimental mediators in a number of clinical settings. An overview of the chemokines MSCs expressed at a transcript and protein level and subsequent immune cell attraction by MSCs under homeostatic and inflammatory conditions is outlined in Figure 7-3.


Homeostatic
Transcript
Protein
Function (in vitro)
Is
Va
Ad
BM
UC
CCL2
CCL3
CCL4
CCL11
CCL13
CCL14
CCL15
CCL18
CCL20
CCL21
CCL24
CCL26
CCL27
CXCL1
CXCL5
CXCL6
CXCL8
CXCL9
CXCL10
CXCL11
CXCL12
CXCL14
CXCL16
Monocytes
Neutrophils
Macrophages
Eosinophils
Dendritic Cells
NK Cells
T Cells
B Cells
Low
Intermediate
High

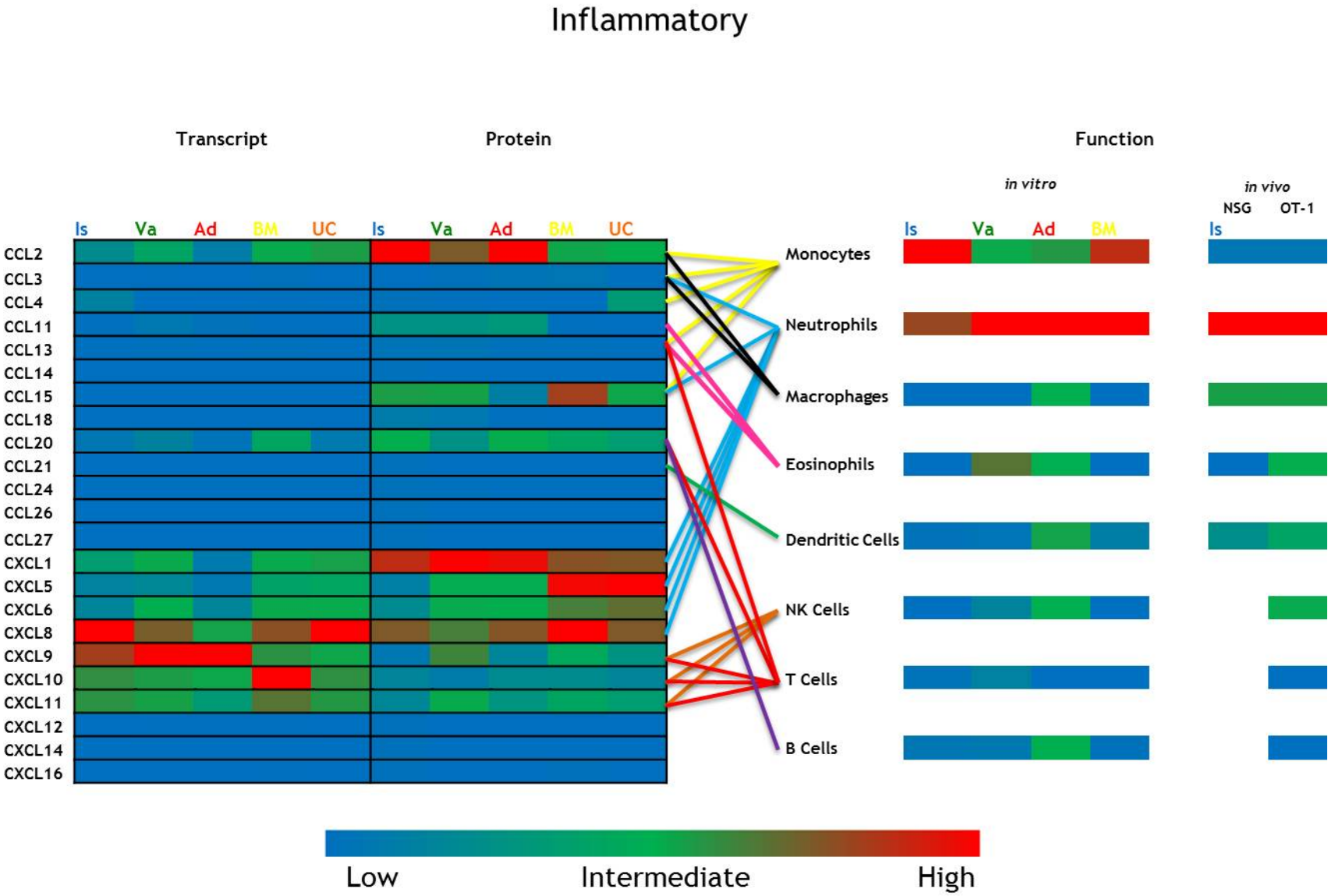

Inflammatory
Transcript
Protein
Function
in vitro
in vivo
NSG
OT-1
Is
Va
Ad
BM
UC
CCL2
CCL3
CCL4
CCL11
CCL13
CCL14
CCL15
CCL18
CCL20
CCL21
CCL24
CCL26
CCL27
CXCL1
CXCL5
CXCL6
CXCL8
CXCL9
CXCL10
CXCL11
CXCL12
CXCL14
CXCL16
Monocytes
Neutrophils
Macrophages
Eosinophils
Dendritic Cells
NK Cells
T Cells
B Cells
Low
Intermediate
High

Figure 7-3 Summary of the CC and CXC chemokines MSCs transcribed, secreted and subsequent immune cell attraction under homeostatic and inflammatory conditions.
A colour coding system was generated to highlight the top transcribed (left hand box) and secreted (right hand box) chemokines by each tissue source of MSC in homeostatic (top) and inflammatory (bottom) conditions. Generally, where low transcription was observed (blue), no chemokine was secreted (blue) under both homeostatic and inflammatory conditions. Equally, if the gene was transcribed at high levels (red) it was often secreted as a protein in high quantities (red/brown). The immune cells that each chemokine could potentially attract have been highlighted by colour coded lines that match specific immune cells. For example, CCL3 &15, CXCL1, 5, 6 & 8 are involved in neutrophil chemoattraction, highlighted by the blue line joining the chemokines to the target cell – neutrophils.

The same colour coding system was implemented (blue-red) based on the number of immune cells MSCs attracted as a percent of the input population, highlighting which immune cells MSCs preferentially attracted *in vitro*. As a worked example: in homeostatic conditions (top box), Is MSCs transcribed and secreted high levels of CCL2 and CXCL8, which in turn attracted high numbers of monocytes and neutrophils, respectively. Under inflammatory stimulation, Is MSCs transcribed high levels of CXCL8 and 9 and moderate levels of CXCL1 and CCL2. Is MSCs secreted the highest levels of CCL2, followed by CXCL1 and CXCL8, whereas CXCL9 was secreted at lower levels. Mirroring the chemokine secretion profile, Is MSCs also attracted large numbers of monocytes and neutrophils, however, T cell and NK cell attraction was not observed. Additionally, pre-inflamed Is MSCs attracted substantial numbers of neutrophils and lower numbers of monocytes *in vivo* in both NSG and OT-1 mouse models. However, it is likely that the monocytes had differentiated into macrophages which were attracted towards Is MSCs at moderate levels *in vivo.*

7.4 Hypothesis

I therefore hypothesise that the tissue origin of MSCs would highly influence MSC *in vivo* behaviour via the differential expression of chemokine receptors and the differential secretion of chemokines and immunomodulatory mediators, which has a direct impact on how MSCs interact with surrounding immune cells. Based on these observations, I believe that it is highly likely that MSC tissue origin predisposes MSCs as being more anti- or pro- inflammatory. Moreover, the current study focussed on the differences in MSC migratory capacity and how they interact with their surrounding environment, however I believe that there will be other undiscovered profound differences between MSCs isolated from different tissue sources that will have a direct impact on their clinical abilities.

7.5 Conclusions

This study provides novel, clinically relevant insights into the phenotypical and functional behaviours of MSCs isolated from the islet, visceral adipose, adipose, bone marrow and umbilical cord. The findings described in this thesis also highlight the extensive difference in MSC behaviour which is dependent upon the tissue origin of MSC and the surrounding environment – homeostatic or inflammatory. To conclude, I will answer the questions outlined in the introduction of this chapter:

1) **Do Is, Va, Ad, BM and UC MSCs have the same phenotype?** With the exception of HLA surface molecule expression, tissue origin does not appear to affect MSC surface molecule phenotype, where, Is, Va, Ad, BM and UC express similar levels of MSC surface markers and present as identically-sized and spindle-shaped adherent cells.

2) **Where could MSCs potentially migrate to and does the tissue origin of MSCs impact this?** Based on the transcript and protein expression of chemokine receptors, MSCs could migrate to the bone marrow, lung, heart, kidneys and lymph nodes and the tissue origin of MSC affects the surface expression of chemokine receptors suggesting that particular tissue sources might be more beneficial to improve homing to specific anatomical sites.

3) **How do MSCs interact with surrounding target (or off-target) tissue(s) and does the tissue origin of MSC impact their behaviour?** MSCs appear to have a chemokine secretion phenotype, where all tissue sources of MSCs secrete CCL2 at moderate levels in homeostatic conditions. This could act as an angiogenic mediator and depending on the tissue source of MSC, could attract monocytes (Is MSCs). After inflammatory stimulation, all tissue sources of MSCs upregulate neutrophil chemoattractants, which was mirrored by an upregulation in neutrophil migration towards all MSCs, however, the tissue origin of MSC dictates the quantities of neutrophils attracted. The immune cell attraction profile of Is MSCs was mirrored exactly *in vivo* in two different mouse strains suggesting that Is MSCs specifically attract neutrophils. Moreover, immunomodulatory mediators are transcribed in homeostatic conditions and upregulated under inflammatory stimulation. The levels of transcription of these mediators are dependent on the tissue origin of MSCs, suggesting that specific tissue sources might possess greater immunomodulatory capacity on the differential numbers of immune cells they attract and therefore highlights that the tissue origin of MSCs predisposes them to have an anti- or pro-inflammatory phenotype.

These data highlight both similarities and differences in MSCs isolated from the Islet, visceral adipose, adipose, bone marrow and umbilical cord. Despite MSCs isolated from various tissues phenotypically presenting as identical cells- based on surface CD molecule phenotype - their *in vivo* function could potentially be very different and this could have huge impact in a number of clinical settings.

7.6 Future Direction

Despite the studies in this thesis revealing some novel and interesting data, the experiments described throughout are not without their limitations and would benefit from more rigorous testing of the systems involved.

To confidently address MSC migratory potential, experiments on the chemokine receptors expressed by MSCs and their function must be thoroughly tested. To assess this, receptor functionality should be tested by intracellular calcium flux assays which would assess if receptors are able to signal upon ligand binding. Moreover, MSC migration assays would test the migratory potential of MSCs *in*

vitro. For example, MSC migratory capacity towards CXCL16 would have to be assayed and compared against other sources of MSCs in order to confidently conclude which tissue source of MSCs possessed greater migratory capacity. Specific migration could be tested via a dose response of MSCs towards increasing concentrations of CXCL16 or via a CXCR6 blocker in a transwell system. Additionally, *in vivo* trafficking and retention of MSCs within specific sites should be address to confidently conclude that these systems are functional *in vivo*. Fluorescent labelling of MSCs, coupled with targeted knock out of a specific MSC chemokine receptor would thoroughly evaluate the chemokine receptors involvement of MSC trafficking to specific sites.

Additionally, despite the chemokines that MSCs secreted matching the immune cells that MSCs attracted *in vitro* and *in vivo*, with an upregulation in the magnitude of response after inflammatory stimulation, it is important to regard the shortcomings of these experiments. Although different tissue sources of MSCs secreted varied levels of chemokines, the levels of chemokine secreted did not mirror the number of immune cells MSCs attracted. For example, CXCL5, 6 and 8 were secreted by Ad MSCs in homeostatic conditions, however, neutrophil chemoattraction here was not observed. This could be evidence to support that Is MSCs attract neutrophils via alternative mechanisms such as VEGF – VEGFR or GM-CSF- GMCSFR. Moreover, Ad MSCs secreted high volumes of CCL2, however, monocyte chemoattraction *in vitro* was not observed. Thus to ensure that monocyte attraction towards MSCs was mediated through CCR2 and that neutrophil attraction was mediated via CXCR1/2 or VEGFR, transwells should be repeated with the appropriate receptor blockers to fully confirm that the chemokines MSCs secrete are fully involved in the immune cell attraction profile we observe.

Although the *in vivo* air pouch model in two individual strains of mice mirrored the exact immune cell attraction profile of MSCs *in vitro*, due to availability of mice, the air pouch model did not have fully adequate controls and therefore the data generated are limited. Assessing the immune cell infiltration towards another cell type with a different chemokine secretion profile such as fibroblasts or keratinocytes in the air pouch model would serve as an appropriate control to confidently conclude that the immune cell attraction into the air pouch was

specific to Is MSCs secreted mediators. To strengthen the air pouch data, the experiment could benefit from the addition of extra time points. In spite of the absence of adaptive immune cell attraction towards MSCs *in vitro*, it is likely that the time point of 24 hours did not allow the full assessment of adaptive immune cell infiltration *in vivo*, as T and B cells would likely infiltrate at a later stage. It would therefore be advantageous to have 2 time points at 24 and 72 hours to assess the infiltration of the innate and adaptive immune systems.

This thesis would also benefit from a full *in vivo* comparison of the immune cell attraction profile of homeostatically vs. inflammatory maintained Is, Va, Ad, BM and UC MSCs into the air pouch, to further confirm and clarify the substantial differences observed in the *in vitro* system. Subsequently, a full phenotypical analysis of the immune cells MSCs attract would strengthen the hypothesis that MSCs isolated from various tissues are pre-disposed to being more pro- or anti-inflammatory.

Lastly, and perhaps beyond the scope of this thesis, this study would greatly benefit from the assessment and comparison of MSCs isolated from various tissues in different clinical models such as mouse islet transplantation and tumour models. This work would clarify if MSCs isolated from different tissues perform differently in clinical settings and would also highlight if the immune cell attraction profile of MSCs remains consistent in varied environments. This could underpin a vital role of MSC immune cell attraction in different clinical settings and further clarify the differences in MSCs isolated from various tissu

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