University of Glasgow

Investigating the Orchestration of the Inflammatory Response: A Role for Chemokine Receptors CCR1, CCR2, CCR3 and CCR5

Robin Bartolini

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

June 2019

University of Glasgow

Institute of Infection, Immunity and Inflammation

120 University Place

G12 8TA

Scotland

Acknowledgments

First and foremost, I would like to thank my undergraduate lecturer and, eventually, PhD supervisor Prof. Gerard Graham, for the support, patience, trust and freedom to pursue interesting side-projects throughout the course of my PhD. This thesis would have been a lot shorter had it not been for Dr. Laura-Medina Ruiz's and Dr. Doug Dyer's (aka, the Dream Team's) unwavering support and carrot and stick approach. I can still hear the whip cracking on my bruised ego. I am also sure Laura will have cuminic PTSD for a good while after I am gone. I would also like to thank the Wellcome Trust for providing the funding that allowed me to undertake this project.

A special thank you to all members of the CRG, working in this group has been fantastic and everyone has always been willing to help, either with experiments or as emotional support. Honourable mentions include: Masterchef Dr. Kelly, PokeMaster Dr. Hayes, Her Majesty Dr. Burgoyne, Dr. Bonita Vidler and our formidable lab manager Dr. Catherine 'Where's Your Lab Coat?' Hughes. A special thanks must go to fellow PhD students Jenny and Sam, this journey wouldn't have been as much fun without you. After the Isle of Skye, Blackpool, the Rough Mudder, Sexy Suzy and legs on the menu, I am sure I will never find better partners in crime.

This work would not have been possible without the psychological/ financial/ emotional/ spiritual support of my parents Lorella and Alberto, my grandmother Nerina and my late grandfather Alfredo. Special thanks also go to my brother, V-dawg, and my Italian friends Giacomo, Francesca, Sara and Maria. Holidays back home would not have been as relaxing without you and seeing you guys was definitely one of the reasons why I am still sane after 4 years of chemokines!

An even bigger thank you goes to my partner Nisar. Chemokines, two PhDs and thousands of kilometres are apparently no big deal, and he's stood by me and supported me the whole way through. Finally, a massive thank you to Scotland, who was my home away from home for nearly a decade. People truly do make Glasgow [tm].

Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature

Printed Name: Robin Bartolini

Abstract

Despite over 20 years of pharmaceutical targeted research, no antagonist of inflammatory chemokine receptors (iCCR) has been licensed for use in treating inflammatory diseases. Marked promiscuity of ligand binding, simultaneous expression of multiple chemokine receptors in individual leukocytes and the inability to generate compound receptor knock out mice make it almost impossible to discern the function of a specific iCCR (especially for inflammatory CC chemokine receptors CCR1, CCR2, CCR3 and CCR5).

To address this complexity, two novel mouse models have been recently developed at the Chemokine Research Group: a full iCCR$^{-/-}$ strain in which the whole chromosomal CC-iCCR cluster has been deleted, (resulting in a compound CCR1, CCR2, CCR3 and CCR5$^{-/-}$ mouse) and a novel iCCR fluorescent reporter strain, which expresses specific fluorescent proteins under the endogenous iCCR promoters.

Experiments on the iCCR$^{-/-}$ have provided a 'clean slate' to assess the development of the immune response in the absence of CCR1, CCR2, CCR3 and CCR5, while studies on the iREP strain allowed for direct visualisation of changes in iCCR expression on the surface of several leukocyte subsets.

The findings presented in this thesis not only validate our current understanding of the crucial roles of CCR2 and CCR3 in the mobilisation of monocytes and eosinophils from the bone marrow to the site of inflammation, but also provide clues on the role of CCR1 in monocyte trans-endothelial migration and describe a novel role for iCCRs in the proliferation and differentiation of dendritic cells and macrophages *in vitro*.

More studies on these new iCCR mouse models will provide clarity on our current understanding of the orchestration of the CC-chemokine driven inflammatory responses, and the results will not only increase our understanding of basic chemokine biology, but could also inform future pharmacological intervention on the chemokine system.

List of Abbreviations

ACK	Ammonium-Chloride-Potassium
ACKR	Atypical Chemokine Receptor
AF	Alexa Fluor
AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen Presenting Cell
APC	Allophycocyanin
BAC	Bacterial Artificial Chromosome
BCR	B-cell Receptor
BM	Bone Marrow
BOS	Bronchiolitis Obliterans Syndrome
BV	Brilliant Violet
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CD	Cluster of Differentiation
CFA	Complete Freud's Adjuvant
CM	Conditioned Media
CO_2	Carbon Dioxide
CpG	CpG oligodeoxynucleotides
CSF-1	Colony Stimulating Factor 1
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
Cy	Cyano Dye
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
EDTA	Ethylemediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ES	Embryonic Stem Cell
FACS	Fluorescence-Activated Cell Sorting

FBS	Foetal Bovine Serum
FcR	Fragment Crystallisable Region
FITC	Fluorescein Isothiocyanate
FLT3L	FMS-like Tyrosine Kinase 3 Ligand
FoxP1	Forkhead Box P1
FSC	Forward Scatter
GAG	Glycosaminoglycans
GDP	Guanine Diphosphate
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GMEM	Glasgow's Minimum Essential Medium
GPCR	G-Protein Coupled Receptor
GTP	Guanine Triphosphate
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
ICAM	Intercellular Adhesion Molecule
iCCR	Inflammatory C-C motif Chemokine Receptor
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
iREP	inflammatory C-C motif Chemokine Receptor Reporter
IRFP	Infrared Fluorescent Protein
IS	Immunological Synapse
Kda	Kilodalton
KO	Knock-out
KSHV	Kaposi's Sarcoma Associated Herpesvirus
LAD	Leukocyte Adhesion Deficiency
LDLr	Low-Density Lipoprotein Receptor
LFA	Lymphocyte Function-Associated Antigen
LIF	Leukaemia Inhibitory Factor
LPS	Lipopolysaccharide
Mac-1	Macrophage-1 antigen
MFI	Mean Fluorescent Intensity
MHC	Major Histocompatibility Complex

mo-DC	Monocyte-Derived Dendritic Cell
MS	Multiple Sclerosis
mTagBFP3	Molecular Tag Blue Fluorescent Protein
NK	Natural Killer Cell
NSAID	Nonsteroidal Anti-Inflammatory Drug
OVA	Ovalbumin
PASI	Psoriasis Area and Severity Index
PBMC	Peripheral Blood Monocytes
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythin
PECAM	Platelet Endothelial Cell Adhesion Molecule
PerCP	Peridinin Chlorophyll Protein Complex
PFA	Paraformaldehyde
Poly I:C	Polyinosinic:polycytidylic acid
PPD	Purified Protein from *M. Bovis*
PT	Pertussis Toxin
RA	Rheumatoid Arthritis
RBC	Red Blood Cells
RiKO	Reversible iCCR Knock-Out
RNA	Ribonucleic Acid
SD	Standard Deviation
SEA	*S. mansoni* Egg Antigen
SSC	Side Scatter
SubC	Subcutaneous
TCR	T-cell Receptor
TEM	Transendothelial Migration
TH1	T-cell Helper 1
TH2	T-cell Helper 2
TLR	Toll-like Receptor
TNF	Tumour Necrosis Factor
vCKBP	Viral Chemokine Binding Protein
VEGF	Vascular Endothelial Growth Factor
WT	Wild Type

List of Figures and Tables

Table of Contents

1 Introduction

1.1 Overview

The development of an effective immune response in a tissue requires coordinated and sequential interactions between leukocytes at the site of inflammation and other immune cells in specialised anatomical compartments. This complex web of interactions between different cell types throughout the body is largely controlled by chemokines, small (8-12kDa) highly conserved cytokines capable of inducing migration of leukocytes to and from tissues[1].

While leukocyte recruitment to the site of infection is a requirement for effective pathogen clearance, aberrant immune recruitment can be the trigger for inflammatory conditions such as allergies, or for chronic inflammatory systemic diseases like rheumatoid arthritis or multiple sclerosis which can ultimately result in life-long debilitating illness, increased mortality and high costs for therapy[2].

Current treatments for inflammatory conditions heavily rely on either glucocorticoids or non-steroidal anti-inflammatory agents (NSAIDs) which have broad inhibitory effects on a wide range of immune responses. While effective, this non-specific approach weakens the whole immune system, leaving patients vulnerable to infections[3].

The discovery of chemokines and their receptors in the 1980s raised the hopes for a more specific approach to treat inflammatory conditions. In theory, it should be possible to interfere with the chemokine system and prevent the recruitment of the leukocytes responsible for a particular pathology to the site of inflammation without affecting other leukocyte subsets. However, despite over 25 years of pharmaceutical targeted research, no antagonist of inflammatory chemokine receptors has been licensed for use in treating inflammatory diseases. This failure as been attributed to an incomplete understanding of the inflammatory chemokine receptor system and confounding effects attributable to the promiscuity of each receptor[4].

1.1.1 Classification of the Chemokine Family

Chemokines (***chemo***tactic cyto***kines***) are defined by structure, and can be divided in two large and two small subgroups depending on the number and arrangement of conserved cysteine motifs in their mature sequence[5]. Even though sequence identity between chemokines varies from about 20% to 90%, their structure overall is highly conserved and most chemokines essentially adopt the same fold[5]. The general chemokine structure consists of a flexible N-terminus and N-terminal loop, followed by a three-stranded antiparallel β-sheet on to which is folded a C-terminal α-helix. Intramolecular disulphide bonds typically join the highly conserved cysteine residues, maintaining the structural integrity necessary for binding to their respective receptor (Figure 1-1)[6].

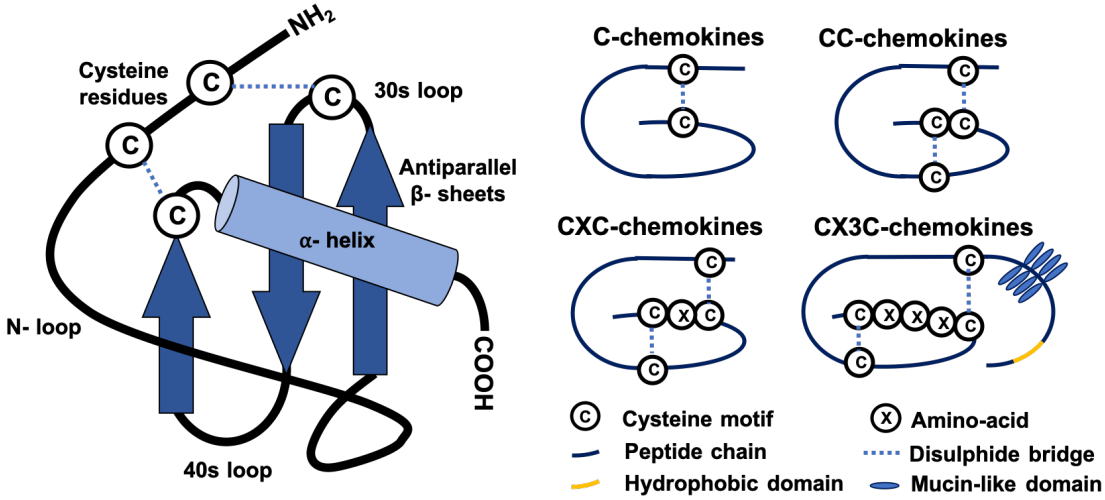

Figure 1-1 General Chemokine Structure- *Graphical representation showing how α-helix, β-sheets and disulphide bonds between cysteine residues interact to give a chemokine its tertiary structure. The figure also shows the position of the cysteine residues in the 4 chemokine subfamilies: C-, CC-, CXC- and CX3C chemokines. Figure adapted from 'Chemokines' by Dr. Habil Kohidai.*

Initially, chemokines were grouped in two major subgroups: the CXC and CC-chemokine families, according to the presence or absence of an amino acid (X) between a pair of cysteine residues near the amino terminus of the protein. As a general rule, members of the CXC chemokines were thought to be chemotactic for neutrophils while the CC-chemokines were found to be chemotactic for monocytes and small subsets of lymphocytes[7]. For example, Interleukin-8 (CXCL8) was regarded as the typical CXC chemokine whereas CCL2, which attracts monocytes and T-cells, was regarded as the typical CC chemokine[8].

Rapid advancement in automated sequencing of human and mouse genomes has uncovered numerous other chemokines [9], expanding the chemokine family and adding two novel subgroups: C-type chemokines that lack the first and third cysteine residue, and CX3C chemokines that have three amino acids between the first two cysteine residues. The only CX3C chemokine discovered to date, CX3CL1, has an extended mucin-like stalk and a transmembrane domain which can be cleaved[10]. This feature allows the chemokine to bind to the surface of certain cells, thereby serving both as a chemoattractant and as an adhesion molecule[11].

Based on expression pattern and function, chemokines can also be grouped as 'inflammatory' or 'homeostatic' according to the biological contexts in which they function. Inflammatory chemokines are typically dramatically induced during an immune response to recruit inflammatory leukocytes to sites of infection or tissue damage. Examples include CCL2, CCL3, CCL5, CXCL1, CXCL2 and CXCL8. In contrast, homeostatic chemokines are continuously secreted at low levels and are involved in controlling migration of cells during normal processes of tissue maintenance or development. Examples of homeostatic chemokines include CCL19, CCL21, CCL27, CXCL12 and CXCL13 [12]–[14].

Nevertheless, classification via structure is generally preferred, as some chemokines can be both pro-inflammatory and homeostatic under different physiological conditions. For example, CCL21, is constitutively expressed on the luminal side of high endothelial venules (HEVs), allowing direct access to lymphoid organs by naïve T and B-cells under homeostatic conditions[15]. However, CCL21 can also be superinduced on afferent lymphatics by inflammatory stimuli[16], thereby promoting recruitment of mature dendritic cells to the draining lymph node and the initiation of the adaptive immune response.

1.2 Chemokine Receptor Structure and Regulation

1.2.1 Chemokine Receptor Structure

Chemokines themselves are recognised by chemokine receptors, G-protein coupled receptors (GPCRs) with 7 transmembrane domains predominantly found on the surface of leukocytes. The human chemokine superfamily currently includes at least 46 ligands, which bind to 18 functionally signalling chemokine receptors and four decoy or scavenger

receptors[17]. Chemokine receptors are denoted by the motif of their ligands: CC chemokine receptors only bind to CC chemokines, CXC chemokine receptors bind to CXC chemokines. Chemokine receptors also share particular features: they measure approximately 350 amino acids in length, have a short acidic extracellular N-terminus which has been shown to be crucial for the initial recognition and selective chemokine binding[18] and an intracellular C-terminus containing serine and threonine residues that act as phosphorylation sites for receptor regulation[19]. The receptor's structure is stabilised by the formation of disulphide bonds between highly conserved cysteines in the first and second extracellular loops, while the G-proteins required for signal transduction are coupled through the C-terminus segment and the intracellular loops[20] (as seen in Figure 1-2). Disulphide bonds also link the N-terminus to the third extracellular loop, forcing the membrane spanning helices into a stable circular conformation (extracellular view, figure 1-2)[18].

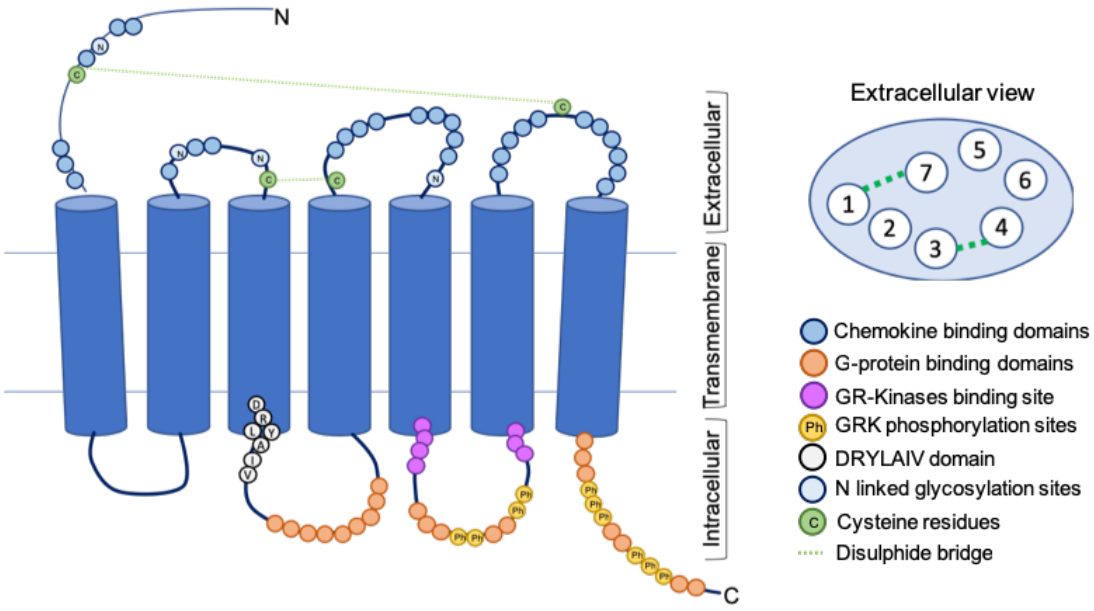

Figure 1-2 General Structure of a CC-chemokine receptor. *The extracellular domain of the receptor is responsible for chemokine engagement. As the N-terminus of the ligand binds to the receptor pocket, a conformational change is induced in the transmembrane region, exposing the DRYLAIV motif. The newly exposed DRYLAIV domain allows GPCR coupling to G-proteins, and the intracellular domain amplifies the signals, initiating a signalling 'domino effect' of secondary messengers that eventually reaches the nucleus and changes gene expression. Disulphide bridges between cysteines stabilise the three-dimensional structure of the receptor. Adapted from Atlas Genetics Ontology and Textbook of Receptor Pharmacology (3rd Edition).*

1.2.2 Chemokine Receptor Activation

Numerous studies have investigated the molecular mechanisms of chemokine receptor binding and activation, leading to the discovery of two distinct regions of chemokines that interact with disparate regions of their receptors. Ultimately, this discovery lead to the two-site, two-step model as a general paradigm for chemokine-receptor interactions [21] [22][23]. This model has been refined recently, with groups proposing a 'three step' model of engagement for specific receptors, such as CCR1 [24] or CXCR4 [25].

In the first step, the core globular domain of a chemokine binds to the N-terminal extension and the second extracellular loop of its receptor. These regions, which are called the chemokine recognition sites, define receptor specificity and affinity[26]. In the second step, the flexible N-terminus of the chemokine interacts with the 'major ligand binding pocket' buried in the cavity formed by the circular arrangement of the receptor's transmembrane domains[24]. This induces an overall change in the receptor conformation that initiates signal transduction.

1.2.3 Chemokine Receptor Signalling

Upon binding of the chemokine to a cognate chemokine receptor, the receptor undergoes a conformational change exposing the highly conserved DRYLAIV domain in the second intracellular loop. The exposed motif allows the receptor to bind to heterotrimeric G-proteins required for signal transduction (Gα, Gβ and Gy subunits)[28]. The interaction with the receptor's intracellular domains activates the G-proteins by stripping the low energy guanidine diphosphate (GDP) from the Gα subunit and allowing guanidine triphosphate (GTP) to bind to it instead[29].

This change forces the separation of the Gα subunit from the Gβ and Gy complex, which can now interact with other signalling effectors such as GPCR kinases (GRKs), ion channels, and phospholipase C-β (PLC-β). The interaction between active G-protein subunits and signalling effectors leads to the production of second messengers.

For example, phospholipase C (PLC) cleaves PIP2 (phosphatidylinositol-bisphosphate) into two second messenger molecules, inositol triphosphate (IP3) and diacylglycerol (DAG), which further propagate the signal transduction pathway [30]. Second messenger IP3 diffuses into the endoplasmic reticulum and binds to the IP3 receptor. The IP3 receptor functions as a calcium channel, and IP3 binding results in the release of calcium ions from the endoplasmic reticulum, which in turn bind to Protein Kinase C (PKC) and activate it[31]

Similarly, increased concentrations of second messenger DAG can also activate PKC directly. DAG/Ca^{2+}-activated PKC in turn activates PI3K, a family of enzymes involved in a variety of cellular functions, including cell growth, motility and survival. PI3K activation phosphorylates AKT (also known as Protein Kinase B) which has a variety of downstream effects, such as increasing the activity of cellular transcription factor CREB[32], inhibiting cell cycle inhibitor protein p27 and recruiting FOXO proteins (a family of transcription factors important in the regulation of cell growth, differentiation and proliferation)[33].

Furthermore, PKC can also activate the NK-kB pathway directly through an IκB-independent cytosolic interaction, which subsequently leads to enhanced p65 phosphorylation and DNA binding affinity [34].

PI3K can also synthesise PIP3 by phosphorylating PIP2 [35]. An increase in PIP3 results in the localised recruitment of signalling proteins containing PIP3-pleckstrin homology (PH) domains[36], proteins that drive actin polymerisation and morphological changes at the leading edge of the cell, causing polarisation and mediating migration towards the highest concentration of chemokine[37].

Although these signalling events are common to all chemokine receptors, it is well known that the activation of further downstream pathways is quite different. Ligand affinity, engagement of multiple receptors simultaneously or even the differentiation state of a leukocyte itself can affect internal chemokine receptor signalling[38]. As such, different leukocytes will respond the same chemokine in different ways, adding an additional level of complexity to understanding the unique role of each receptor.

1.2.4 Chemokine Receptor Regulation

Chemokine receptor signalling can be regulated by the process of receptor internalisation and desensitisation: a feedback mechanism which protects cells from ligand overstimulation[39]. Following chemokine-chemokine receptor binding and release and activation of the G protein subunits, G-protein coupled receptor kinases phosphorylate the receptor's intracellular domains, enhancing their ability to bind to β-arrestins. This reaction induces a conformational change that releases β-arrestin's C-terminal tail which contains binding sites for a variety of endocytic proteins, including trafficking regulators such as clathrin and the clathrin adaptor AP2 [40].

Thus, β-arrestins act as scaffolding intermediates with components of the endocytic machinery, stabilising the receptor's association with clathrin-coated pits (CCPs) and targeting it for internalisation via endosomes for lysosomal degradation[41]. In some cases, endocytosed receptors are dephosphorylated by endosomal-associated phosphorylases and recycled back to the cell surface in a process called resensitisation[42].

Different chemokines active at a given receptor may have different effects on its internalization: CCR1, CCR3 and CCR5 are efficiently internalized by CCL5, but the trafficking of the receptors follows different pathways. CCR5 is entirely recycled back to the cell surface[43], CCR3 is partially degraded and recycled[44], while CCR1, once internalised, cannot be recycled and has to be synthesised *de novo*[45]. The strength and stability of the interaction between the chemokine receptor and β-arrestins appears to be critical in determining if a chemokine receptor is internalised: receptors that preferentially bind to β -arrestin 2 with low affinity and dissociate from it upon internalization are rapidly recycled back to the cell surface, while receptors that bind both β-arrestins 1 and 2 with high affinity and remain β-arrestin-bound inside the cell and are eventually degraded[46].

1.2.5 Atypical Chemokine Receptors

Conventional chemokine receptors are not the only receptors capable of binding chemokines. At least four other receptors exist: ACKR1, ACKR2, ACKR3 and ACKR4[47]. These molecules structurally resemble conventional chemokine receptors but cannot

initiate classical chemokine receptor signalling responses upon ligand binding as they lack the canonical DRYLAIV motif necessary for G-protein coupling and induction of classical signalling pathways[48][49]. This led to their exclusion from the systematic chemokine receptor nomenclature and they are thus referred to as atypical chemokine receptors (ACKRs).

An important GPCR feature retained by atypical chemokine receptors is their ability to efficiently internalize their chemokine ligands, affecting chemokine availability and shaping chemokine gradients during an immune response. For example, ACKR2 displays promiscuous binding of inflammatory CC chemokines and acts as a scavenger receptor for its ligands, internalising them and targeting them for intracellular destruction. As exaggerated inflammation is seen at all sites of normal ACKR2 expression in *Ackr2*$^{-/-}$ mice [50], this suggests ACKR2 has an important role in the resolution of chemokine-driven inflammatory responses in the tissues in which it is expressed[48].

The fate of internalised ligands is not the same for every atypical receptor. Upon internalisation, ACKR1 does not target its ligands for lysosomal degradation, but instead transports the chemokines across biological barriers in a process called transcytosis[51]. ACKR1 is thus part of an active chemokine transendothelial transport mechanism by which soluble chemokines are intercepted and carried to other microanatomical domains, altering chemokine gradients and at the same time preventing the spreading of chemokine-mediated inflammatory stimulation into distal organs[52].

Due to their role in regulation of chemokine availability in inflamed tissues, atypical chemokine receptors tend to be expressed on the surface of non-leukocyte cell types, such as erythrocytes, lymphatic or vascular endothelial cells, ensuring tight control of chemokine availability necessary to attain gradients for efficient leukocyte recruitment and for resolution of inflammation[53].

1.3 General Role of Chemokine Receptors

Leukocyte migration requires multiple chemokine-mediated signals for both migration to, and permanence within, the site of inflammation. In broad terms, an infectious or inflammatory stimulus rapidly triggers the release of alarmins and other cytokines which cause localised activation of epithelial and other tissue resident cells, including vascular endothelial cells. In response to inflammation, these endothelial cells upregulate expression of several leukocyte binding proteins, including intercellular adhesion molecules ICAM-1, ICAM2, vascular cellular adhesion molecules (VCAM), as well as members of the selectin family of adhesion molecules, E and P-selectins[54]. P-selectins are found in endothelial cells and platelets and are stored intracellularly. Exposure to inflammatory mediators such as histamines, TNFs or LPS induces their rapid translocation to the plasma membrane in as little as two minutes[55]. On the other hand E-selectins are produced 'on demand', with peak expression happening just four hours after the initial inflammatory insult[56].

1.3.1 Arrest in Shear Flow

E-selectins mediate initial low affinity interaction between leukocytes and the walls of blood vessel, a process described as leukocyte rolling. This slows down the leukocyte, increasing its mechanical resistance to shear stress and reducing its velocity in the blood stream from 1,000-4,000 μm/s to 5-40 μm/s [57]. This 100-fold decrease in speed allows the leukocytes to further sample the vascular walls for other signals.

Epithelial cells and tissue resident leukocytes exposed to an inflammatory stimulus also rapidly release pro-inflammatory cytokines and various chemokines, which diffuse from the tissue and enter circulation. While the blood carries a fraction of these chemokines away from the site of inflammation, ultimately inducing migration and activation of distal leukocytes residing in the periphery, chemokines are also retained at the site of inflammation by binding to glycosaminoglycans (GAGs) on the surface of endothelial cells[58].

1.3.2 Interaction with Glycosaminoglycans (GAGs)

GAGs are long, linear polysaccharides which display varying patterns of sulphation, which in addition to carboxyl groups, give these proteins one of the highest negative charge densities of any known biological macromolecules[59]. This negative charge is a critical determinant of chemokine binding, as chemokines are generally positively charged[60] and will bind to GAGs via electrostatic interactions[59].

As a result, leukocytes rolling along the vasculature encounter clusters of GAG-immobilised chemokines, recognised by specific sets of chemokine receptors on the leukocyte's surface. Engagement of the chemokine receptors initiates a signalling cascade which, in a few milliseconds[61], causes surface integrin molecules to switch from a default 'low affinity' state to a 'high affinity' state, immobilising the leukocyte. Additional chemokine signals are implicated in crawling of leukocytes across the endothelium in search of a suitable site for extravasation[62], which usually happens at endothelial cell borders where expression of adhesion molecule PECAM is highest[63].

1.3.3 Transendothelial Migration (TEM)

Leukocyte rolling, activation and adhesion are generally reversible, with most leukocytes that attach to the venules at the site of inflammation ultimately separating and re-entering the circulation. Indeed, this series of adhesion events almost resembles a 'combination lock'[63]: the correct molecule must interact in the correct order within a short temporal window for transmigration to occur. This tightly controlled system restricts recruitment, allowing trans-endothelial migration (TEM) only in leukocytes that are expressing the right combination of receptors. The combination of chemokine receptor engagement, integrin binding and homophilic interaction between leukocyte PECAM and endothelial border PECAM[64] induce a signalling cascade which ultimately results in the cytoskeletal rearrangement required to squeeze through endothelial cell junctions. Like PECAM, JAMs (Junctional Adhesion Molecules) are also enriched at the endothelial cell junctions, and their inhibition via neutralising antibodies was shown to reduced trans-endothelial migration both *in vivo*[65] an *in vitro*[66].

This method of trans-endothelial migration is also known as 'paracellular' or 'junctional migration', as the leukocyte migrates through endothelial cell junctions to reach the site on inflammation. There is however increasing evidence that under certain conditions, leukocytes can also pass *through* the endothelial cells directly, in a process termed transcellular route[67]. This method of trans-endothelial migration relies on adhesion molecules ICAM-1, VCAM-1 and PECAM, but also requires the leukocyte to extend pseudopodia into the endothelial cells[68] and the involvement of metalloproteinases which help remodel the leukocyte-endothelial cell interaction to facilitate the formation of trans-cellular channels required for migration[69].

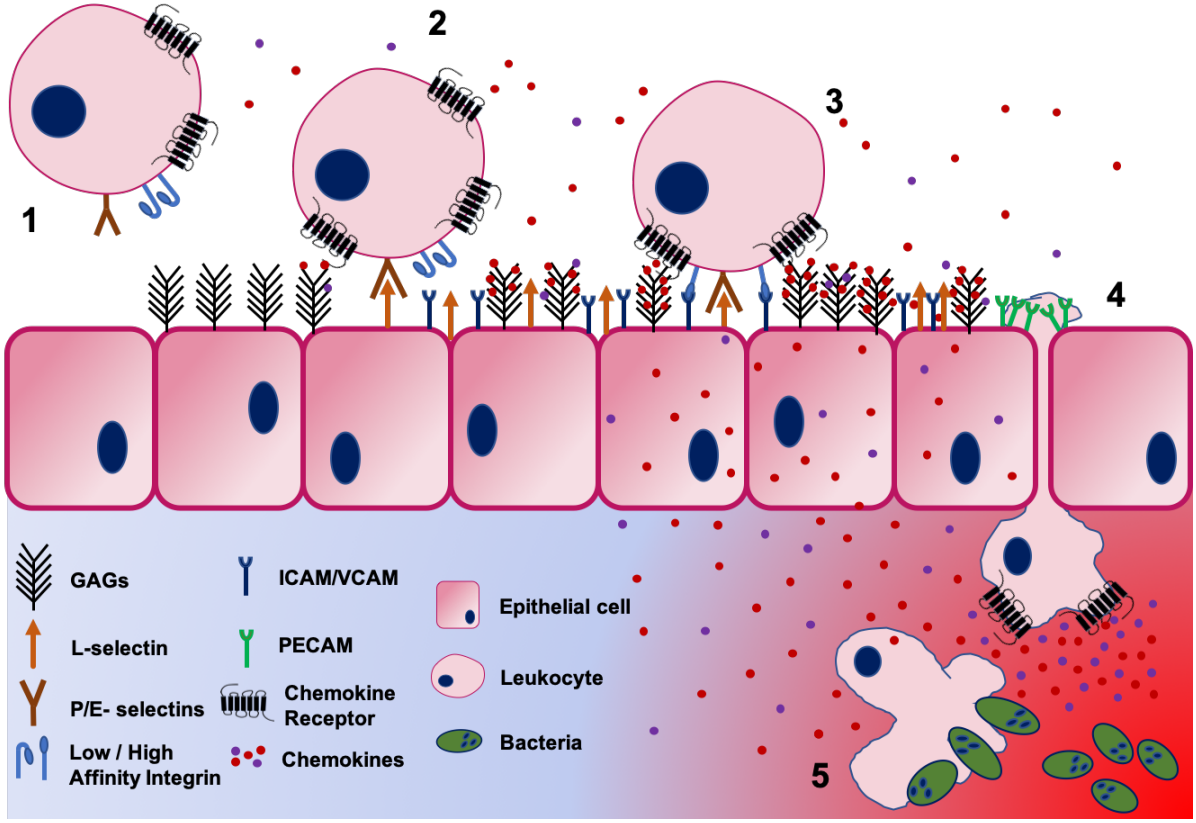

Figure 1-3 Leukocyte Trans-endothelial Migration- *This figure shows a simplification of the different stages and steps required for effective exit of leukocytes from the bloodstream into the site of inflammation.* ***1****- Circulating leukocytes express chemokine receptors, low affinity integrins and selectins.* ***2****-Tethering and rolling: L-selectins and P/E selectins on the epithelial cells bind to each other, slowing down the leukocyte by a factor of 100 and allowing the leukocyte to come into interaction with chemokine-bound GAGs.* ***3****- Tight Adhesion: engagement of the chemokine receptor activates integrins, strengthening adhesion.* ***4****- Trans-endothelial migration: Signalling cascades induce actin cytoskeleton reorganisation which allows the cell to squeeze through epithelial cells at PECAM rich sites.* ***5****- Differentiated leukocytes reach the site of inflammation following other chemotactic cues. Adapted from 'Leukocyte Recruitment from Vasculature'* [70]

1.3.4 Localisation within Inflamed Tissues

Once diapedesis has occurred, local chemokine and inflammatory cytokine gradients are responsible for directional migration and positioning of the leukocytes within the inflamed tissue. In these conditions, migrating leukocytes will probably encounter multiple overlapping chemoattractant signals. While it is still unclear how a leukocyte 'decides' which chemotactic signal to follow, studies on neutrophils have shown evidence of 'multistep navigation'[71], a mechanism by which cells can use chemokine receptors sequentially to move along different chemotactic gradients.

The switch from one receptor to the next appears to be determined by receptor desensitisation, a process by which repeated stimulation of the same chemokine receptor decreases its responsiveness[36]. This would allow for different chemokine receptors to take over and contribute to overall migration[39] and would also explain why leukocytes generally express several types of chemokine receptors, as each one is required to perform distinct steps in the process of extravasation and localisation within tissues.

1.4 Biological Role of the Main Inflammatory Chemokine Receptors (iCCRs)

1.4.1 Role of CCR2

CCL2 was first characterized as a monocyte-chemoattracting protein [72]. Its receptor, CCR2, is highly expressed on monocytes and is critical for bone marrow egress of classic monocytes and trafficking to sites of inflammation [73][74]. Subsequent studies showed that CCR2 is also expressed on activated and memory T cells[75], including both T_H1 and T_H2 cells[76][77][78], and mediates, to different extents, chemotaxis in a variety of other cell types[79] including mast cells[80], neutrophils (only in mice)[81] NK cells[82] and Immature B-cells [83]. In basophils, CCR2 engagement is strongly linked to histamine release and leukotriene secretion [84] while in endothelial cells it promotes proliferation and may play a central role in angiogenesis and wound healing[85]. Overexpression of CCR2 mRNA was also found in prostate cancer metastatic cells[86], and more aggressive cancer

cells were found to express greater levels of CCR2 compared to less aggressive cancer or non-neoplastic cells[87], suggesting that CCR2 is a key regulator of chemotaxis.

1.4.1.1 Phenotype of Ccr2$^{-/-}$ mice

Although *Ccr2*$^{-/-}$ mice are developmentally normal[88], it was initially observed that deletion of *Ccr2* reduces the number of macrophages recruited into the peritoneum after thioglycolate administration[88]. Further studies have uncovered a much wider range of effects ranging from susceptibility to infections[89], to pain[90], metastases of certain tumours[87] and neurodegenerative disorders[91]. Generally, deletion of *Ccr2* results in protection in non-infectious models of inflammation, where aberrant and sustained leukocyte accumulation is responsible for the pathology. Indeed, *Ccr2*$^{-/-}$ mice are protected from the development of EAE (experimental autoimmune encephalomyelitis) pathology, with reduced infiltration of both monocytes and T-cells into the central nervous system[92]. Selective absence of CCR2 decreases atherosclerotic lesion formation in ApoE$^{-/-}$ mice without any effects on plasma lipid or lipoprotein concentrations [93], while *Ccr2*$^{-/-}$ mice are protected from DSS (Dextran Sulfate Sodium)-induced intestinal adhesions and mucosal ulcerations[94]. In addition, *Ccr2*$^{-/-}$ mice showed 70% reduction in pain behaviour after intraplantar formalin injection[95], mediated by a reduced infiltration of monocytes/macrophages into the sciatic nerve and dorsal root ganglion and less activation of microglia in the spinal cord.

1.4.1.2 Resistance to Inflammation vs Susceptibility to Infection

Lack of CCR2 has also been shown to protect mice from acute transplant rejection, as both *Ccr2*$^{-/-}$ mice, and WT mice treated with neutralizing antibodies to CCL2, showed significantly reduced recruitment of mononuclear phagocytes following tracheal transplantation and an attenuation of bronchiolitis obliterans syndrome (BOS), a major limitation to survival after lung transplantation[96]. Furthermore, CCR2 inhibition reduces the transmigration of patient-derived sarcoma cells *in vitro* and reduces the metastatic burden in the lungs of treated mice[97].

However, lack of CCR2 is deleterious when the pathology is mediated by an infectious agent. Decreased recruitment and retention of immunocompetent cells in the bone

marrow[74] promotes survival and spread of pathogens worsening symptoms and increasing lethality. For example, the accumulation of blood-derived macrophages at the site of infection is critical for controlling bacterial growth of *L. monocytogenes* in infected organs. *Ccr2*$^{-/-}$ mice are unable to clear *L. monocytogenes* infection, and eventually succumb by day 5, while WT mice show no obvious symptom [98].

Genetic deficiency in *Ccr2* also markedly increases mortality in West Nile virus encephalitis, with *Ccr2*$^{-/-}$ mice showing sustained monocytopenia, reduced accumulation of monocytes in the brain and an increase in cerebral viral load [99]. Granuloma formation is also diminished in *Ccr2*$^{-/-}$ mice in both PPD (purified protein from *M.bovis*) and SEA (*S. mansoni* egg antigens) experimental models, suggesting defects in clearance of intracellular pathogens [100]. Indeed, further studies showed that *Ccr2*$^{-/-}$ mice are highly susceptible to i.v. *M. tuberculosis* infection, with significantly higher bacterial loads by 14 days post-infection, severe pathology, and increased mortality [89]. A dramatic reduction in IFNy levels and TH1 cytokine response in the draining lymph nodes of *Ccr2*$^{-/-}$ mice was also observed, suggesting that CCR2 might have a direct role in T-cell function[88].

Further studies have however shown that this effect is primarily due to a defect in the trafficking of monocytes to the site of immunisation. Reduced numbers of monocytes differentiating *in situ* results in fewer antigen-loaded antigen presenting cells (APC) reaching the draining lymph nodes, ultimately affecting the maturation and polarisation of the T-helper cell response[101]. These findings were also supported by the discovery that *Ccr2*$^{-/-}$ CD11c+ DCs display a persistent activation/maturation defect, with decreased MHCII and CD40 expression and abrogated cytokine production[102]. Indeed, while monocytes seem to be directly affected by CCR2 inhibition, other cell types show defects in recruitment and function only because of the lack of monocyte-derived signals at the site of infection. For example, even if neutrophils express low levels of CCR2, their recruitment kinetics seem to be unaltered[103][104]. Although some other studies have reported a slight delay in neutrophil recruitment and a modest decrease in neutrophil numbers at the site of infection in *Ccr2*$^{-/-}$ mice[105], this is probably caused by a decrease in monocyte-derived chemoattractants and not a direct effect of CCR2 on neutrophil chemotaxis[106].

1.4.2 Role of CCR1

CCR1 was originally cloned in 1993 and was shown to be expressed by neutrophils, T cells, B lymphocytes, natural killer (NK) cells, monocytes, and CD34+ bone marrow cells [107].

Ccr1$^{-/-}$ mice develop normally, show no histologic differences in lymphoid organs, peripheral blood counts or any altered susceptibility to spontaneous infections, but the receptor has been implicated in the development of the immune response after experimentally induced renal injury. Indeed, *Ccr1*$^{-/-}$ mice showed 35% fewer neutrophils and 45% fewer macrophages in injured kidneys compared to wild type controls[108]. This reduction in inflammatory leukocyte infiltration reduced oedema and improved renal function, suggesting that treatment with CCR1 antagonists could protect against experimentally induced renal injury. Indeed, administration of CCR1 agonist reduced macrophage and T cell infiltrates, reducing tubular injury and interstitial fibrosis as a result. CCR1 blockade with the small molecule antagonist BX471 also reduced inflammation in mice with glomerulosclerosis and nephrotic syndrome [109].

Inhibition of CCR1 also resulted in protection against Arthus reaction (an immune complex mediated type III hypersensitivity induced by the accumulation of antigen/antibody complexes in the vascular wall and glomeruli) by preventing recruitment of leukocytes to the kidney and reducing inflammatory damage to surrounding tissues[110]. The Arthus reaction is a mouse model for several other IC (immune complex) mediated diseases such as vasculitis syndrome, systemic lupus erythematosus, RA and cryoglobulinemia, suggesting that CCR1 could be involved in several immune complex mediated autoimmune conditions.

CCR1 expression can also be increased on the surface of certain leukocytes during particular inflammatory conditions. Mononuclear phagocytes have been shown to upregulate CCR1 in multiple sclerosis plaques [111] and in endometriosis[112], while the upregulation of CCR1 during inflammation has also been reported in T-cells of mice exposed to respiratory syncytial virus (RSV). Its inhibition prevented T-cell recruitment to the infected lung, but the lack of recruitment to the lung was actually protective, as it decreased the number of infection-permissive leukocytes at the site, ultimately limiting viral spread[113].

1.4.3 Role of CCR3

CCR3 is mainly expressed by eosinophils and basophils[114], with some expression also reported in mast cells[115] and Th2 cells[116]. *Ccr3*$^{-/-}$ mice show normal development, but impaired eosinophil trafficking to the lung, skin and intestinal mucosa. *Ccr3*$^{-/-}$ mice also failed to recruit eosinophils to the skeletal muscle in response to *Trichenella spiralis* inoculation, resulting in inability to clear the pathogen and a 2-fold increase in muscle larval cysts when compared to WT controls[117]. Recruitment of eosinophils to lung parenchyma and bronchoalveolar lavage (BAL) fluid is also severely impaired in *Ccr3*$^{-/-}$ mice, which also fail to develop AHR (airway hyperresponsiveness) in response to antigen inhalation, suggesting that targeting CCR3 may offer a possible therapy for allergic airway diseases and asthma [118]

Normal numbers of eosinophils in *Ccr3*$^{-/-}$ blood and bone marrow suggest that there are multiple chemokine/chemoattractant factors involved in sequestration of eosinophils out of the bone marrow and into the bloodstream[119]. However, histological studies on *Ccr3*$^{-/-}$ lungs using OVA as model antigen showed the presence of large numbers of 'trapped' eosinophils within the blood vessels themselves (under the endothelial cells and on top of elastic lamina), suggesting that CCR3 is largely required for the 'final steps' of the migratory process[119].

1.4.4 Role of CCR5

CCR5 belongs to a diverse group of chemokine receptors which have sparked interest not just for their chemotactic functions, but as highly versatile players that fine tune immune responses by functioning as co-receptors[120]. CCR5 (and CXCR4) are required for correct T-cell activation by stabilising the immunological synapse (IS) between an antigen presenting cell and the T-cell itself. The adhesins interact with the chemokine receptor at the IS, keeping the cells in close proximity, facilitating and ensuring that additional activating signals in the form of cytokines are delivered to the target cells and not lost in the surrounding area, preventing aberrant activation of bystander cells[120].

Ccr5 polymorphism also influences the severity of progression of various inflammatory conditions, suggesting that blocking this receptor might ameliorate symptoms. Indeed, studies on a specific CCR5 variant with a 32 base pair deletion of the *Ccr5* gene (*Ccr5*Δ32) have uncovered several unexpected of phenotypes, both protective and deleterious. This deletion involves a frameshift mutation with the inclusion of seven novel amino acids following amino acid 174 and a stop codon at amino acid 182 and results in the transcription a non-functional version of CCR5[121].

Homozygotes for *Ccr5*Δ32 show reduced incidence of renal transplant rejection[122], reduced incidence of childhood asthma[123], increased survival in IgA nephropathy[124], reduced incidences of Sjogren syndrome[125] and rheumatoid arthritis[126] and a delay in onset of myocardial infarction [127]. As CCR5 also acts as a viral co-receptor that facilitates entry of HIV (Human Immunodeficiency Virus) into the cell, individuals homozygous for *Ccr5*Δ32 are protected against HIV infection whereas those heterozygous for *Ccr5*Δ32 have lower pre-AIDS viral loads and delayed progression to AIDS[128].

In contrast, *Ccr5* polymorphism is responsible for increased susceptibility to sarcoidosis and more severe progression of the disease[129], doubles mortality rates in patients with MS [130], increases susceptibility to *Mycobacterium tuberculosis*[131], worsens prognosis in patients with Bechet's disease (a form of systemic vasculitis)[132] and increases the risk of symptomatic West Nile virus infection[133].

1.5 Chemokine-Independent Migration

Chemokine receptors are not the only regulators of chemotaxis, and many other GPCRs receptors and soluble mediators assist leukocyte migration during the immune response.

1.5.1 Formyl Peptide Receptors

These receptors were originally identified by their ability to bind to N-formyl peptides that are secreted by invading pathogens or passively released from dead and dying host cells after tissue injury [134]. Engagement of these GPCRs elicits a wide variety of cellular responses, ranging from chemotaxis to an increased ability to phagocytose and degrade pathogens.

FPR1 and FPR2 are critical for neutrophil chemotaxis in the early stages of *Listeria monocytogenes* infection, and their engagement also increases the generation of reactive oxygens species and the secretion of proteolytic enzymes necessary for effective pathogen clearance[135]. In human primary macrophages, FPR1 engagement result in polarisation towards a pro-inflammatory M1 state, characterised by an increase inflammatory cytokine secretion and increased chemotactic activity *in vitro* towards FPR1 ligands[136]

1.5.2 Leukotriene Receptors

Leukocytes exposed to formyl peptides (or other inflammatory stimuli) also increase the production of leukotrienes, inflammatory mediators synthesised in leukocytes by the oxidation of arachidonic acid (AA) via the 5-lipoxygenase pathway[137]. This pathway is predominant in leukocytes such as mast cells, eosinophils, neutrophils, monocytes, and results in the production of leukotriene A4 (LTA_4), an unstable epoxide[138] which serves as a 'parent molecule' for the production of other types of leukotrienes. Cells expressing LTA hydrolase (such as neutrophils and monocytes) can covert LTA4 into LTB4, a potent chemoattractant that dramatically amplifies formyl peptide-mediated neutrophil polarization[139].

On the other hand, cells expressing LTC4 synthase (such as mast cells and eosinophils) convert LTA4 in LTC4 and LTD4, leukotrienes responsible for the increase in vascular permeability and contraction of bronchial smooth muscle associated with acute allergic reactions or asthma attacks[137]. The production of leukotrienes is usually also accompanied by the production oh histamine and prostaglandins, which also act as inflammatory mediators.

1.5.3 Prostaglandins

Prostaglandins are also produced from arachidonic acid, but, unlike leukotrienes, are produced by most nucleated cells and their production requires the action of cyclooxygenase (COX) enzymes and terminal prostaglandin synthases to produce four principal bioactive prostaglandins, namely PGE2, PGI2, PGD2 and PGF2a [140].

Prostaglandin production is generally very low in uninflamed tissues and regulated by the enzyme COX-1, while inducible cyclooxygenase COX-2 produces prostaglandins through stimulation, especially during an immune response[141]. Prostaglandins also exert their effects by binding to G protein-coupled receptors (GPCRs), and are powerful locally acting vasodilators and inhibitors of platelet aggregation. This increase in microvascular permeability and arterial dilation result in the characteristic 'redness' and swelling during inflammation. Prostaglandins E2 and I2 can also function as neurotransmitters on peripheral sensory neurons, delivering the 'pain' signal also associated with inflammation[142]. Prostaglandins E2 has also been shown to increase integrin expression on endothelial cells, allowing recruited leukocytes to stick to the vessel walls and initiate trans-endothelial migration[143]

1.5.4 Histamines

Another class of potent vasodilators are the histamines, nitrogenous compounds produced by basophils and mast cells residing in connective tissue. The effects of histamines are mediated by four different histamine receptors, H1R, H2R, H3R and H4R, and together coordinate a wide variety of functions including cell differentiation, haematopoiesis and cell regeneration[144].

Upon engagement of their IgE receptor by an antigen, basophils and mast cells rapidly release histamines stored in granules, activating nearby cells. Histamine disrupts the endothelial barrier by acting on H1R on vascular cells, increasing vascular permeability[145]. Engagement of H1R on endothelial cells also increases arachidonic acid metabolism, resulting in increased prostaglandin production, synthesis of nitric oxide and contraction of smooth muscle cells[146]. Binding of histamine to the H4 receptor on eosinophils directly increases the expression of integrins Mac-1 and adhesion molecule ICAM-1, promoting eosinophil chemotaxis and trans-endothelial migration[147].

Furthermore, histamines can also modulate leukocyte maturation and differentiation directly at the site of inflammation. Indeed, *in vitro* studies have shown that histamine binding to H1R can directly increase IL-6 secretion by human primary macrophages and co-stimulatory molecule expression in dendritic cells[148]. Histamine release from mast cells can also be directly induced by C5a, a product of the complement protein C5[149].

1.5.5 Anaphylatoxins (Complement Peptides)

The complement system consists of numerous proteins that are present in either soluble form or in bound form at local inflammatory sites and in cell membranes. These soluble proteins normally exist in an inactive form that can be rapidly activated and amplified by a series of sequentially acting proteases that are tightly regulated[150]. Complement activation ultimately leads to the formation of the membrane attack complex (MAC), a structure that embeds itself in lipid bilayers on the surface of a pathogen forming a pore that can cause cell death by osmotic flux[151]. Some by-products of the complement cascade, such as C5a and C3a, can act as potent chemoattractants: administration of C5a *in vivo* induced a rapid activation-dependent adhesion of both rolling neutrophils and eosinophils[152], while C3a was shown to be a potent attractant for mast cells[153].

The receptor for C5a, C5aR, has been found on a wide range of leukocytes, such as neutrophils. eosinophils, monocytes, and T-lymphocytes, and has been shown to mediate phagocytosis and the release of cytotoxic granules and generation of oxidants.

1.6 The Evolution of the Chemokine Receptors

Chemokine receptors can share very high levels of homology across different species, suggesting an ancient origin. While fruit flies, sea urchins and sea squirts have no identifiable chemokines or chemokine receptors[154][155],more complex organisms, such as chickens (*G.gallus*), frogs (*X. tropicalis*), zebrafish (*D.rerio*) and pufferfish (*F.rubripes*) all share chemokine receptors which have very high similarities to their human counterparts. For example, human CCR7, the chemokine receptor present on dendritic cells which allows them to migrate to the lymph node, shares 82% of its sequence with chicken CCR7, 80% with frog and 64% with Zebrafish[156].

1.6.1 The Original Chemokine Receptor

Amphioxus, the closest living invertebrate relative to the vertebrates, has no chemokine receptors while lampreys, one of the earliest vertebrates, have a single chemokine-chemokine receptor pair: CXCL12 and CXCR4. Thus, the CXCR4-CXCL12 was identified as the 'primordial chemokine-chemokine receptor pair' [157][158] and the sequence of CXCR4 was analysed and compared across different species to understand how and why chemokine receptors evolved[159]. By tracking the mutations of this conserved chemokine receptor back through time, some groups have placed the origins of ancestral chemokine receptors between 650 and 564 million years ago, at the emergence of the vertebrate lineage[156][160]. Indeed, this period is marked by the evolution of neural crest tissue, a closed circulatory system, blood-based oxygen transport, and a hematopoietic system- all of which require some sort of controlled primitive cell migration.

1.6.2 Diversification of the Chemokine Receptors

The CXCL12-CXCR4 axis is still essential today for the proper migration of haemopoietic stem cells to the bone marrow, both late in development and in the adult[161] and for the migration of primordial germ cells to the genital ridge during development[162][163]. From this single gene and its defined biological role, the family has expanded through gene duplication throughout our evolutionary history, giving rise to all the other chemokine receptors. Specifically, the main inflammatory CC-chemokine receptors CCR1, CCR2, CCR3

and CCR5 evolved very recently in evolutionary terms, diversifying from a primordial CC-chemokine receptor and coinciding with the emergence of mammals [156]. The emergence of the mammalian lineage also corresponds with the establishment of homeothermy, lymph nodes and a more sophisticated adaptive immune response.

While a higher body temperature provided a fitness advantage against fungal infections during the massive 'fungal bloom' that followed the mass-extinction event that wiped out up to 90% of cold-blooded reptiles and all non-avian dinosaurs at the end of the Cretaceous [164][165], it is also believed that the increase in body temperature and metabolism may have made certain infections by thermotolerant bacteria more rapid and severe, adding a selective pressure on the primordial CC chemokine receptor to diversify into the inflammatory CC chemokine receptors (iCCRs) we see today.

1.6.3 Pathogen Perturbation of the Chemokine System

Several pathogens have evolved the ability to either degrade host, or secrete their own 'decoy' chemokines to avoid being detected by the immune system[166]. Natural selection in viruses, helminths, and ticks has resulted in the convergent evolution of a wide range of proteins and soluble factors that are capable of interfering with the chemokine system by disrupting glycosaminoglycan binding or preventing chemokine binding to its receptor.

1.6.3.1 Viruses

Many viral chemokines and chemokine receptors generally share a high degree of similarity with host proteins, suggesting that the virus has acquired them from the host and modified them in a process termed viral piracy[167][168]. These proteins, termed viral chemokine binding proteins (vCKBP), are secreted by infected cells and inhibit chemokine activity by preventing formation of the chemokine gradient or by blocking chemokine-chemokine receptor interaction[169].

The genome of *M. contagiosum*, a member of the poxvirus family responsible for the formation of small skin tumours in children and young adults, encodes for MC148, a protein capable of binding to CCR1, CCR5, CCR2, CCR8, CXCR2 and CXCR4 [170]. Studies suggest MC148 functions by masking the chemokine's own receptor interaction site, resulting in

decreased binding and biological activity impairment[171]. Similarly, both vaccinia virus and cowpox virus express a secreted chemokine binding protein that binds to CC chemokines (but not CXC or C) with high affinity, blocking their interaction with chemokine receptors and inhibiting cell migration *in vitro*[172].

Other viruses employ a completely different tactic: promote leukocyte recruitment to ensure dissemination throughout the host. Viral chemokine U83A, secreted by cells infected with human herpesvirus 6 (HHV-6), shows highly potent chemokine activity for CCR1, CCR4, CCR5, CCR6 and CCR8. These receptors are present on a wide array of cell types, including monocytes, DCs and skin-homing T-cells, suggesting that the virus is capable of actively recruiting leukocytes to promote its dissemination[173]. Similarly, Kaposi's sarcoma herpesvirus (KSHV) encodes three secreted chemokines; vCCL1, vCCL2 and vCCL3, which activate CCR8, CCR3 and CCR4, respectively[174]. This set of chemokines antagonizes the recruitment of antiviral Th1 and NK cells, skewing the immune response towards an antibacterial/antiparasitic Th2 profile which allows the virus to survive and disseminate[175].

1.6.3.2 Bacteria and other Parasites

While viruses generally produce receptor and chemoattractant mimics, parasites and bacteria such as *S. aureus*[176] and *S. pyogenes*[177] can secrete proteins that affect inflammatory chemokine receptor (iCCR) signalling by directly antagonising the receptors and cleaving signalling messengers, thus preventing stimulus generation[178].

Pertussis toxin (PT), secreted by *B. pertussis* (a small gram-negative bacterium that infects the human respiratory tract and causes whooping cough), is not only capable of inhibiting GPCR signaling[179], but can also inhibit early chemokine gene expression in alveolar macrophages and lung epithelial cells. This in turn causes a delay in neutrophil recruitment, allowing *B. pertussis* to establish itself early during colonisation[180]. Soluble chemokine binding proteins have also been identified in the human parasite *S. mansoni* [181], while hookworms have been shown to be capable of directly cleaving chemokines, such as CCL11, via metalloproteinase expression[182].

Ticks, uniquely, have two classes of chemokine binding proteins called evasins that have evolved to bind to either CC or CXC chemokines. '8-Cys' tick chemokine binding protein (CBK) contains 8 cystein residues and targets CC chemokines specifically, while the 6-Cys evasin targets CXC chemokines. Binding of evasins to their cognate chemokine prevents chemokine-glycosaminoglycan (GAG) interactions, reducing leukocyte recruitment and inflammation at the site of the bite and allowing for continued feeding[183].

The result of this evolutionary arms race between pathogens and hosts has resulted in the diversification and expansion of the chemokine-chemokine receptor system: by expressing many different chemokines and chemokine receptors, the immune system can rely on other signals to correctly move to the site of infection, even if the pathogen has managed to disable a few.

In addition, different copies of the same chemokine receptor gene can evolve independently and develop specialised functions, allowing for discrete recruitment of specialised leukocytes to the site of infection. For example, while CCR2 and CCR3 derive from the same CC primordial receptor and share 78% of amino acid structure homology, CCR3 controls eosinophil recruitment, while CCR2 controls monocyte and dendritic cell migration.

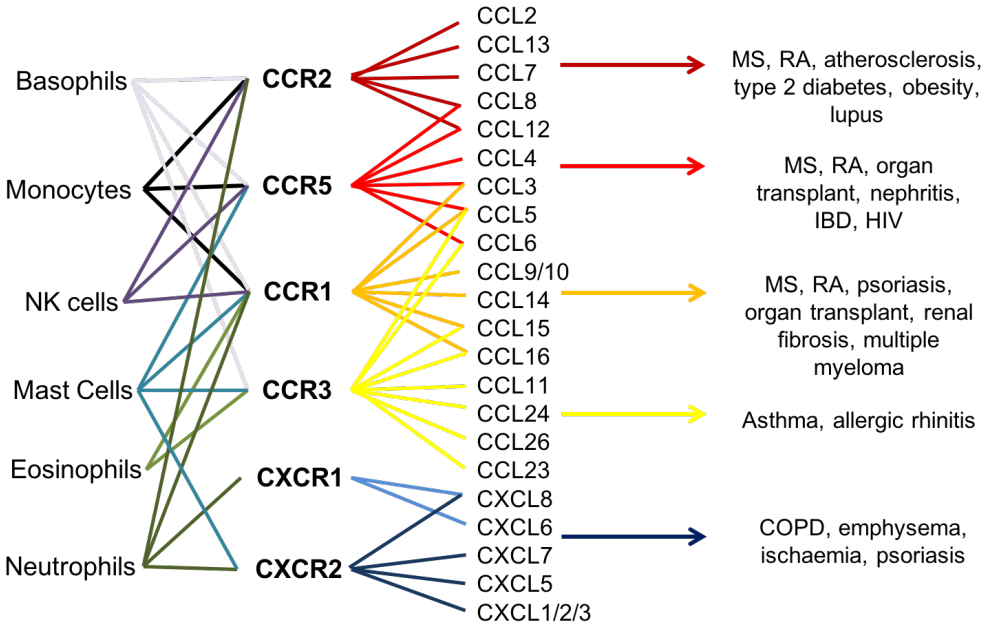

Figure 1-4- Apparent Redundancy in the Inflammatory Chemokine System. *Chemokines are redundant in their action on target cells and promiscuous in receptor usage. Moreover, certain cells simultaneously produce several chemokines with an overlapping spectrum of action.*

1.7 Problems with iCCR Studies

As gene duplication is the major driver of chemokine and chemokine receptor evolution[156], related chemokines share high sequence homology with each other and high degrees of similarity in structure[23]. Thus, many chemokine receptors can bind to multiple chemokines, creating an apparent redundancy in the inflammatory chemokine system where several ligands bind and activate multiple receptors and each receptor displays marked promiscuity of ligand binding (Figure 1-4).

Although redundancy, promiscuity and overlapping gene expression patterns in the chemokine system may protect from possible pathogen induced perturbation of chemotactic signals, this complexity has hindered research on the individual role of each inflammatory chemokine[184]. More specifically, the amino acid sequence of CCR2 shares 51% of identity with CCR1, 78% with CCR3 and 71% with CCR5[185], which makes it hard to develop specific antibodies for flow cytometry or immunohistochemistry which do not cross-react with other chemokine receptors. In addition, gene duplication has resulted in related chemokines being found in 'clusters' throughout the genome.

1.7.1 Gene Clustering

There are two major clusters of CC chemokine genes and two of CXC genes, plus numerous non-clustered or mini-cluster genes of both types, in both the mouse and human genomes [17]. Similarly, inflammatory CC-chemokine receptors *Ccr1*, *Ccr2*, *Ccr3* and *Ccr5* are all found on the same 170kb tight cluster on murine chromosome 9 (Figure 1-5). This 'clustering' of similarly sequenced chemokines and chemokine receptors makes it extremely difficult to create compound knock-out mice, as their proximity does not allow for easy genetic recombination during crossing over events.

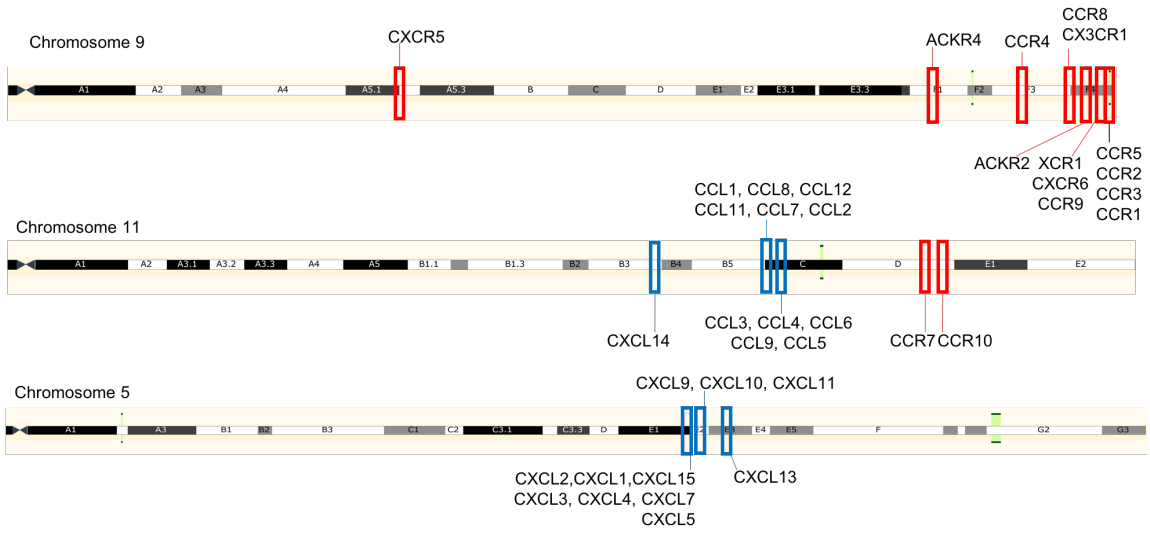

Figure 1-5- Genes for chemokines and chemokine receptors are found in clusters across the genome. *There are two major clusters of CC chemokine genes and two of CXC genes, plus numerous non-clustered or mini-cluster genes of both types, in both the mouse and human genomes. This schematic representation is adapted from Ensembl genome database project data*

1.7.2 Fluctuating Chemokine Receptor Expression

A further factor complicating studies on inflammatory chemokine receptors is that expression of chemokine receptors in leukocytes is not static, but fluctuates depending on activation, differentiation state and localisation. For example, differentiation of monocytes into macrophages is achieved by exposure to CSF-1, which is secreted by epithelial cells exposed to cytokines such as IL-1 or TNF-α [186]. Not only does this terminal differentiation alter the behaviour of the monocyte by inducing an inflammatory state characterised by elevated phagocytic rates, proteolytic activity and up-regulation of pro-inflammatory surface markers[187], but chemokine receptors expressed on the surface also change as differentiation progresses (*Ccr2* expression is shut down, while *Ccr1* and *Ccr5* are upregulated [188][189]).

This coordinated regulation of chemokine receptor expression and altered functional responsiveness during differentiation adds even more complexity to the chemokine system, as a pharmacological antagonist might only be effective at blocking migration at a specific temporal window in the life of a leukocyte. As a result, research so far has failed to pin-point the relative contribution of each single iCCR, resulting in sometimes very costly failed attempts to pharmacologically alter the chemokine system in clinical trials (see below).

1.8 Role of CC-Chemokines in Inflammatory Conditions

While studies on single iCCR knock out murine strains have identified multiple roles for CCR1, CCR2, CCR3 and CCR5 in the development of the immune response, they fail to address how different chemokines and chemokine receptors interact with each other to drive a particular pathology. Indeed, both acute and chronic inflammatory conditions are generally characterised by simultaneous expression of a wide variety of chemokines and chemokine receptors. Thus, targeting multiple chemokines at once might be the only way to fully explore the combinatorial mechanisms of the chemokine network.

Acute inflammation is a short-term process occurring in response to tissue injury, usually appearing within minutes or hours. Characterised by the five cardinal signs of inflammation (pain, redness, loss of function, swelling and heat), the acute phase response is generally initiated by resident immune cells already present in the involved tissues and relies on vasoactive substances released by activated endothelial cells[190]. CXCL8, one of the most potent neutrophil chemoattractants in inflammation[191], is consistently among the first chemokines expressed and released by various cell types involved in inflammation and, via CXCR1 and CXCR2, induces specific intracellular signalling cascades that result in rapid neutrophil recruitment[192].

On the other hand, CC-chemokine receptors are generally expressed predominantly by monocytes/macrophages and T-cells, leukocyte subsets largely associated with chronic inflammation occurring over weeks or years[193]. Chronic inflammatory diseases like rheumatoid arthritis and atherosclerosis are characterised by continued leukocyte infiltration into the inflammatory site, driven in large part by excessive chemokine production[194].

1.8.1 CC-chemokines in Atherosclerosis

Atherosclerosis is a chronic inflammatory disease of medium and large size arteries, characterised by accumulation of oxidised low-density lipoprotein (oxLDL) within the arterial wall and a progressive accumulation of inflammatory cells[195]. One of the key

initiating events in atherogenesis is believed to be endothelial dysfunction[196], which can occur as a result of hypertension, diabetes, smoking, or elevated plasma low-density lipoprotein (LDL) levels[197].

Endothelial dysfunction leads to decreased nitric oxide (NO) production, a potent vasodilator and inhibitor of platelet aggregation and leukocyte adherence[198]. Reduced bioavailability of NO results in increased vasoconstriction, coagulation, production of inflammatory cytokines and expression of adhesion molecules on the arterial walls[197]. The enhanced permeability of the endothelium and high levels of chemokines, such as CCL2 and CCL5, presented on the arterial wall promote the recruitment of monocytes from the blood into the innermost layer of the arteries, where they differentiate into macrophages[199]. Differentiated macrophages then phagocytose the oxLDL in the vessel wall via scavenger receptors, leading to accumulation of cholesterol droplets in the cytoplasm and the generation of the canonical 'foam cells' that are typical of early atherosclerotic lesions[200].

In turn, activated foam cells release TNF and other pro-inflammatory cytokines and chemokines[201] which fuel the inflammatory cycle by recruiting T cells, B cells and more monocytes into the lesion site[202]. Continued recruitment of inflammatory cells and accumulation of modified lipids on the arterial walls leads to the generation of an atherosclerotic plaque composed largely of recruited macrophages, T-cells, modified lipids and smooth muscle cells[203]. As the plaque increases in size over the years, it develops a necrotic core composed of dead and dying cells, as well as extracellular cholesterol. The atherosclerotic plaque then either continues growing, eventually occluding the lumen of the artery and leading to angina or ischemic attacks, or ruptures, releasing the contents of the necrotic core into circulation and forming a sudden blood clot that can lodge into the coronary arteries resulting in a heart attack, or in the brain causing a stroke[204].

Flow cytometric analysis of plaques in the aorta of *ApoE*$^{-/-}$ (Atherosclerosis-prone apolipoprotein E-deficient) mice revealed several monocyte/macrophage subsets differentially employing CCR2, CCR5 and CXCR1 to accumulate within atherosclerotic plaques[205]. Histologic analysis of lesions in *Ccr2/Apo E* double deficient mice revealed that the absence of CCR2 inhibited plaque formation and reduced macrophage infiltration into the vessel wall[206], while bone marrow transfer of *Ccr5*$^{-/-}$ bone marrow in lethally

irradiated low-density lipoprotein-receptor (LDLr)-deficient mice resulted in decreased plaque macrophage content and reduced collagen degradation[207]. In addition, overexpression of CCL2 in *ApoE*$^{-/-}$ mice was shown to increase the progression of atherosclerosis by increasing both macrophage numbers and oxidized lipid accumulation[208]. Overall, these studies hint at a central role for CC-chemokines in the development of atherosclerosis.

1.8.2 CC-chemokines in Rheumatoid Arthritis

Rheumatoid Arthritis is also a chronic inflammatory disease, characterised by massive infiltration of synovial tissue and synovial fluid with immune cells and mediated by chemokines and adhesion molecules[209]. Although the exact cause of RA remains unknown[210], recent findings hint at a genetic basis for disease development as more than 80% of patients express disease-associated alleles in the HLA-class II region, namely DRB1*0101, DRB1*0401, DRB1*0404 and DRB1*0405 [211]. The HLA gene complex encodes for the antigen presenting protein MHC-II, suggesting that these disease associated alleles may allow antigen presenting cells (such as macrophages, dendritic cells and B-cells) to present arthritis-related peptides to the immune system, leading to the stimulation and expansion of autoantigen-specific T cells in the joints and lymph nodes[212].

Activation of T-cells via arthritis-associated antigens stimulates CD4 T-cell proliferation and differentiation, characterised by increased secretion of IL-2 and IFNy in the synovial fluid[210]. Activated T-cells in turn support B-cell differentiation and autoantibody production[213]. Secreted autoantibodies can form larger immune complexes that can further stimulate the production of pro-inflammatory cytokines, including TNF-α, through complement and Fc-receptor activation on macrophages and dendritic cells[214]. This increased production of cytokines and chemokines activates more leukocytes, leading to a feedback loop for additional T-cell, macrophage and B-cell recruitment.

Exposure of synoviocytes (synovial fibroblasts) to pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α also results in the secretion of matrix metalloproteinases (MMPs) which are involved in tissue degradation and irreversible destruction of the cartilage, tendon, and

bone[215]. Similarly, *ex vivo* studies stimulating synovial fibroblasts with IL-1, TNF-α or IFNy revealed that these cells are the major source of monocyte chemoattractant CCL2[216]. These findings indicate that synoviocytes are central players in the creation of the inflammatory feedback loop that sustains the progression of the pathology. Other chemokines expressed at high levels in inflamed rheumatoid synovium include monocyte chemoattractants CCL3, CCL4 and CCL5 and neutrophil chemoattractants CXCL8 and CXCL10[217][218].

Interestingly, a meta-analysis on *Ccr5* polymorphism and rheumatoid arthritis revealed a negative association between the non-functional CCR5-Δ32 receptor and RA progression and severity[219], suggesting pharmacological inhibition of CCR5 might be a strategy for treatment of this degenerative autoimmune conditions.

1.9 Failure of Therapeutic iCCR Blockade in Clinical Trials

Despite over 25 years of pharmaceutical targeted research, no antagonist of inflammatory chemokine receptors has been licensed for use in treating inflammatory diseases such as rheumatoid arthritis or other conditions in which excessive inflammation is the main driver of the pathology (Figure 1-6).

Receptor	Company	Clinical Trial Phase	Compound	Indication	Status
CCR1	Berlex/Schering	II	BX471	MS, psoriasis	**No efficacy**
CCR1	Pfizer	II	CP481	RA	**No efficacy**
CCR1	Millennium/Aventis	II	MLN 3701	RA/MS	**No efficacy**
CCR1	Millennium	II	MLN 3897	RA	**No efficacy**
CCR2	Merck	II	MK 0812	RA/MS	**No efficacy**
CCR2	Pfizer	II	PF-4136309	Pain	**No efficacy**
CCR2	Incyte	I	INCB8696	MS, Lupus	**No efficacy**
CCR3	GlaxoSmithKline	I	776884	Asthma	**No efficacy**
CCR3	Bristol-Myers	II	DCP-168	Asthma	**No efficacy**
CCR3	Topigen	II	TPI-ASM8	Asthma	**No efficacy**
CCR5	GlaxoSmithKline	III	Aplaviroc	HIV	**Toxicity**
CCR5	Pfizer	II	Maraviroc	RA	**No efficacy**

Figure 1-6- Pharmacological Intervention with antagonists against the main inflammatory chemokine receptors has resulted in several failed clinical trials. *Despite over 20 years of pharmaceutical targeted research, no antagonist of inflammatory chemokine receptors (iCCR) has been licensed for use in treating inflammatory diseases. Adapted from 'International Union of Basic and Clinical Pharmacology. Update on the Extended Family of Chemokine Receptors and Introducing a New Nomenclature for Atypical Chemokine Receptors by Bachelerie et al.*[47]

1.9.1 Pharmacological Inhibition of CCR2

The CCL2-CCR2 axis is attractive for pharmacological intervention as it is involved in a wide range of human pathologies such as Alzheimer's disease[220], ischemic brain injury [221], asthma[222], myocardial infarction[223] and multiple sclerosis[224] and its inhibition drastically affects monocytes/macrophages while leaving other cell types relatively unaffected. Therefore, inhibition of CCR2 in pathologies where monocytes are the key players and regulators of the disease (such as RA) should be effective and have few side-effects.

While attempts to inhibit CCR2 pharmacologically to treat inflammatory conditions in mice with CCR2 antagonists [225]–[228] and neutralising antibodies[229] have been successful to varying degrees, they have ultimately resulted in disappointing clinical trials which were mostly discontinued due to lack of efficacy [230][231]. For instance, Merck's CCR2 inhibitor MK-0812 inhibited chemokine binding *in vitro* [232] but failed to demonstrate any improvement in the treatment of either RA or MS in Phase II clinical trials. Likewise, treatment with Millennium's CCR2 neutralising antibody MLN1202 did not result in amelioration of synovial inflammation in active RA [231].

Like RA, Multiple Sclerosis (MS) is an example of an extremely heterogeneous disease which consists of at least four distinct patterns of demyelination: macrophage mediated, antibody mediated, distal oligodendroglopathy and primary oligodendrocyte damage with secondary demyelination[4]. These different stages are characterised by different leukocytes and driven by different chemokine receptors. CCR1, CCR2, CCR5 and CXCR3 have all been implicated in the development and progress of MS[233]. Thus, similarly to RA, depending on the expression and activation of these receptors in patients with the disease, targeting more than one receptor might be necessary to show any efficacy in a clinical trial.

1.9.2 Pharmacological Inhibition of multiple iCCRs

As inhibition of CCR2 alone did not decrease disease progression in patients with MS and RA, clinical studies began to focus on simultaneous inhibition of several iCCRs. Monocytes are thought of as the effector cells in the pathogenesis of RA, and as their numbers in

affected joints usually correlate with clinical signs and symptoms[234], several clinical trials were set in place to inhibit recruitment of monocytes to inflamed joints by inhibiting the chemokine receptors responsible for monocyte chemotaxis: CCR2, CCR5 and CCR1. Although CCR2 and CCR5 receptor blockade had shown positive results in RA animal models, using chemokine antagonists to block these receptors was not effective in patients affected by RA [231]. *In vivo* and *in vitro* studies had also shown a positive correlation between blocking CCR1 ligands and inhibition of chemotaxis and reduction of synovial inflammation [235], but clinical trials using a CCR1 antagonist gave conflicting results, with some groups reporting a modest trend towards clinical improvement [236] and others reporting no efficacy at the Phase II stage [237]. Initially, it had been suggested that the redundancy in the chemokine system could have explained the failed trials using CCR2 and CCR5 antagonists, as both CCR2 and CCR5 share common ligands[238].

However, another trial in which both chemokine receptors were blocked simultaneously using antibodies showed no inhibition of monocyte recruitment to the inflamed joint, possibly indicating that other chemokine receptors are at play[209]. In addition, lack of efficacy of treatment by CCR5 antagonists could be explained by inhibition of regulatory T-cell (T-reg) recruitment to the site of inflammation. T-regs also use CCR5 in chemotaxis and are responsible for suppressing the immune response by scavenging IL-2 (necessary for proliferation of activated leukocytes) and releasing anti-inflammatory IL-10 and TGF-β. By preventing recruitment of T-regs to the inflamed joint, the resulting excessive inflammation might counter-balance the positive effect of reduced monocyte recruitment [239]. Several reasons have been suggested as to why clinical trials blockading chemokine receptors have not worked, ranging from off-target effects to redundancy of the target.

1.9.3 iCCR Antagonist Cross-Reactivity

Antagonist dosage might have also been to blame for the failed CCR1 blockade trials, as a group using a daily 10mg dose of CCR1 antagonist MLN389 showed no clinical efficacy[238][240], while another group reported clinical improvements after administration of 300mg of another CCR1 antagonist CP-481715 every eight hours[237]. These results suggest that CCR1 blockade may be sufficient to inhibit monocyte recruitment to the synovial compartment only in the presence of high levels of receptor

occupancy[241]. However, off-target effects have been considered as the potential reason behind the modest protection observed at very high antagonist concentrations. At such high levels (almost 1g of antagonist/day), there was sufficient drug present to cross-react effectively with other GPCRs, even those for which the compound had low affinity. It is therefore possible that the protective effects reported by the group could have been mediated through these other GPCRs. This was the case with Compound 1, a highly potent human CCR1 inhibitor which could cross react with other GPCRs including adenosine A and dopamine D2[242]. The compound was not developed further as it was discovered that the protective effects were not mediated by CCR1 inhibition but by affecting dopamine, which is known to attenuate T-cell functions and secretion of Th1 cytokines that are involved in the pathophysiology of multiple sclerosis[243].

Although studies have suggested that it is possible to alter monocyte recruitment pharmacologically, the examples elucidated so far underline the complexity of the chemokine system and the unanticipated and sometimes ineffectual consequences of downregulating the expression of a single receptor. Before any attempt can be made to treat inflammatory conditions by targeting critical chemokine-ligand combinations, it is necessary to fully understand and determine how the various chemokine receptors and their ligands interact with each other and which one (or combinations of) is necessary for the correct functioning of a specific leukocyte subset.

1.10 Aims of the Project

Studying the contribution of CCR1, CCR2, CCR3 and CCR5 to the development of the immune response presents various hurdles. Simple crossing of single knock out mice to obtain double chemokine knock outs for analysis is not possible, as inflammatory chemokine receptors CCR1, CCR2, CCR3 and CCR5 are all found on the same cluster on chromosome 9. Their proximity prevents chromosomal recombination and this has hindered research, especially as the use of antibodies to target specific chemokine receptors can induce potent changes to the cell by cross linking the monocyte's Fc receptors and altering their phenotype[244]. To investigate the contribution each iCCR has on the development of the inflammatory immune response, this project has focused on characterising two novel mouse models recently developed by the Chemokine Research Group, an iCCR$^{-/-}$ and an iCCR reporter strain.

iCCR$^{-/-}$: a novel knock-out model in which the whole iCCR cluster on murine chromosome 9 has been deleted via CRE-LoxP recombination, resulting in an iCCR$^{-/-}$ (CCR1, CCR2, CCR3, CCR5 knock-out) mouse. Removing all iCCRs gives us the opportunity to investigate the inflammatory response when the main inflammatory chemokine receptors are completely absent and therefore unable to compensate for each other. Not only does this model allow for assessment of the importance of CCR1, CCR2, CCR3 and CCR5 in leukocyte chemotaxis, it also allows us to determine to what extent leukocytes require inflammatory chemokine receptors to modulate their activity and differentiation, highlighting the importance of these iCCRs in the correct functioning of the inflammatory system and the effect of their absence on both inflammation and cancer progression.

iCCR Reporter: this novel murine iCCR reporter strain possesses a BAC (bacterial artificial chromosome) inserted in its genome, which encodes for the iCCR locus, but the coding sequences for the iCCRs themselves have been replaced with genes encoding for fluorescent proteins. Thus, these reporter mice express both the inflammatory chemokines receptors and specific fluorescent proteins under the same conditions, which allows for direct tracking of leukocytes with intra-vital microscopy and a direct visualisation of chemokine expression in leukocytes. This model also allows us to assess whether leukocytes express all iCCRs at the same time, if some chemokine receptors are preferentially expressed at different stages or if their expression is stochastic and follows random probability distribution.

Inflammatory chemokine receptors are regarded as major contributors to *in vivo* inflammatory cell recruitment. However, the totality of their combined contribution to the overall orchestration of the inflammatory responses, and residual inflammatory cell recruitment in their absence, has not yet been defined. We hypothesise that the severe immune dysregulation caused by the lack of iCCRs will be protective in acute inflammatory models in which the pathology is driven by excessive leukocyte recruitment, but will be deleterious and increase the severity of chronic conditions due to the inability to resolve inflammation and to clear pathogens.

This study will provide some clarity on our current understanding of the orchestration of the chemokine-driven inflammatory response. The results will not only increase our understanding of basic chemokine biology, but could also inform future pharmacological intervention on the chemokine system.

2 Materials and Methods

2.1 Mice

Animal experiments conformed to the animal care and welfare protocols approved by the University of Glasgow, carried out under UK Home Office Project Licence (70/8377).
The mice used in these experiments (regardless of strain) were all females of 7-8 weeks of age. The mice were bred in-house at the specific pathogen-free facilities at the Beatson Institute for Cancer Research, Glasgow.

iCCR$^{-/-}$: C57BL/6NTac (iCCR KO) (Taconics, UK) The 'Wild-Type' mice used in the experiments were of the same age, sex and background as those lacking the iCCR deletion.

CCR1/CCR2/CCR5: C57BL/6J. The 'Wild-Type' mice used in the experiments of the same age, sex and background as those lacking the specific CCR deletion.

CCR3: BALB/CJ. The 'Wild-Type' mice used in the experiments were of the same age, sex and background as those lacking the specific CCR deletion.

GGiREP: C57BL/6NTac. The 'Wild-Type' mice used in the experiments were of the same age, sex and background as those lacking expression of fluorescent proteins.

2.2 Data Normalisation across murine strains

Most of the experiment in this project involved comparing the iCCR knock-out strain to the single *Ccr1*, *Ccr2*, *Ccr3* and *Ccr*5$^{-/-}$. However, the different knock-out strains are on different backgrounds, making it difficult to draw meaningful conclusions when comparing data across strains as different backgrounds have slightly different immune system compositions. For example, B-cells in the C57BL/6J strain account for 45% of blood leukocytes, while only comprising 20% in the BALB/CJ strain. To compare different strains, each knock-out was first normalised to its own WT (knock-out sample/ average WT) (Figure 2-1). Only after transforming all values into 'fold of WT', can the data then be pooled and compared across strains.

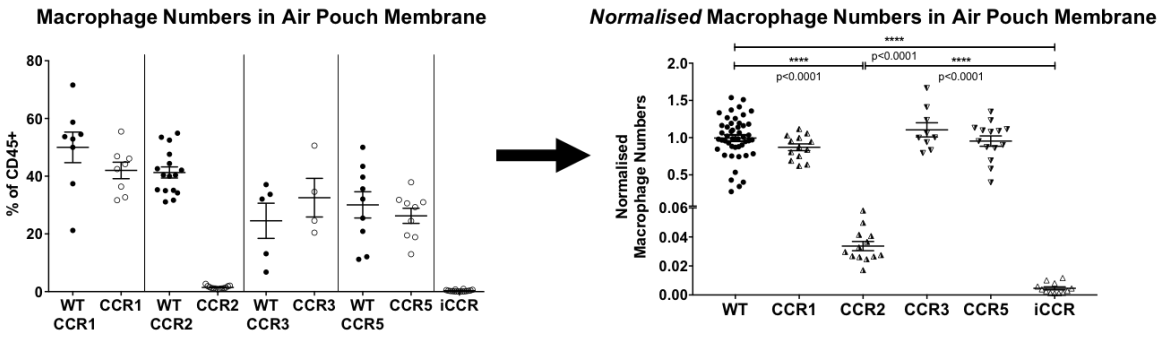

Figure 2-1 Example of Data Normalisation when comparing changes in leukocyte populations across different murine strains. *For each experiment, all samples were divided by the average WT value to transform all samples in 'fold of WT'. Normalised data can then be pooled across experiments and strains to uncover the contribution each iCCR has on leukocyte recruitment.*

2.3 *In Vivo* Models of Inflammation

2.3.1 Imiquimod Application to Skin

To simulate local skin inflammation and induce a dermatitis-like pathology, Aldara cream was used as a model of inflammation[245]. The active ingredient, Imiquimod, is a TLR-7 agonist which induces the localised production of IL-1β, TNFα and other pro-inflammatory cytokines. ¼ of the content of an Aldara cream sachet (5% Cream, approximately 3.12mg of Imiquimod) was applied to the shaved back of a mouse every day for four days. On the fifth day, mice were culled, and the shaved skin was removed. The tissue was then cut coarsely and digested enzymatically to achieve a single cell suspension which could be stained and analysed via flow cytometry.

2.3.2 Air Pouch Model

To study acute inflammation in the synovial cavity, the air pouch model was selected[246]. Briefly, mice were anaesthetised in an isoflurane chamber and once unresponsive, an air pouch was produced by subcutaneous injection of 3ml of sterile air into the back of the mouse (Figure 2-2). The process was repeated every two days for a total of 3 times (day 0, day 2, day 4). The day after the last air injection (day 5), the inflammatory irritant carrageenan (Sigma, C1867-5G) was resuspended in PBS (1% w/v), autoclaved and 1ml injected (always under isoflurane anaesthesia) into the air pouch to induce local inflammation. 48 hours after the addition of carrageenan to the air pouch, the mice were culled with rising CO_2 concentration. 3ml of PBS were then injected in the air pouch, removed using the same syringe and spun down at 300g for 5 minutes to extract cells that had migrated inside the cavity. The membrane surrounding the pouch was also collected,

weighed and digested enzymatically to produce a single cell suspension which was then stained and analysed via flow cytometry.

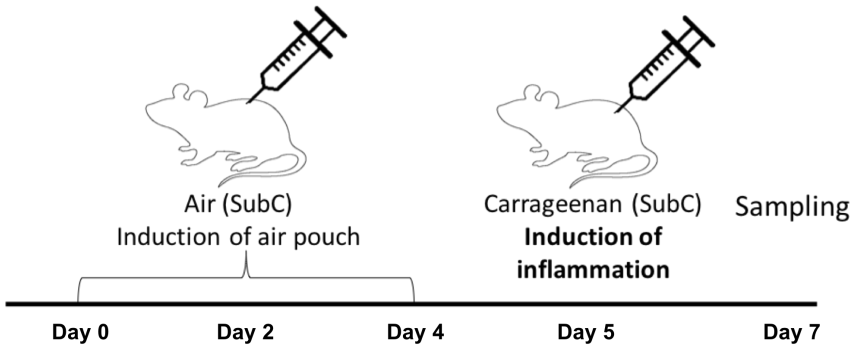

Figure 2-2- Air Pouch Generation. *Diagram describing the outline of the air pouch model. 3ml of sterile air are injected subcutaneously 3 times over a 6-day course. The resulting air pouch stabilises and forms a membrane which can then be filled with any mediator that induces inflammation. The air pouch generated provides a localized environment in which to study cell trafficking and the inflammatory response.*

2.3.3 LPS Lung Challenge and Broncho-alveolar Lavage

The LPS lung challenge was chosen as a model for lung inflammation. Briefly, mice were anaesthetised in a chamber with isoflurane gas. Once unresponsive, the mice were quickly removed from the chamber and 30µl of 250ng/ml of LPS was dropped onto the nostrils using a pipette. Once the LPS was breathed in, the mice were placed into a separate cage to recover from the anaesthesia, before placing them back to their respective housing cages. Mice were culled 48 hours later using rising CO_2 concentrations. Once culled, a catheter was surgically placed into the trachea through the neck, and 1ml of PBS with 2mM EDTA was injected into the lungs. The fluid was then taken out using the same syringe. Overall, the lungs were washed three times (1ml each time), with a final BAL (broncho-alveolar lavage) volume of around 3ml. Cells were counted, spun down, resuspended, and stained for flow cytometry.

2.4 Tissue Processing for Flow Cytometry

2.4.1 Skin Preparation

Digestion Mix= in Hanks BSS (Gibco, 14175.053), 1mg/ml of collagenase D (10mg/ml, Roche), 500µg/ml of dispase II (10mg/ml, Roche or Invitrogen) and 100µg /ml of DNase I (10mg/ml, Roche)

The dorsal skin of the mice was shaved and 8 dorsal skin disks (5mm) were cut into smaller pieces and submerged in 1ml of digestion mix in 2ml Eppendorf tubes and placed on a shaker for 30 minutes at 37°C at 1000rpm. After 30 minutes, 1 extra ml of digestion mix was added to the Eppendorf tubes and incubated under the same conditions for an additional hour. The digested tissue was then passed through a 70µm nylon mesh strainer, washed in RPMI (Gibco) + 10%FBS (Gibco) to inactivate digesting enzymes and resuspended in FACS buffer (PBS (Invitrogen) +2mM EDTA (Ambicon) + 0.5% FBS (Gibco)).

2.4.2 Lung Tissue Preparation

Digestion mix= in RPMI (Gibco), dispase II (1600µg /ml, Roche), collagenase P (200µg /ml, Roche), DNase I (100µg /ml, Roche)

Murine lung tissue was extracted and coarsely minced with scissors. Lung pieces were resuspended in 5ml of enzymatic digestion mix and incubated in a water bath at 37°C for 40 minutes. The digested tissue was then passed through a 40µm nylon mesh strainer, washed in RPMI before resuspending in 1ml of red blood cell lysis buffer (Ebiosciences) for 1 minute. Cells were then washed twice in FACS buffer (PBS (Invitrogen) +2mM EDTA (Ambicon) + 0.5% FBS (Gibco).

2.4.3 Spleen Preparation

The spleens from the mice were collected, carefully removing any visible fat or connective tissue. The spleens were inserted in 2mL Eppendorf tubes containing 1ml of RMPI and coarsely cut in smaller pieces mixing roughly. The pieces were then forced through a 70µm nylon mesh strainer using the internal plunger of a 5ml syringe. The nylon filter was then washed with 5ml of PBS. The cell suspension was then spun down at 300g for 5 min and the red blood cells in the pellet lysed with 1ml of ACK (Ammonium-Chloride-Potassium) lysis buffer (Gibco) for 1 minute. 9ml of RPMI were added to stop the reaction, and the cell suspension was again passed through a 70µm filter to obtain a single cell suspension.

2.4.4 Blood Preparation

Before blood extraction, 1ml syringes with a 26G ½" needle were soaked in 0.5M EDTA (Invitrogen) to prevent blood coagulation during extraction. Blood was then collected by inserting a 1ml syringe into the inferior vena cava and placed in a 1.5ml Eppendorf tube containing 100µl of 0.5M EDTA. The blood samples were then spun down at 300g for 5 minutes. The plasma component was carefully removed for multiplex analysis of circulating

cytokines and stored at -80°C. The pellet containing the leukocytes and red blood cells was resuspended in 1ml of ACK lysis buffer and incubated for 5 minutes. The lysis reaction was stopped by adding 0.5ml of RPMI and the samples were spun down again. Blood samples underwent a second round of RBC lysis with 1ml of ACK lysis buffer for 2 minutes to ensure that all RBCs had been removed. The remaining leukocytes were then resuspended in FACS Buffer (PBS (Invitrogen) +2mM EDTA (Ambicon) + 0.5% FBS (Gibco)).

2.4.5 Bone Marrow Preparation

Tibia and Femurs were collected from culled mice and excess tissue was removed. Both ends of each bone were cut, and the bone marrow was flushed out using 5ml of RPMI-1640 (Sigma) (10% FBS) in a 5ml syringe with a 23G needle. The cell suspension was collected and forced through the same syringe to reduce clumps, filtered through a 70µm cell strainer and spun down for 5 minutes at 300g. The resulting pellet was incubated for 1 minute with 1ml of ACK lysis buffer (Gibco) to remove red blood cells. Cells were spun down again and the pellet resuspended in FACS Buffer.

2.4.6 Air Pouch Membrane Preparation

The membrane surrounding the airpouch was dissected and digested for 1 hour at 37˚C with shaking in 1ml of HBSS containing 0.44 Wünsch units of Liberase (Roche). Liberase was then deactivated by adding 20µl of FBS and membrane cell suspensions were passed through 70µm nylon mesh filters and washed in PBS.

2.5 Flow Cytometry

2.5.1 Surface Antigen Staining

Starting from single cell suspensions from various tissues (spleen, skin, lung and lymph node), 300.000 cells per condition were added to polystyrene tubes (BD Falcon) and washed with FACS buffer (PBS (Invitrogen) +2mM EDTA (Ambicon) + 0.5% FBS (Gibco)). Cells were then spun down at 300g and resuspended in 100µl of Zombie Aqua Viability Dye at a 1:1000 dilution in PBS and incubated for 15 minutes on ice. Cells were then spun down, washed in FACS buffer, and incubated for 15 minutes at room temperature with 100µl of Fc Block (Macs Miltenyi Biotech) at a 1:100 concentration in FACS buffer. Cells were washed again and stained with the appropriate antibodies (Figure 2-3) on ice for 30 minutes. Cells

were washed one last time with FACS Buffer and resuspended in Fixation Buffer (Biolegend, 420801), incubated at room temperature for 20 minutes, and once fixed, cells were washed and resuspended in 200µl of FACS buffer and filtered through a nylon mesh to remove any clumps before being analysed. Data were acquired using either the MACSQuant (Miltenyi) or LSRII (BD Biosciences) flow cytometer and analysed using MACSQuantify version 2.5 software (Miltenyi Biotec). Voltages and compensation were determined using UltraComp eBeads (Bioscience, 01-2222-42) as single controls. Positive staining was determined using Fluorescence Minus One controls.

Leukocyte Panel (Blood)

Antibody	Fluorophore	Supplier	Clone Number
Ly6C	BV 421`	Biolegend	HK1.4
CD11b	V500	BDHorizon	M1/70
CD8	FITC	eBiosciences	53 6.7
CD3e	PE	biolegend	145-2CII
CD45	Per/cp	biolegend	30-FII
CD19	Pe/Cy7	eBiosciences	eBio1D3
F480	APC	eBiosciences	BM8
Live/dead	efluor 780	eBiosciences	

Leukocyte Panel (Bone Marrow)

Antibody	Fluorophore	Supplier	Clone Number
Ly6C	BV 421`	Biolegend	HK1.4
B220	V500	BDHorizon	RA3-6B2
Ly6G	FITC	biolegend	IA8
pDCA1	PE	biolegend	127103
CD45	Per/cp	biolegend	30-FII
CD11B	Pe/Cy7	eBiosciences	M1/70
CD11c	APC	Biolegend	N418
Live/dead	efluor 780	eBiosciences	

Reporter (GGiREP) Panel (all tissues)

Antibody	Fluorophore	Supplier	Clone Number
CD11b	bv605	biolegend	M1/70
CD45	APC-Cy7	biolegend	104
F480	Pe/Cy7	eBiosciences	BM8
CD19	Pe/Cy7	eBiosciences	eBio1D3
Ly6C	AF700	biolegend	HK1.4
Siglec F	PerCP Cy 5.5	eBiosciences	1RNM44N
Live/dead	efluor 780	eBiosciences	

T-cell Panel (tissues)

Antibody	Fluorophore	Supplier	Clone Number
NK1.1	BV421	Biolegend	PK136
Live/Dead	BV 510	Biolegend	ZombieAqua
F480/B220	FITC	Biolegend	BM8/RA3-6B2
yd	PE	Biolegend	eBioGL3
CD4	Pe/cy7	Biolegend	RM4.5
TCRb	PerCp/cy5.5	Biolegend	30.FII
CD45.2	APC	Biolegend	104
CD44	APC-Cy7	Biolegend	IM7

Bone Marrow/ ES derived Macrophages and DC

Antibody	Fluorophore	Supplier	Clone Number
CD115	PE	biolegend	AFS18
CD64	percp.cy5.5	biolegend	x54-5/7.1
MHC II	bv421	biolegend	i-a/1-e
CD11b	v500	bd biosciences	m1/70
CD11c	APC	biolegend	n418
CD86	percp	biolegend	gl-1
CD40	FITC	biolegend	3-- 23
CD80	PE	biolegend	16-10A1
Ly6C	FITC	biolegend	hk1.4
F480	pe.cy7	ebioscience	bm8
CD206	AF700	biolegend	C068C2
CD11A	FITC	biolegend	m17/4
CD11A	PE	biolegend	2D7
CD18	PE	biolegend	M18/2
Live/Dead	V510	Biolegend	ZombieAqua

Monocyte Panel (tissues)

Antibody	Fluorophore	Supplier	Clone Number
CD64	BV421	Biolegend	X54-5/7.1
MHC II	BV605	Biolegend	M5/114.15.2
Live/Dead	V510	Biolegend	ZombieAqua
Ly6G	FITC	biolegend	IA8
Siglec F	PE	BD Biosciences	E50-2440
F480	Pe/Cy7	eBiosciences	BM8
CD45	PerCP/Cy5.5	Biolegend	30.F11
CD11c	APC	Biolegend	N418
CD11b	APC-Cy7	eBiosciences	M1/70
Ly6C	AF700	Biolegend	HK1.4

Macrophage Activation

Antibody	Fluorophore	Supplier	Clone Number
MHC II	BV421	Biolegend	M5/114.15.2
CD11b	V500	BD Horizon	M170
CD40	FITC	Biolegend	44986
CD103	PE	biolegend	2E 4
Siglec F	PE	BD Biosciences	E50-2440
F480	Pe/Cy7	eBiosciences	BM8
CD86	PerCP/Cy5.5	Biolegend	GL-1
CD11c	APC	Biolegend	N418
CD206	AF700	Biolegend	C068C2

Figure 2-3 List of Antibodies for flow cytometry- *Supplier, clone number and fluorophore of all the different antibodies used to stain different organs and cell types.*

2.5.2 Intracellular Staining

2.5.2.1 Proliferation Marker Ki67

The day before intracellular staining, a Falcon tube of 70% Ethanol was chilled at -20°C overnight. The next day, after normal surface antigen staining, cells were spun down and vortexed for a few seconds in 500µl of cold ethanol. After resuspension, each sample received an additional 1ml of cold ethanol and 1 hour incubation at -20°C. Cells were then spun down at 300g for 5 minutes and washed in FACS buffer. The pellet was then resuspended in 150µl of FACS Buffer+ Ki67 antibody (Biolegend, 652403, clone 16A8, 1/220 dilution) and incubated in the dark at room temperature for 30 minutes. Cells were then washed x2 with FACS Buffer and analysed on the MacsQuant on the PE channel. Positive staining was determined using an isotype control (IgG2ak, PE Rat 400507, Biolegend).

2.5.2.2 Forkhead Box transcription Factor FoxP1

After normal surface antigen staining, cells were spun down and resuspended in 500µl of Fixation/Permeabilisation Buffer provided in the Intracellular Staining Buffer Set (Miltenyi, 130-093-142). Samples were incubated for 30 minutes at 4°C in the dark. After incubation, each sample received additional 500µl of FACS buffer and was spun down at 300g for 5 min. Samples were then resuspended in 110µl of FoxP1 antibody (Miltenyi, 130-110-417, clone REA682) in Permeabilisation Buffer solution (100µl Buffer+10µl antibody) and incubated for 30 minutes at 4°C. After incubation, samples were washed twice in FACS Buffer and analysed on the MacsQuant on the PE channel. Positive staining was determined using an isotype control

2.6 Tissue Culture

2.6.1 Collecting Bone Marrow Stem Cells

Tibia and Femurs were collected from culled mice and excess tissue was removed. The bones were taken to a sterile hood and briefly dipped for 1 minute in 70% ethanol to kill any bacteria. Bone marrow was extracted and the red blood cells were lysed using the same method for preparing bone marrow samples for flow cytometry. The cells in the single cell suspension were counted and resuspended in an appropriate volume of media depending on the cell type required for analysis.

2.6.2 L929 Conditioned Media- Source of CSF-1

L929 CM (conditioned media) was produced by growing L929 cells (kindly provided by Dr. Helen Taylor, University of Edinburgh) to confluency in T225 flasks (CytoOne, Starlabs) in DMEM/F12 with Glutamax (1:1) (Gibco, 31331) supplemented with Heat Inactivated FBS (Gibco, 0270, 56°C for 30min) and Primocin (Invivogen). A vial was first thawed and cells allowed to recover in a vented T75 flask. Once confluent, cells were detached using Trypsin/EDTA 0.005%, spun down and counted. 9 million cells were used to seed each T225 flask in 90ml of media (100,000 cells/ml). Cells were then allowed to grow undisturbed for 4 days. Medium was changed (90ml) and cells were left for an additional 7 days. After 1 week, the medium (now containing CSF-1) was collected, filter sterilised with a 0.22µm Millipore sterile vacuum (EMD, Millipore) and frozen at -80°C to be added as a source of CSF-1 in GMEMdiff and GMEMcult.

2.6.3 Embryonic Stem Cell Derived Macrophages

The murine embryonic stem cells (ES) used in the *in vitro* experiment are from cell line E14 derived from inbred mouse strain 129/OLA. ES cells were kindly provided by Dr. Derek Gilchrist from the University of Glasgow.

Murine Embryonic Stem cells (ES) were cultured in GMEMsr with LIF (Leukaemia Inhibitory factor) in a T25 flask to maintain their undifferentiated state. Before confluency was reached, ES cells were washed and detached with trypsin/EDTA 0.05%. Cells were counted and resuspended in GMEMdiff at a concentration of 37.500 cells/ml. A square petri dish was then filled with 15ml of PBS and 27µl drops of cell suspension (containing approximately 1000 ES cells/drop) were pipetted on the lid. Each lid can hold a total of 120-150 individual drops. The lid was turned slowly but decidedly over the square petri dish, and the ES cells were left to grow undisturbed for 4 days (Figure 2-4).

Upon withdrawal of LIF and addition of IL-3 (Peprotech, 2131310UG) and CSF-1 (L929 conditioned medium), ES cells start differentiating in embryoid bodies which increase in size over the next few days. By day 4, embryoid bodies were collected from the lids and allowed to settle by gravity in a 50ml falcon. The media was carefully removed and replaced with 20ml of fresh GMEMdiff and the cell suspension placed in a petri dish. Media was changed again at Day 6.

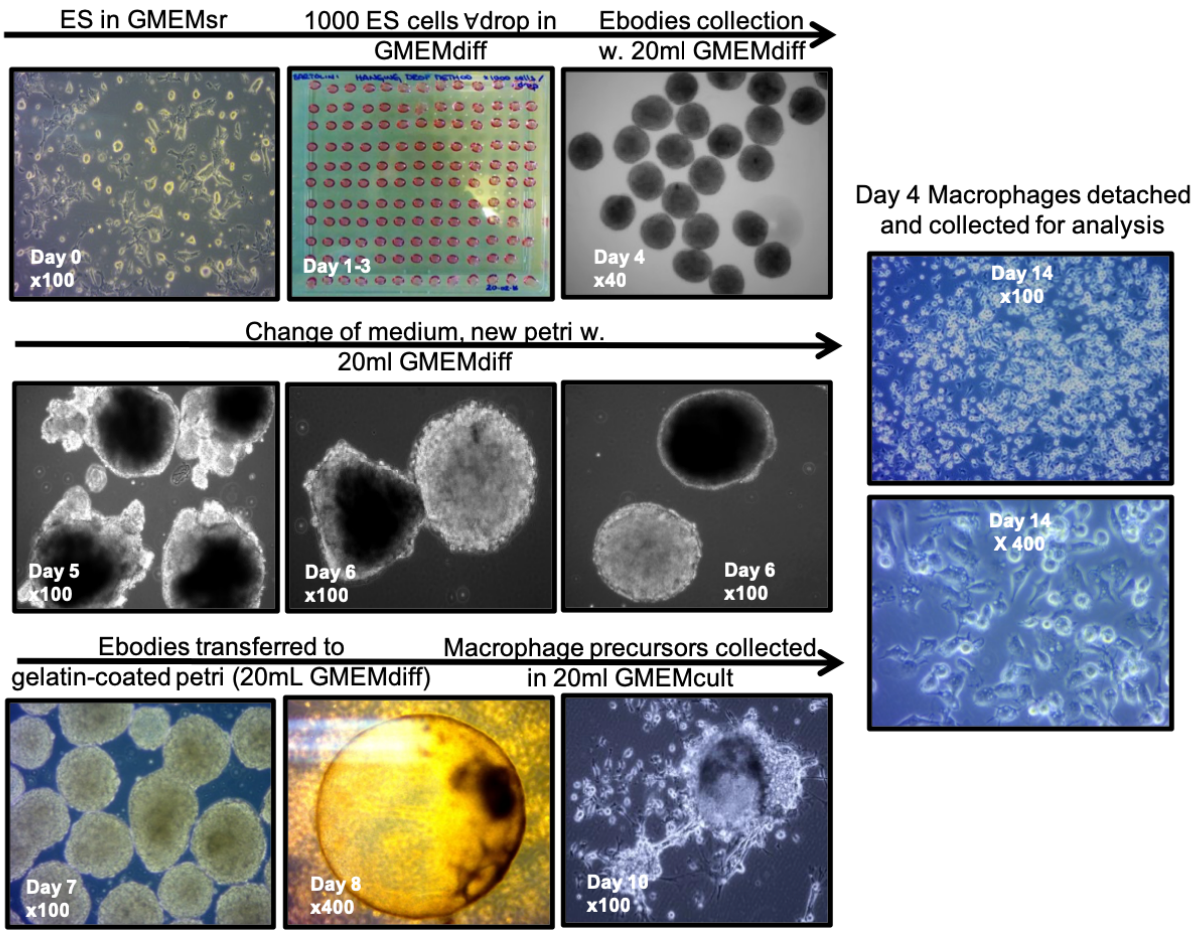

Figure 2-4 Generating ES-derived Macrophages- *A visual summary of the stages of macrophage differentiation protocol from murine embryonic stem cells used in this study from day 0 to day 14, acquired via phase contrast microscopy. Briefly, ES cells are maintained in GMEMsr (Day 0), while differentiation into embryoid bodies requires GMEMdiff containing IL-3 and CSF-1 from day 1 to day 10. Macrophage precursors can be collected every 2 days from day 12 to day 20. The collected precursors are then grown in GMEMcult, and differentiated macrophages can be collected from day 3 to day 5 post-precursors collection.*

By day 8, Embryoid bodies were collected and re-plated in a gelatin-coated (EmbryoMax, Millipore) petri dish, where they adhered and released macrophage precursors in the medium. Every two days (day 10, 12, 14, 16, 18), macrophage precursors were collected, pelleted and resuspended in 20ml GMEMcult. Differentiated macrophages were collected from day 3 to day 5 after replating using TrypLe Select (Gibco, A12177.01) as detachment buffer.

GMEMdiff (differentiation)		
GMEM	Gibco	21710.025
10% FBS	Gibco	0270
1% Non Ess. AA	Gibco	11140-50
2mM L-glutamine	Gibco	25030.024
1mM sodium pyruvate	Gibco	11360-070
0.1mM b-mercapto.	Gibco	31350.01
1ng/mL IL-3	PeproTech	213.13.10UG
15% L929 CM	**	

GMEMsr (self renewal)			GMEMcult (MΦ culture)		
GMEM	Gibco	21710.025	GMEM	Gibco	21710
10% FBS	Gibc	0270	10% FBS	Gibco	0270
1% Non Ess. AA	Gibco	11140-50	1% Non Ess. AA	Gibco	11140-50
2mM L-glutamine	Gibco	25030.024	2mM L-glutamine	Gibco	25030
1mM sodium pyruvate	Gibco	11360-070	1mM sodium pyruvate	Gibco	11360-070
0.1mM b-mercapto.	Gibco	31350.01	0.1mM b-mercapto.	Gibco	31350
100 U/mL LIF	Millipore	ESgro mLIF	15% L929 CM	**	

Figure 2-5 List of Reagents for ES-derived Macrophage Generation– *A description of the contents of the three different media required at different stages of macrophage differentiation. ** L929 conditioned media generation described previously.*

2.6.4 Bone Marrow Derived CSF-1 Macrophages

Bone marrow cells were isolated using the method described above, counted and $5x10^6$ cells were resuspended in 10ml of GMEM cult in a 90mm petri-dish. At day 4, cells were washed with 1X PBS to remove cell debris and undifferentiated cells and 10ml of fresh GMEMcult was added to cultures. Differentiated macrophages can be collected from day 5-7 after replating using TrypLe Select (Gibco, A12177.01) as detachment buffer. Macrophages were generally collected at Day 5, stimulated for 24 hours with cytokines/growth factors and collected at days 6-7 for analysis.

2.6.5 Bone Marrow Derived DCs and GM-CSF Macrophages

Bone Marrow was collected as above and 10^7 cells were resuspended in 10ml of RPMI-1640 (Sigma) (+10% FBS, L-glutamine, 50µM β-mercaptoethanol, primocin and 20ng/mL of murine recombinant GM-CSF (Peprotech). Cells were placed in tissue culture treated petri dishes. At Day 3, supernatant containing cells in suspension was collected, spun down at 300g for 5 minutes and the pellet re-plated in a new petri dish with 10ml fresh medium. Macrophages left stuck to the petri dish were discarded. This was repeated again at Day 5 and Day 7. After collection at Day 7, dendritic cells were counted and replated as required for phagocytosis and cross presentation assays.

2.6.6 Growing FLT3 Dendritic Cells

Bone Marrow was collected as described above and $1.5x10^7$ cells were resuspended in 10ml of RPMI-1640 (Sigma) (+10% FBS, L-glutamine, 50µM β -mercaptoethanol, primocin and 100ng/ml of murine recombinant FLT3L (Peprotech)). Cells were placed in tissue culture-treated petri dishes. At Day 5, 2/3 of the supernatant was collected and replaced with fresh FLT3L containing media. At Day 8 non-adherent and loosely adherent cells were collected, replated and stimulated for 24/48 hours and analysed via flow cytometry and ELISA.

2.6.7 *In vitro* Macrophage and Dendritic Cell Stimulation

The following cytokines/inflammatory signals (Figure 2-6) were used to stimulate macrophages and dendritic cells in numerous assays. Generally, cells were always resuspended at 10^6/ml before exposure to various cytokines at the concentrations shown below.

In Vitro Macrophage/ DC Stimulation			
Cytokine	**Concentration**	**Supplier**	**Code**
LPS	100ng/mL	Invivogen	Tlrl-eblps
CpG	10ug/mL	Invivogen	Tlrl-1585
Poly I:C	10ug/mL	Invivogen	Tlrl-picw
IL-10	20ng/mL	Peprotech	210-10-2ug
IL-6	50ng/mL	Peprotech	216-16-2ug
IL-4	20ng/mL	Peprotech	214-14-20ug
IFNy	100ng/mL	Peprotech	315-05-20ug
TNFa	50ng/mL	Peprotech	315-01A-5ug
IFNa	25ng/mL	Peprotech	752802
IL-17	50ng/mL	Peprotech	210-17-5ug
GMCSF	20ng/mL	Miltenyi Biotech	130-095-739
FLT3L	100ng/mL	Peprotech	250-31L-50ug
UCB 35625 (CCR1/3 inhibitor)	20uM	Tocris Biosciences	2757
Maraviroc (CCR5 inhibitor)	20uM	Tocris Biosciences	3756
BMS CCR2 22	20uM	Tocris Biosciences	3129

Figure 2-6 Macrophage and DC Stimulation- *List of reagents and the concentrations used for in vitro macrophage and dendritic cell stimulation*

2.7 Analysing GM-CSF Derived Proliferation Clusters

Bone Marrow was collected as above and 10^7 cells were resuspended in 10ml of RPMI-1640 (Sigma) (+10% FBS, L-glutamine, 50µM β-mercaptoethanol, primocin and 20ng/ml of murine recombinant GM-CSF (Peprotech). Cells were placed in tissue culture treated petri dishes. At day 3, medium was removed and replaced gently with fresh media, taking care not to disrupt growing proliferation clusters. Clusters were then either examined under light contrast microscopy and imaged by selecting consistent fields of view (Figure 2-7A) and analysing cluster number using Fiji Software's Particle analyser (detection of particles >6000µm^2) (Figure 2-7 B,D) or collected by vigorous pipetting at day 4-5 and analysed via Flow Cytometry.

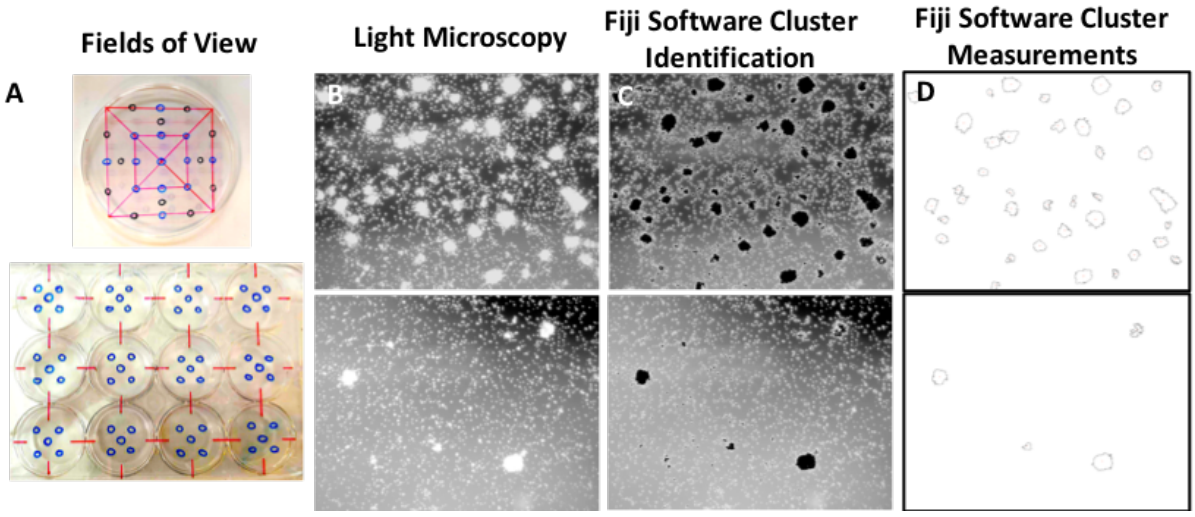

Figure 2-7 Cluster Identification- A- *Diagram showing fixed fields of view to acquire unbiased images for cluster quantification of cultures. Petri cultures have 25 fields of view. If cultures were grown in 12 well plates, each well had 5 fixed fields of view.* ***B-*** *Pictures were taken at x4 magnification on a light microscope for each field of view.* ***C-*** *Bright field images were analysed with the 'Particle Count' program on Fiji Image Software to determine the number of clusters larger than 6000µm^2.* ***D-*** *At the end of the analysis, Fiji provides an outline and a size for each identified cluster.*

2.8 *In Vitro* Assays

2.8.1 Adhesion Assay

The outlines of three circular coverslips were drawn onto a glass slide with an ImmEdge Pen as shown in Figure 2-8A. Recombinant mouse ICAM-1-Fc Chimera (553004, Biolegend) was resuspended in PBS to a final concentration of 10µg/ml. 50µl of ICAM-1 were then pipetted on top of the coverslips and incubated overnight at 4°C. The following day, the

ICAM solution was gently removed and the coverslips were washed x3 with 200µl of PBS. To block any residual binding sites, 50µl of 1% BSA in PBS were pipetted onto the coverslips and incubated at room temperature for 30 minutes. Coverslips were then washed again x3, and 50µl of GM-CSF cell suspension (200,000/ml) from WT or iCCR$^{-/-}$ cultures were added to the coverslips. The slides were then incubated at 37°C for 20 minutes to promote attachment of cells to ICAM-1. Before counting, the coverslips were again gently washed x3 with 200µl of PBS. 5 fields of view were recorded for each coverslip with a light microscope at a x10 magnification, and cell quantification was performed via Fiji Software Particle Analyser Program (Figure 2-8 B,D). Setting for detection: particles >50µm.

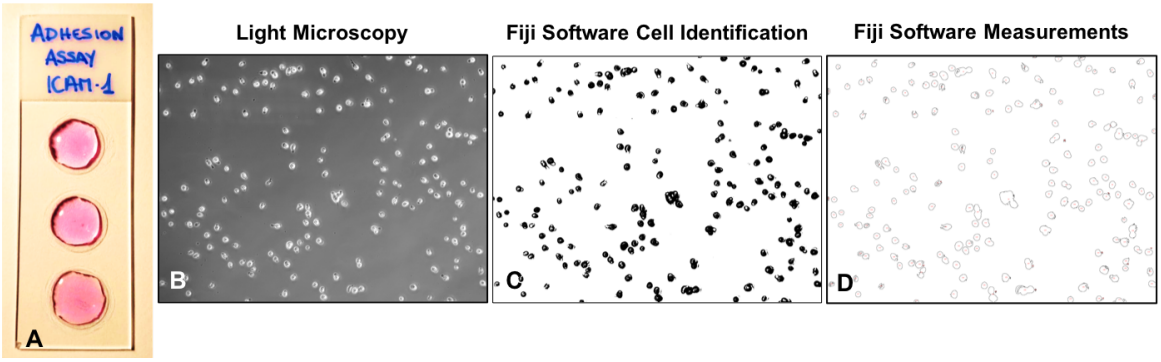

Figure 2-8 Static Adhesion Assay- A*- Diagram showing the arrangement of coverslips on the slide. 5 fields of view were taken for each coverslip.* ***B****- Pictures were taken at x10 magnification on a light microscope for each field of view.* ***C****- Bright field images were analysed with the 'Particle Count' program on Fiji Image Software to determine the number of cells larger than 50µm^2 D- At the end of the analysis, Fiji provides an outline and a size for each identified cell.*

2.8.2 Phagocytosis Assay

2.8.2.1 Ovalbumin AF488 –

Day 8 Dendritic cells were incubated at 37°C with Ovalbumin Alexa Fluor 488 Conjugate (Invitrogen) at various concentrations (60µg/ml – 15µg/ml) for 30-45 minutes. Controls were performed at 4°C. Fluorescence on the FITC channel was detected on the MACS Quant and analysed via FlowJo.

2.8.2.2 pHrodo Red E. coli BioParticles Conjugate-

Day 8 Dendritic cells were incubated with 250µg/ml of pHrodo *E.coli* BioParticles for 30 minutes at 37°C. pHRodo Bioparticles do not fluoresce but emit bright green light detectable on the FITC channel when exposed to pH change in lysosomes. Cells were

washed x3 with ice-cold PBS before analysis, and fluorescence on the FITC channel was detected on the MACS Quant and analysed via FlowJo.

2.8.3 Antigen Presentation and Cross Presentation Assay

BM derived Dendritic cells were grown in RPMI supplemented with 10% FBS, primocin and 20ng/ml of recombinant GM-CSF for 7 days. The protocol was adapted from an established procedure described by Alloatti et al [247]. Briefly, DCs were then incubated with ovalbumin coated beads (simulating particulate antigen), stimulated with LPS, fixed, and then co-incubated for 24 hours with cognate T-cells. Supernatants were then collected and analysed via ELISA for IL-2 to assess the extent of T-cell activation.

2.8.3.1 Coating Beads

The day before the assay, 250µl of 3µm latex beads (Polysciences) were spun down (19.000g) and resuspended in 3ml of Ovalbumin solution in PBS (10mg/ml). The beads were then incubated overnight on an agitator at 4°C to promote attachment of ovalbumin to their surface. The next day, the beads were spun down again at 19,000g, washed in PBS x3 to remove any unbound ovalbumin protein, and resuspended in 400µl of RPMI. 10µl of bead suspension was then added to the DCs to simulate particulate antigen (200:1 bead/DC ratio)

2.8.3.2 OVA Peptide Controls

Specific ovalbumin peptides were chosen as controls to determine if the detected changes in antigen presentation are due to defects in the cell's antigen processing capabilities or just decreased expression of MHCI/II or other costimulatory molecules. The peptides are fragments of ovalbumin protein that bind directly to either MHCI (OVA 323-339) or MHCII (OVA 257-264), without requiring any processing by cell's antigen degradation machinery.

2.8.3.3 OVA-Specific T-cells

The T-cells used in this assay come from the lymph nodes of OT-1 and OT-2 mice. The OT-1 mouse strain contains transgenic inserts for Tcra-V2 and Tcrb-V5 genes[248]. Thus, most CD8 T-cells in this murine line recognise ovalbumin residues 257-264 in the context of MHCI

(mimicking antigen cross presentation). On the other hand, the OT-2 strain expresses the mouse alpha-chain and beta-chain T cell receptor that pairs with the CD4 coreceptor and is specific for ovalbumin 323-339 [249]. Thus, most CD4 T-cells in this murine line recognise ovalbumin residues 323-339 in the context of MHCII (mimicking antigen presentation). To extract the T-cells for co-incubation with OVA-loaded DCs, cervical and inguinal lymph nodes were extracted from OT-1 and OT-2 mice, mechanically disaggregated through a 70µm nylon mesh and resuspended in complete RPMI at a concentration of $2x10^6$/ml.

2.8.3.4 Antigen Presentation Assay

After 7 days of culture, 100µl of GM-CSF cells were incubated for 5 hours with OVA-coated 3µm latex beads (Polysciences) (particulate antigen, 200:1 bead/DC ratio), and OVA control peptide binding to either MHCI or MHC II (OVA 257-264, OVA 323-339) in tissue-culture treated 96 well plates (Costar, 3799) at a density of 10^6/ml. After incubation, cells were washed extensively (x3 in PBS) to remove unbound beads and stimulated with LPS overnight to allow the cells to process ovalbumin and present it on MHC together with co-stimulatory molecules. The next day, cells were washed and fixed with 50µl of 0.008% glutaraldehyde (G7526-10ml, Sigma-Aldrich) in PBS for 3 minutes on ice. Immediately afterwards, cells were quenched to remove any unreacted aldehyde with 50µl of 0.4M PBS-glycine. Cells were then spun down at 300g for 5 minutes, washed one more time with 100µl of PBS-glycine 0.2M and resuspended in 100µl of RPMI. 100µl of OT-1 or OT-2 T-cell suspension in RMPI was then added to each well (cell suspension= $2x10^6$/ml → 200.000 T-cells/well → 2:1 T-cell/DC ratio), bringing the final volume of each well to 200ul. After 24 hours of co-incubation of fixed DCs with cognate T-cells at 37°C, the supernatant was collected and IL-2 levels were measure via ELISA.

2.9 ELISA

To determine the activation status of ES-derived macrophages, bone marrow GM and M-CSF macrophages, GM-CSF and FLT3 derived DCs, cells were collected and replated in tissue-culture treated 96 well plates (Costar, 3799) at a density of 10^6/ml. Their supernatant was collected after overnight stimulation with LPS or other inflammatory mediators. The following cytokines were measured via Affymetrix's ELISA Ready-SET-GO! Kits according to

manufacturer's instruction: IL-6, IL-10, IL-1β, IL-12p70, TNFα, GM-CSF, IL-2, CCL2. CCL5 levels were instead measured using R&D DuoSet ELISA Kit. Samples were measured in triplicate. Briefly, wells of a 96 well plate were coated at 4°C overnight with a specific capture antibody. The next day, wells were washed with PBS Tween 0.05%, blocked for 1.5 hours with assay diluent, and the supernatant was added and incubated at room temperature for 2 hours. Wells were then washed, and a monoclonal detection antibody specific to the cytokine in question was added to the samples and incubated for 1 hour. This second antibody is linked to HRP-peroxidase, an enzyme which induces a colour change when a substrate (TMB) is added. Colour develops in proportion to the amount of cytokine bound in the initial step, and the reaction is stopped with 50µl of H_2SO_4. The final optical density of the solution in each well was measured using a microplate reader set to 450nm (Sunrise, Tecan). A best-fit curve was constructed by plotting mean absorbance of the standards on the Y-axis against their known concentrations on the X-axis. The concentration of the samples was extrapolated using the equation of the line and substituting for X.

2.10 RNA extraction and cDNA generation

ES-derived macrophages, DCs or GM-CSF clusters were detached, pelleted, resuspended in RLT Buffer and spun through a QIAshredder (QIAGEN, #79654). The resulting elute was placed in a spin column from an RNeasy Mini Kit (QIAGEN, #74104) and the manufacturer's instructions were followed to obtain RNA in a final 40µl volume. RNA concentration was measured via Nanodrop 1000 (Thermo Scientific). cDNA was then obtained using ThermoFisher's High Capacity RNA to cDNA Kit according to manufacturer's instructions and using the following PCR programme (37°C 60 min, 95°C 5min, hold at 4°C) run on the Veriti 96 well Thermal Cycler (Applied Biosystems, #4947). cDNA samples were stored at -20°C.

2.11 qPCR

qPCR reactions were performed in 384 well –plates, providing quadruplicate values for each cDNA sample. Each well contained 10µl of reaction volume (1µl cDNA, 5µl SYBR Green Mix (Quanta Biosciences, #013590), 0.15µl primer mix (1:1) and 4µl nuclease free H_2O). Expression levels of inflammatory chemokine genes CCR1, CCR2, CCR3 and CCR5

were assessed via Real Time PCR analyser (Applied Biosystems, #7900HT). GAPDH was used as a housekeeping gene to normalise samples. The primer sequences are shown below in Figure 2-9.

	FORWARD PRIMER	REVERSE PRIMER
CCR1	GCCCTCATTTCCCCTACAA	CGGCTTTGACCTTCTTCTCA
CCR2	TGTGGGACAGAGGAAGTGG	GGAGGCAGAAAATAGCAGCA
CCR3	TTTCCTGCAGTCCTCGCTAT	GGAAAGAGGCAGTTGCAAAG
CCR5	TTTGTTCCTGCCTTCAGACC	TTGGTGCTCTTTCCTCATCTC
GAPDH	TGTCTCCTGCGACTTCAA	TGCAGCGAACTTTATTGAT

Figure 2-9 Primer Sequences- *Primer sequences of the inflammatory chemokine receptors CCR1, CCR2, CCR3, CCR5 and housekeeping gene GAPDH.*

2.12 PCR Array

To further characterise the gene expression of GM-CSF monocyte derived DCs and macrophages, a PCR array (RT2 Profiler PCR Array, PAMM-406ZE-4, Qiagen) was used to simultaneously determine the expression of 84 genes involved in dendritic cell maturation and activation, including cytokines, chemokines, cell surface receptors and signal transduction molecules. Genes involved in antigen uptake, processing and presentation were also represented in the array. Briefly, cells were collected, washed in PBS, spun down and lysed with a QiaShredder column at 23,000g for 1 minute. The lysate was then added to an RNeasy column for RNA extraction

2.12.1 RNA extraction and cDNA synthesis

RNA was isolated using an RNeasy spin column and the RNA was cleaned of genomic DNA contamination with a brief incubation with DNAse. cDNA was prepared using 400ng of purified RNA from both WT and iCCR$^{-/-}$ samples as template using the RT_2 First Strand Kit The cDNA was then mixed with RT_2 Sybr Green and RNAse-free water according to manufacturer's instructions, and 10µl of PCR component mix was added to each well containing primers specific for each gene of interest. Each 384-well plate contains 4 replicates of 84 genes, enough for 2 WT repeats and 2 iCCR$^{-/-}$ repeats. The array was therefore repeated twice, thus bringing the number of repeats for both WT and iCCR$^{-/-}$ cells to 4. The cycling conditions were chosen specifically for the BIORAD machine and the results analysed via the Web-based PCR Array Data Analysis Software after applying a stringent cycle threshold of 30.

3 Results- iCCR Knock-Out Strain

3.1 Introduction

The main inflammatory chemokine receptors CCR1, CCR2, CCR3, and CCR5 are expressed constitutively on a variety of leukocytes, and their expression can be upregulated or change altogether after differentiation or during inflammation. 'Classical' inflammatory monocytes in the bone marrow initially express high levels of CCR2, but its expression rapidly decreases as maturation progresses. Conversely, CCR1 and CCR5 expression increases continuously with ongoing differentiation, reaching its highest values in terminally differentiated macrophages[188]. CCR5 expression in T-cells also fluctuates, increasing during differentiation of naive CD8 T cells to memory CD8 T cells and then decreasing during differentiation into effector CD8 T cells[250]. Even neutrophils, whose migration is largely dependent on CXC chemokine receptors CXCR1 and CXCR2[251], have been shown to increase expression of CCR1 and CCR2 during inflammation[252]–[254].

While studies on single knock-outs have highlighted the importance of each receptor, what has emerged is that no single iCCR is completely responsible for the migration of a particular subset. Deletion of CCR3, the main chemokine receptor expressed on eosinophils[118][255], reduces the accumulation of these cells in the lung during inflammation by around 80%, but the residual perivascular and peribronchial eosinophils still contribute to disease[256]. Similarly, although CCR2 is expressed by virtually all 'classical' Ly6C+ inflammatory monocytes, CCR2 deficient mice do not show a complete ablation of monocyte migration, but only an 80% reduction. Some groups suggest that this finding is an indication that CCR2 does not mediate migration *per se*, but rather promotes monocyte attachment to endothelium, thus allowing other receptors on the surface of the monocyte to interact and begin migration in a CCR2 independent manner[257]. It could also be argued that the absence of CCR2 increases the levels of CCR2 ligands in the circulation (such as CCL2, CCL7, CCL8 and CCL12) making them available for interaction with lower affinity receptors such as CCR5, preserving some level of cellular migration. Furthermore, CCR2 and CCR5 have significant overlap in signalling machinery[258][38], and engagement of CCR5 alone could mediate some of the effects performed by CCR2, again promoting migration in its absence.

These findings imply that some compensatory mechanism may also be regulating iCCR expression, suggesting that any phenotype emerging from the inhibition of a single receptor could be 'masked' by the upregulation of others. It is only by generating a mouse in which the whole iCCR locus has been deleted that the issue of ICCR redundancy/compensation can be fully addressed.

One of the main concerns in generating an iCCR $^{-/-}$ mouse was the effect that a whole cluster deletion would have on murine development. Some leukocytes (such as monocytes) are necessary for vital processes of tissue formation and maintenance in embryogenesis, and while studies in single knock-outs have shown no obvious developmental abnormalities, a developmental phenotype might only emerge if the migration of these cells is completely abrogated when all the main inflammatory chemokine receptors are inhibited simultaneously.

3.2 Role of Monocytes in Development

Before being able to replenish tissue macrophages and DCs in the steady state, mediate direct antimicrobial activities and affect tissue development, monocytes and dendritic cell precursors (MDPs) have to egress from the bone marrow into circulation[259].

Generally, classical monocytes can remain in circulation for up to two days expressing high levels of CCR2 to respond to localised CCL2 induced by tissue injury or infection. If they have not been recruited to tissues, monocytes undergo apoptosis and are removed from the circulation[189]. If diapedesis does occur, then other cytokines in the microenvironment alter the monocyte's fate. Generally, terminal differentiation of monocytes to macrophages is achieved by exposure to their primary growth and differentiation factor CSF-1, which is secreted by a number of cell types, including epithelial cells [186].

Alternatively, classical CCR2+ Ly6Chi monocytes recruited to sites of infection can also differentiate into dendritic cells that produce TNFα and iNOS which contribute to innate defence against pathogens such as *L. monocytogenes, M. tuberculosis* and various protozoa (*Plasmodium, Toxoplasma* and *Leishmania*)[260].

Remodelling deficiencies in the absence of macrophages have been noted in several tissues, including the mammary gland, kidney and pancreas, suggesting a general requirement for macrophages in tissue patterning and branching morphogenesis[261]. Skin-resident macrophages have recently been implicated in epithelial hair follicle stem cell activation during the murine hair cycle and their ablation resulted in hair growth inhibition [262]. Depletion of uterine and ovarian macrophages demonstrated their importance in normal progesterone synthesis in early pregnancy, which ultimately results in complete implantation failure[263]. In stem cell biology, similar roles for macrophages have been suggested in the maintenance of intestinal integrity and hepatic regeneration after damage [264].

Unexpected roles for macrophages in development can also be observed in erythropoiesis, where maturing erythroblasts surround macrophages that ingest the extruded erythrocyte nuclei[265]. In their absence, erythropoiesis is blocked and lethality soon ensues. In addition, spleen and liver macrophages are responsible for maintaining the haematopoietic steady state through engulfment of neutrophils and erythrocytes and depleting these macrophage populations results in neutropenia, splenomegaly and reduced body weight[266]. Macrophages also regulate angiogenesis through a number of mechanisms. Specifically, in the retina, macrophages secrete WNT, which in turn induces the expression of soluble VEGFR1. This ultimately reduces the availability of VEGF to the vascular endothelium, reducing branching and vascular complexity so that the vascular system is appropriately patterned[267]. Brain development is also controlled by macrophages, as microglia have been shown to promote neuron viability, modulate neuronal activity[268] and prune synapses during development[269], as well as expressing a range of neuronal growth and survival factors, including NGF[270].

While the role of macrophages in development is becoming clearer, our understanding of the role of inflammatory chemokines in these processes is still at an early stage and limited to the study of single chemokine receptors knock outs.

3.3 Effect of iCCR Deletion on Murine Development

Homozygous iCCR$^{-/-}$ mice were born at the expected Mendelian frequency from het/het crosses and were healthy and fertile. No differences in embryo size, foetal liver monocytes or cKIT+ haematopoietic progenitor cells were observed[271], suggesting that iCCRs are not essential for embryonic developmental and postnatal survival, and their deletion does not result in gross developmental abnormalities. Once confirmed that no overt developmental phenotype was present, various iCCR$^{-/-}$ adult tissues were collected, processed and analysed (both at rest and under various inflammatory stimuli) to assess the extent of cellular migration and distribution of cellular subpopulations to specific tissues in the absence of CCR1 CCR2 CCR3 and CCR5.

3.4 Effect of iCCR Deletion on Resting Tissues

3.4.1 Bone Marrow

The expression of iCCRs in the bone marrow and their role in leukocyte mobilisation from this niche has been addressed by several studies in the past, often resulting in conflicting information and incompatible mechanisms of action. For example, while the role of CXCR4 in hematopoietic stem cell migration has been known for some time[272][273], more recently some groups have uncovered a potential role for CCR3[274], CCR1[275] and CCR2[276]. Similarly, a study by Flaishon et al. suggested that expression of CCR2 in immature B-cells is required to retain these cells in the bone marrow, as CCR2$^{-/-}$ B-cells exhibit up-regulation of chemokine-induced actin polymerisation, migration and increased homing of immature B-cells to the lymph node[83]. While in this study CCR2 expression was assessed by PCR, other groups failed to find functional CCR2 protein on those same cells[277], suggesting that even if CCR2 is detectable at the transcript level, there would not be enough protein to mediate any effects.

In contrast to B-cell precursors, the roles that specific iCCRs have in regulating migration and function of other leukocyte subsets is a lot more obvious and unambiguous. For example, the role of CCR2 in monocyte mobilisation from the bone marrow is well described[257] both in resting conditions[73] and inflammation[74]. Deletion of CCR2 ligands, CCL2 or CCL7, was shown to diminish the recruitment of monocytes during

infection by approximately 50% [278]. Lack of CCR2 engagement does not allow for actin remodelling required for bone marrow extravasation, resulting in an accumulation of Ly6Chigh leukocytes in the bone marrow, severe impairment of the peripheral reconstitution of inflammatory monocytes and increased mortality in infectious models of inflammation[279][74].

To assess the effects of deletion of the 4 main iCCRs on the cellular composition of the bone marrow, iCCR$^{-/-}$ bone marrow was extracted and compared to WT, CCR1, CCR2, CCR3 and CCR5 single knock-out mice.

In all strains, neutrophils were the most prominent bone marrow leukocyte subset, representing approximately 35% of all live cells recovered. Immature B-cell numbers were the second most prominent subset, representing approximately 10% of all live cells in all strains examined. None of these lineages were affected in the iCCR$^{-/-}$. Similarly, T-cell precursor numbers (~3%) and dendritic cells were also not affected by the iCCR deletion (Figure 3-1A).

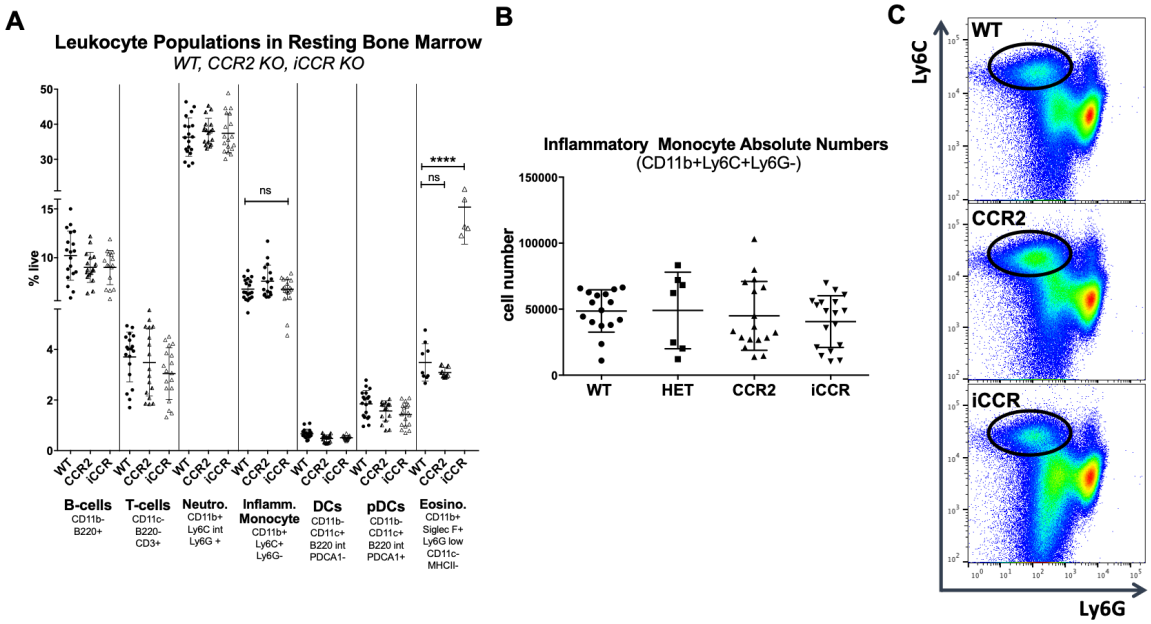

Figure 3-1 Leukocyte Subpopulations in Resting Bone Marrow. A *– Bone marrow leukocyte subpopulations in resting bone marrow from WT, CCR2$^{-/-}$ and iCCR$^{-/-}$ mice.* ***B****- Normalised Inflammatory Monocyte numbers in WT, Het, CCR2$^{-/-}$ and iCCR$^{-/-}$ bone marrow.* ***C****- Representative FACS plots showing inflammatory monocytes (CD45+ CD11b+Ly6C++Ly6G-) in CCR2 and iCCR$^{-/-}$ bone marrow compared to WT. Ordinary one-way ANOVA with Dunnett's multiple comparisons test was performed to determine statistical significance, with a p value of 0.05 determined as significant, n>3.*

Curiously, even inflammatory monocytes were not affected, with no significant accumulation observed in the iCCR$^{-/-}$ (WT: 6.79%, iCCR$^{-/-}$: 7.15%) and in the CCR2$^{-/-}$ bone marrow (7.86%) (Figure 3-1A,C). As this was unexpected, further experiments using

counting beads to determine actual numbers of inflammatory monocytes confirmed that there was no difference in the number of inflammatory monocytes at rest in WT, CCR2 and iCCR$^{-/-}$ bone marrow (Figure 3-1B), in contrast with previous studies on CCR2$^{-/-}$ mice[74][280].

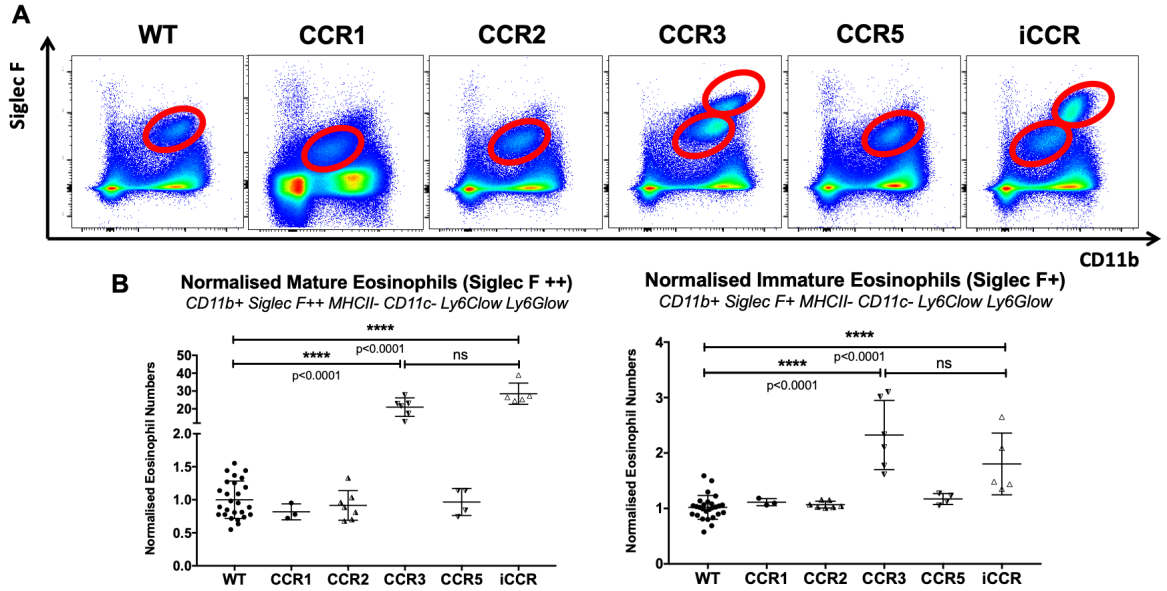

Figure 3-2 Eosinophil Subpopulations in Resting Bone Marrow- A*- Representative FACS plots showing mature (Siglec F++) and Immature (Siglec F+) Eosinophil populations in WT, CCR1, CCR2, CCR3, CCR5 and iCCR$^{-/-}$ bone marrow.* ***B****- Graphs summarising normalised mature (Siglec F++) and immature (Siglec F+) eosinophil numbers in resting bone marrow in each iCCR$^{-/-}$ strain. Ordinary one-way ANOVA with Dunnett's multiple comparisons test was performed to determine statistical significance, with a p value of 0.05 determined as significant, n>3.*

Eosinophils were the only major leukocyte subset showing profound differences when comparing WT, CCR2 and iCCR$^{-/-}$ resting bone marrow sub-populations. As seen from the representative FACS plots in Figure 3-2A, 2 populations of eosinophils can be found in the resting bone marrow of iCCR$^{-/-}$ mice, a Siglec F+ and Siglec F bright population. Analysis of bone marrow from CCR1, CCR3 and CCR5 single knock-out mice showed that the phenotype observed in the iCCR$^{-/-}$ is attributable to CCR3 deletion only (Figure 3-2B,C), as the CCR3$^{-/-}$ also shows the presence of two eosinophil populations (Figure 3-2A). These results suggest that CCR3 might be required for bone marrow egress of mature (Siglec F bright) eosinophils. Furthermore, the absence of this Siglec F bright populations from WT, CCR1, CCR2 and CCR5$^{-/-}$ bone marrow may suggest that CCR3 is upregulated only when eosinophils mature from Siglec F+ to Siglec F++. As soon as a mature eosinophil expresses CCR3, extravasation into the blood can occur, explaining why only a single Siglec F+ eosinophil population was detectable in the CCR1, CCR2 and CCR5$^{-/-}$ strains.

3.4.2 Blood

Analysis of peripheral blood leukocytes showed a marked reduction in CD11b+ F480 low cells in iCCR$^{-/-}$ circulation (FACS plots Figure 3-3A). Further gating with Ly6C showed that this overall reduction is attributable to a decrease in two main subpopulations (Figure 3-3B,C), the inflammatory monocytes (CD11b+ F480 low Ly6C++), and patrolling monocytes (CD11b+ F480 low Ly6C low) both of which reportedly express CCR2 [276][187].

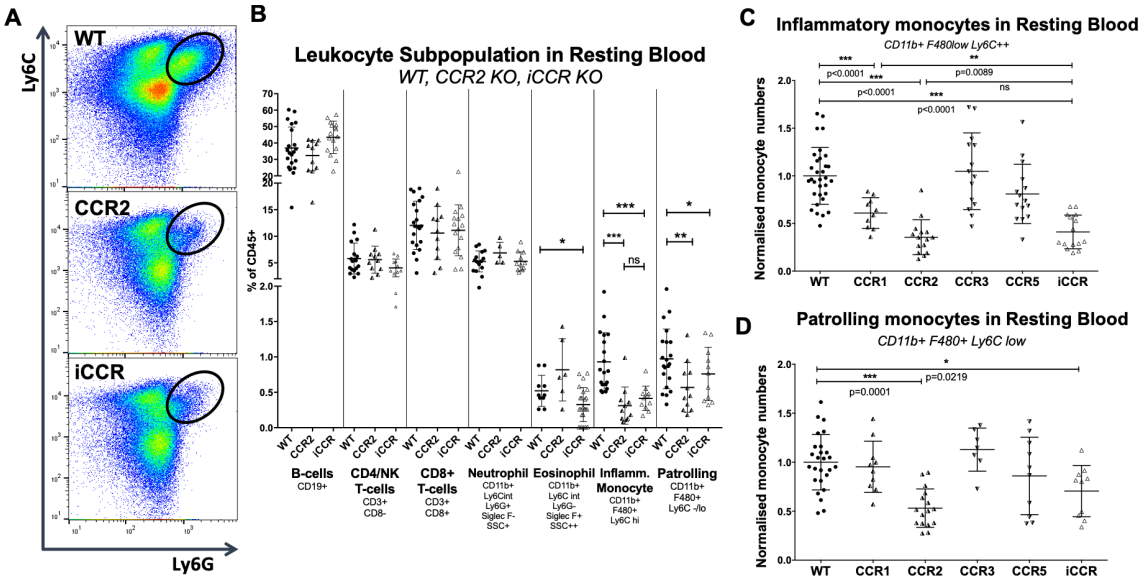

Figure 3-3 Leukocyte Subpopulations in Resting Blood- A- *Representative FACS plots showing the reduction in CD11b+F480 low cells in CCR2$^{-/-}$ and iCCR$^{-/-}$ circulation.* ***B-*** *Graph summarising the distribution of several leukocyte subsets in WT, CCR2 $^{-/-}$ and iCCR$^{-/-}$ resting circulation.* ***C****- Summary of normalised inflammatory monocyte (F480 lo Ly6C++) numbers from WT, CCR1$^{-/-}$, CCR2$^{-/-}$, CCR3$^{-/-}$ and CCR5$^{-/-}$ blood.* ***D****- Summary of normalised patrolling (F480+Ly6C low) monocyte numbers from WT, CCR1$^{-/-}$, CCR2$^{-/-}$, CCR3$^{-/-}$ and CCR5$^{-/-}$ blood. Ordinary one-way ANOVA with Dunnett's multiple comparisons test was performed to determine statistical significance, with a p value of 0.05 determined as significant, n>3*

CCR2$^{-/-}$ mice displayed a similar reduction in both circulating Ly6C++ monocytes (WT: 0.93%, CCR2$^{-/-}$: 0.31%, iCCR$^{-/-}$: 0.41%) and patrolling macrophages (WT: 0.97% , CCR2$^{-/-}$: 0.58%, iCCR$^{-/-}$: 0.76%), indicating that, in terms of peripheral blood leukocyte subpopulations, iCCR$^{-/-}$ mice are essentially a phenocopy of CCR2$^{-/-}$ mice.

There was also a modest but significant reduction in circulating Ly6C++ monocytes in the blood of CCR1$^{-/-}$ mice (WT: 1.23%, CCR1$^{-/-}$ 0.81%). While this defect has been mentioned in the literature before [281], the relevance is unclear as the overall defect observed in iCCR$^{-/-}$ mice can be wholly attributed to CCR2, with no evidence of an additive reduction brought by the absence of CCR1 in iCCR$^{-/-}$ circulation. Eosinophils were also reduced in iCCR$^{-/-}$ circulation (Figure 3-3B).

While the data presented so far compared blood and bone marrow across all knock out strains, the remaining analysis on resting tissues will focus on WT, CCR2$^{-/-}$ and iCCR$^{-/-}$ KO samples. A more complete analysis including CCR1, CCR3 and CCR5$^{-/-}$ skin, lung and spleen samples was performed by Dr. Dyer and Dr. Medina-Ruiz[271].

3.4.3 Skin

Various organs, such as skin, lung and spleen were also analysed to determine if the observed defects found in iCCR$^{-/-}$ blood and bone marrow would amplify as the leukocytes continue to migrate towards their target organ. Indeed, just by looking at the number of CD45+ cells in resting skin samples it was apparent that iCCR$^{-/-}$ skin contained fewer leukocytes when compared to the WT (WT: 10.5% of live cells, iCCR$^{-/-}$: 3.5%, Figure 3-4A). While CCR2$^{-/-}$ skin also had a reduction in skin CD45+ cell content (6.5%, Figure 3-4A,C), the defect was more pronounced in iCCR$^{-/-}$ skin, suggesting an additional role for CCR1, CCR3 and CCR5.

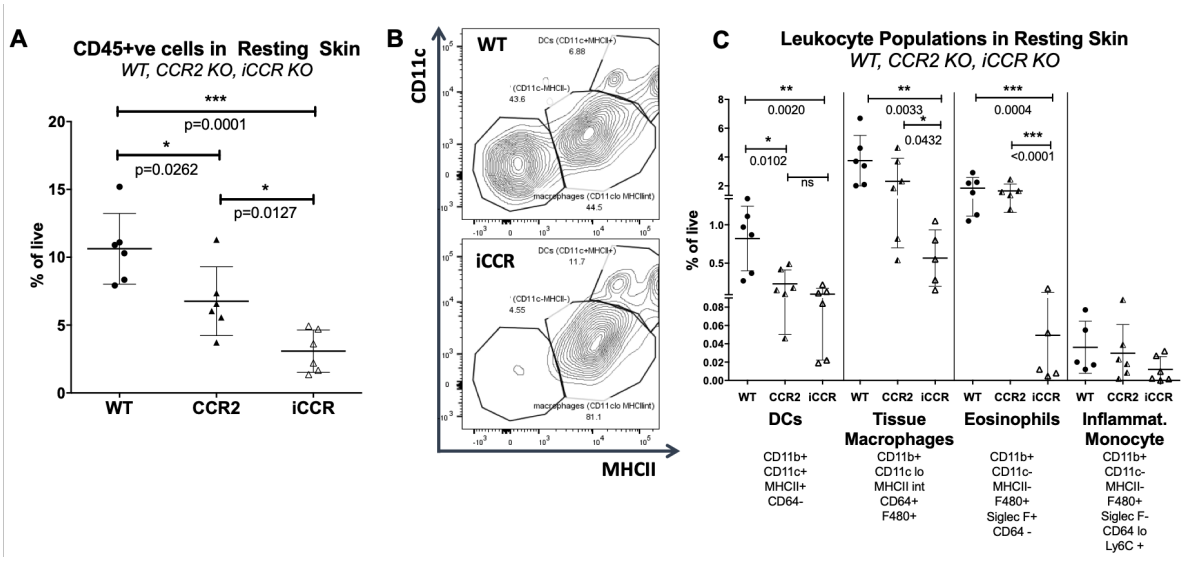

***Figure 3-4 - Leukocyte Subpopulations in Resting Skin. A**- Number of CD45+ cells (expressed as % of live) in excised resting skin from WT, CCR2 and iCCR$^{-/-}$. **B**-- Representative FACS plots (after CD45 and CD11b gating) showing 3 main subpopulations in WT and iCCR$^{-/-}$ skin: CD11c+MHCII+, CD11c+ MHCII int and CD11c-MHCII- representing dendritic cells, macrophages, and monocytes/eosinophils respectively. **C**- Graph summarising the distribution of different leukocyte subpopulations in WT, CCR2 and iCCR$^{-/-}$ resting skin. Data representative of one experiment with n>4. Ordinary one-way ANOVA with Dunnett's multiple comparisons test was performed to determine statistical significance, with a p value of 0.05 determined as significant.*

Further flow cytometric analysis on CD45+ CD11b+ cells revealed a substantial reduction in 3 distinct populations in iCCR$^{-/-}$ skin: CD11c-MHCII-, CD11cloMHCII int and CD11c+MHCII++, corresponding to monocytes/eosinophils, tissue macrophages and dendritic cells respectively (Figure 3-4B). Whilst both iCCR$^{-/-}$ and CCR2$^{-/-}$ skin displayed a similar ~50% decrease in DC numbers, the reduction observed in macrophage numbers was significantly greater in iCCR$^{-/-}$ mice (~65% reduction against 80% reduction in the full knock-outs) (Figure 3-4C)

Furthermore, while the defects in eosinophils were also seen in CCR3$^{-/-}$ skin[271], tissue macrophage and DC numbers were unaltered in CCR1, CCR3 and CCR5$^{-/-}$ [271], indicating that no single receptor can account for the substantial reduction in the aforementioned leukocyte subsets. Together, these data suggest that multiple iCCRs can contribute to the effective establishment of myeloid leukocyte subpopulations in the skin, hinting at some degree of redundancy of receptor involvement which was not observed in either blood or bone marrow. No difference was detected in any lymphoid cell subtype in resting skin of any of the single receptor null mice[271].

3.4.4 Spleen and Lung

Leukocyte distribution in resting spleen generally recapitulated the same defects observed in resting circulation, with lymphoid cell populations unaffected and a decrease in Ly6C++ inflammatory monocytes, observed in both iCCR and CCR2$^{-/-}$ mice (Figure 3-5 A,B). Unlike tissue macrophages in the skin however, red pulp macrophage numbers (a tissue specific set of tissue macrophages) were unaffected by iCCR deletion. This was expected, as it has been shown that red pulp macrophages differ from other resident macrophage populations not just in function but also origin[282].

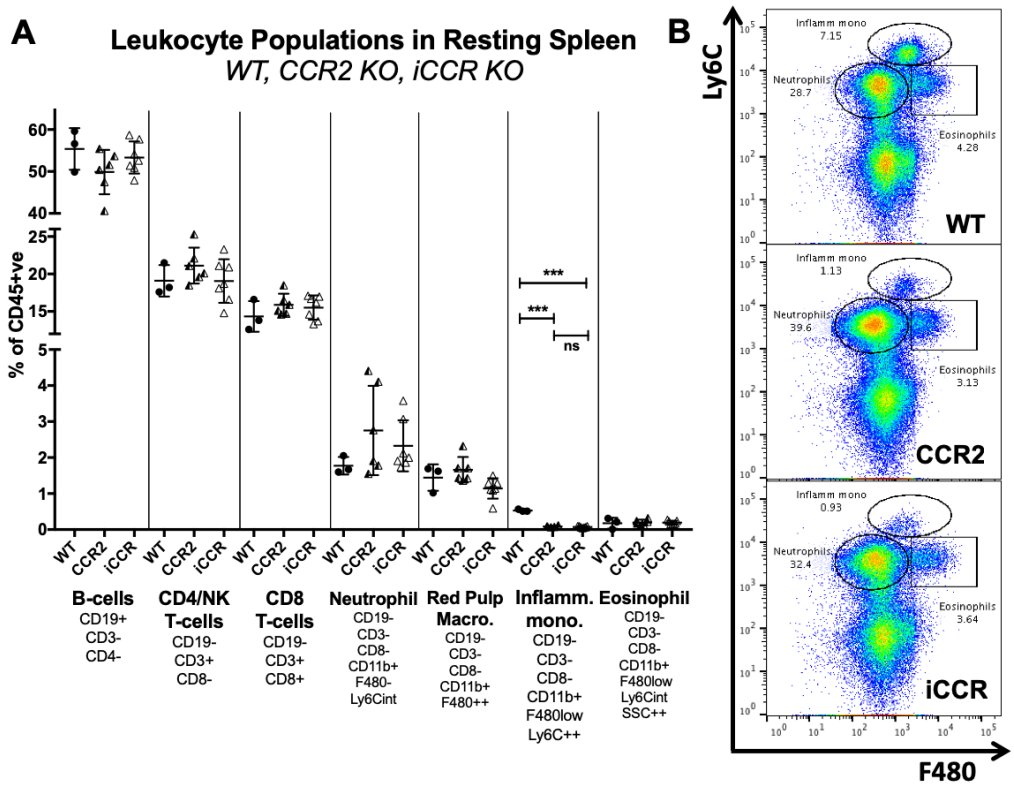

Figure 3-5- Leukocyte Subpopulations in Resting Spleen. A*- Graph summarising leukocyte distribution in the spleen of resting WT, CCR2 and iCCR $^{-/-}$ mice.* ***B-*** *Representative FACS plots showing spleen Inflammatory monocytes (Ly6C++ F480 low) and eosinophils (Ly6Cint F480+). Data representative of one experiment with n>4. Ordinary one-way ANOVA with Dunnett's multiple comparisons test was performed to determine statistical significance, with a p value of 0.05 determined as significant*

During foetal development, macrophages derived from embryonic yolk sac and foetal liver precursors are seeded throughout various tissues in a CCR2 independent manner [283][284][285] and persist in the adult as resident, self-maintaining populations. These include microglia in the brain, red pulp macrophages in the spleen and alveolar macrophages in the lung, all of which turn over locally under steady state conditions and perform a variety of clearance and organ-specific trophic functions, such as pruning of the neuronal synapses[269] or clearance of damaged or senescent erythrocytes in the spleen[282].

After birth, CCR2+ bone marrow-derived blood monocytes replenish resident macrophage populations with high turnover[286] in organs with direct contact with the external environment, such as the skin, lung or gut. These inflammatory monocytes are recruited in larger numbers following infection or injury, giving rise to infiltrating activated tissue macrophages. It is therefore unsurprising that red pulp macrophage numbers in the spleen (Figure 3-5A) and alveolar macrophage numbers in the lung (Figure 3-6A) were unaffected by iCCR deletion, while both organs also showed a lack of Ly6C+ inflammatory monocytes

(Figure 3-6C, Representative FACS plots Figure 3-6B). Similarly to the resting spleen, resting lung lymphoid cell numbers were also unaltered (Figure 3-6A).

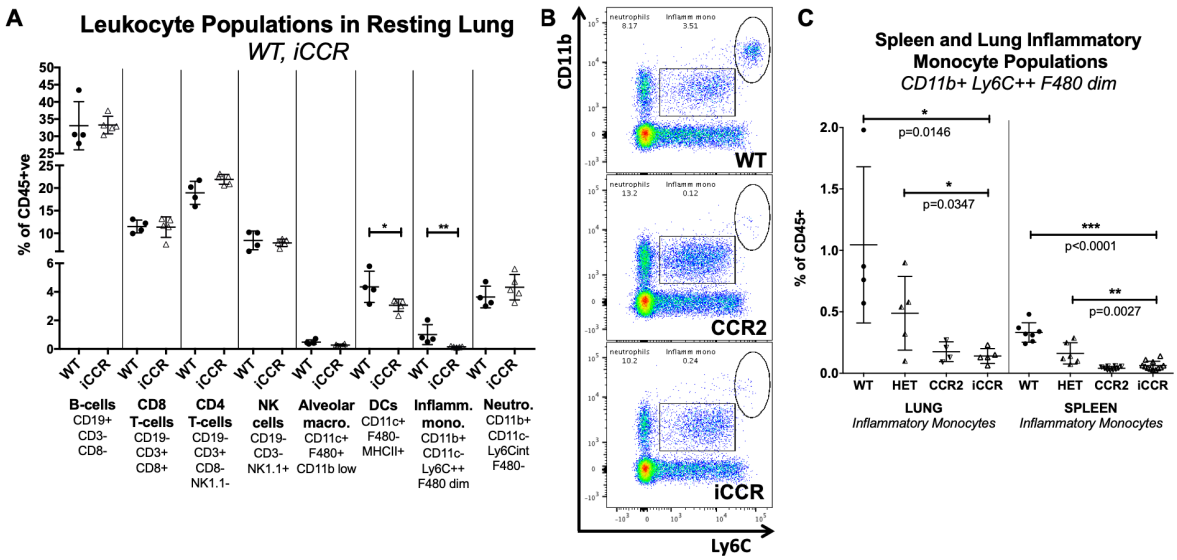

***Figure 3-6- Leukocyte Subpopulations in Resting Lung**. **A**- Graph summarising leukocyte distribution in the lung of resting WT and iCCR $^{-/-}$ mice. **B**- Representative FACS plots showing lung Inflammatory monocytes (CD11b+ Ly6C++) in WT, CCR2 and iCCR KO mice. **C**- Summary and comparison of spleen and lung inflammatory monocytes (CD11b+ F480 low Ly6C++) from WT, CCR2, iCCR $^{-/-}$ and iCCR $^{-/+}$ heterozygous mice. Data representative of one experiment with n>4. Ordinary one-way ANOVA with Dunnett's multiple comparisons test was performed to determine statistical significance, with a p value of 0.05 determined as significant.*

Dendritic cell numbers were also found reduced in iCCR$^{-/-}$ lung tissues (Figure 3-6A), and this minor defect was also observed in CCR2$^{-/-}$ lungs[271]. While a defect in chemotaxis of DC precursors might explain this phenotype, other groups have also reported altered DC activation and function in CCR2$^{-/-}$ lungs characterised by abrogated MHCII and CD40 expression in these cells[102], hinting at an additional role for CCR2 in supporting function, maturation and proliferation of dendritic cells *in situ*.

3.4.4.1 Lung Alveolar Macrophages

Broncho-alveolar lavages of iCCR$^{-/-}$ and WT lungs confirm that local tissue resident populations are unaffected by iCCR deletion (Figure 3-7 Ai, Representative FACS plots 3-7Aii). While the number of cells recovered from lung lavages after exposure to LPS was lower in iCCR$^{-/-}$ lungs compared to WT (Figure 3-7A), flow cytometric analysis of the cellular content revealed this decrease was attributable to impaired inflammatory monocyte and dendritic cell recruitment (Figure 3-7B), while the numbers of neutrophils and alveolar macrophages were unaffected by iCCR deletion (Figure 3-7B and Representative FACS plots 3-7C).

While there have been reports in the literature of recruited inflammatory monocytes replacing alveolar macrophages during inflammation[287], the fact that the number of alveolar macrophages recovered from WT and iCCR$^{-/-}$ lung lavages is identical (Figure 3-7B) suggests that alveolar macrophages are maintained via self-renewal, with minimal input from recruited blood inflammatory monocyte in cases of acute inflammation[288][289].

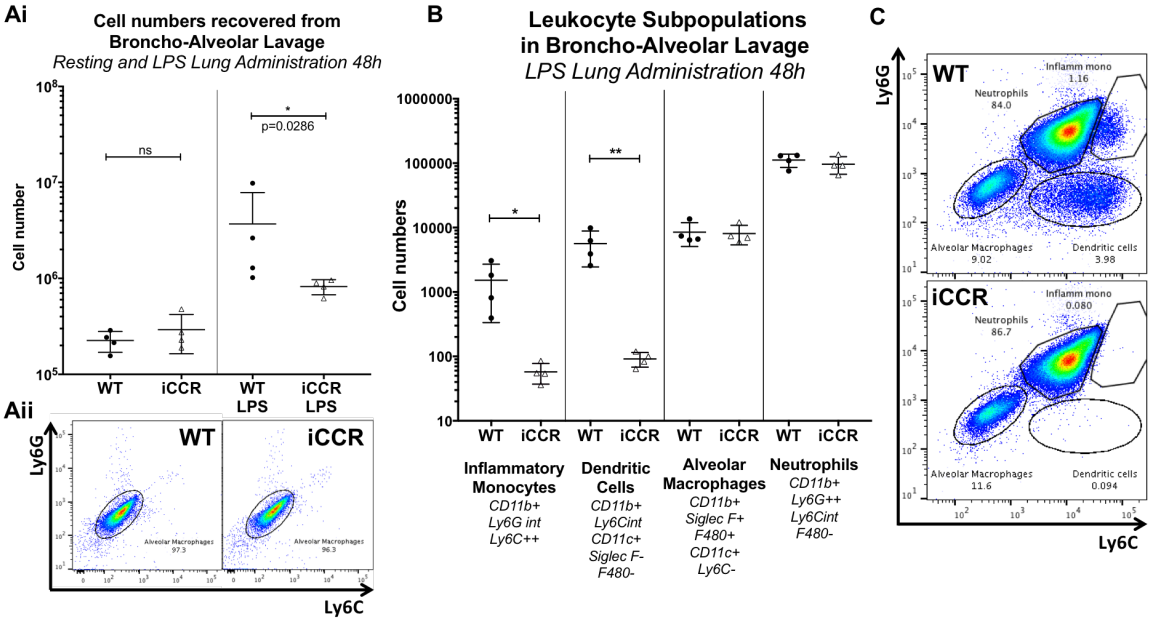

Figure 3-7- Alveolar Macrophage Populations in iCCR$^{-/-}$ Lungs. ***A****- Pooled cells recovered after 3 1ml broncho-alveolar lavages (BALs) with PBS+EDTA of WT and iCCR $^{-/-}$ lungs.* ***Aii****- Representative FACS plots of resting BAL in WT and iCCR $^{-/-}$ lungs showing alveolar macrophages (CD11b+ Ly6C- Ly6G- F480+ SiglecF+ CD11c+) are the only cell type present in the alveolar space under resting conditions.* ***B****- Graph showing the different cell types recovered from BALs in WT and iCCR $^{-/-}$ lungs 48 hours after the lungs were exposed to a single dose of LPS.* ***C****- Representative FACS plots showing an absence of dendritic cells and inflammatory monocytes in BALs of inflamed iCCR $^{-/-}$ lungs compared to WT. Ordinary one-way ANOVA with Dunnett's multiple comparisons test was performed to determine statistical significance, with a p value of 0.05 determined as significant. Data representative of one experiment with n=4.*

Overall, the data so far show that resting iCCR$^{-/-}$ tissues are characterised by comprehensive depletion of a range of myelomonocytic cell subtypes which varies according to tissue type. Inflammatory monocyte and eosinophil numbers are profoundly affected, although the absence of CCR2 and CCR3 are generally enough to explain all the phenotypes observed in the full iCCR$^{-/-}$. The only evidence so far of a possible contribution of other iCCRs in directing leukocytes to different organs was found in the skin, where a decrease in skin macrophages in iCCR$^{-/-}$ mice could not be completely explained by the absence of CCR2. Tissue resident populations with distinct phylogenetic origin, such as alveolar macrophages in the lung or red-pulp macrophages in the spleen, were not affected by iCCR deletion.

At this stage however, it was still unclear if impaired chemotaxis was wholly responsible for the observed phenotype (i.e. decreased chemotaxis when CCR1, CCR2 and CCR5 are all absent), or if the absence of certain iCCRs is preventing maturation and proliferation of certain leukocyte subsets, further exacerbating the defect. In other words, while the absence of CCR2 would explain 65% of the reduction in skin tissue resident macrophages, it is unclear if the 80% reduction seen in the full knock-out is caused by impaired chemotaxis caused by the absence of CCR1 and CCR5, or if the lack of CCR1 and CCR5 decreases the ability of skin tissue macrophages to mature and proliferate, reducing their overall numbers.

3.5 Effect of iCCR deletion on Inflammation

3.5.1 Skin Inflammation- Overview

After establishing the 'resting' phenotype of iCCR$^{-/-}$ leukocyte subpopulations in most organs, the mice were then exposed to a variety of inflammatory agents to uncover the effect of iCCR deletion on the development of the inflammatory response.

Aldara cream was initially chosen as a model for skin inflammation, mainly due to ease of application and the brief time between the first application and analysis[245]. Briefly, the back of the mice was shaved and ¼ of a sachet of Aldara cream was applied to the shaved area daily for 4 consecutive days. Mice were then culled, and various tissues (including the skin at the site of direct application) were collected, digested, and analysed via flow cytometry.

Aldara is routinely used as a topical treatment for a variety of skin conditions, including genital warts and small superficial skin cancers[245]. The active ingredient, Imiquimod, is a TLR7 agonist. Toll-like-receptor 7 is usually expressed in the endosomes of macrophages and dendritic cells, and detects single stranded RNA, a common feature of viral genomes that have either been endocytosed directly by the cell or are already present inside the cytoplasm after infection. Activation of TLR7 induces a potent anti-viral response, characterised by the production of IL-1β, IFNy, TNFα and other pro-inflammatory cytokines that activate local cells and induce the recruitment of blood leukocytes to the

site of inflammation[290]. This strong and localised inflammatory response can override immune evasion mechanisms used by viruses or small cancers to persist in the skin, ultimately causing warts and basal cell carcinomas to regress and fade away[291].

3.5.2 Imiquimod Induced Skin Inflammation

After 4 days of repeated Aldara cream application and before analysing tissues, skin thickness from iCCR$^{-/-}$, CCR2$^{-/-}$ and WT mice was measured using a caliper. As shown in Figure 3-8A, iCCR$^{-/-}$ skin showed reduced thickness when compared to inflamed WT and CCR2$^{-/-}$ skin, suggesting reduced localised inflammation. Histological analysis of involved skin performed by Dr. Dyer also showed a reduction in the number of inflammatory cells in the dermis [271], suggesting either reduced recruitment, lack of proliferation at the site of Aldara cream application or a combination of both. iCCR$^{-/-}$ mice also show reduced Spleen/Body mass ratio compared to WT, indicating reduced systemic inflammation after 4 days of Aldara cream application to the skin (Figure 3-8B).

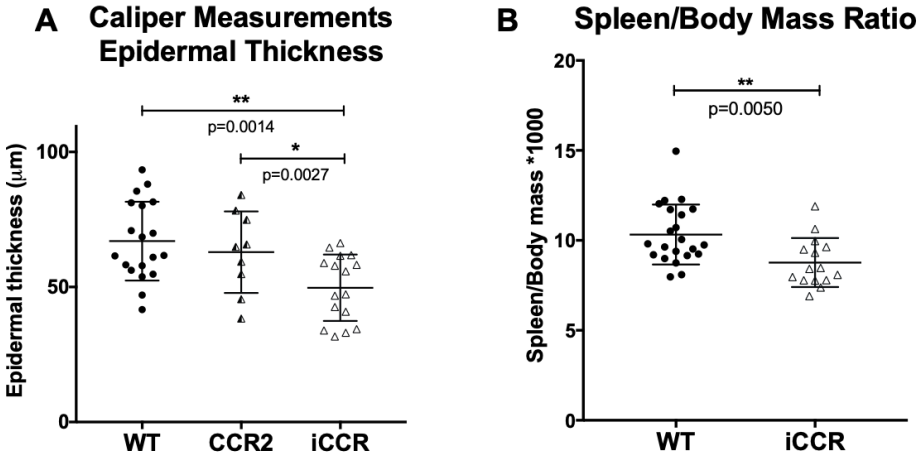

***Figure 3-8- Effects of Imiquimod Treatment to Murine Skin**. **A**- Caliper measurements of skin thickness after Aldara treatment to the skin in WT, CCR2 and iCCR $^{-/-}$ mice. **B**- Graph summarising spleen to body mass ratio from WT and iCCR Imiquimod treated mice.*

The site of skin application was also assessed for redness (erythema), scaling and thickness, the three main indices used to measure the severity of psoriasis. The scores for each were then combined to determine a single PASI score (Psoriasis Area and Severity Index) that would represent the severity of the lesion of the affected area on a scale from 0 (no disease) to 72 (maximal disease). As seen in Figure 3-9, iCCR$^{-/-}$ skin showed reduced erythema, scaling and thickness, with a combined average PASI score at day 5 of 21, while WT skin scored an average of 27. While iCCR$^{-/-}$ skin generally scored lower than WT skin, the results were highly variable and overall difference was not statistically significant.

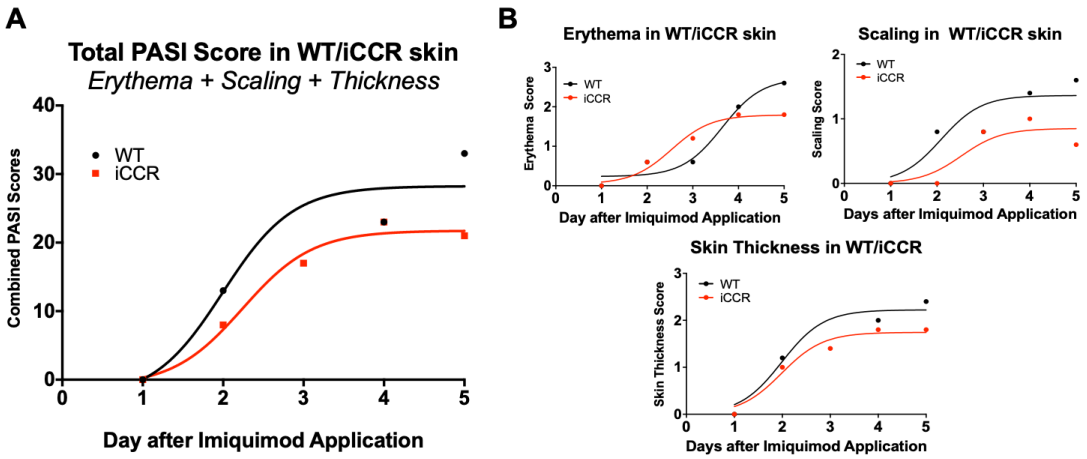

Figure 3-9 Severity of Erythema, Scaling and Thickness of Imiquimod Inflamed Skin in WT and iCCR $^{-/-}$ mice- A*- Daily summary of the progression of severity of skin inflammation in WT and iCCR$^{-/-}$ mice, measured as a combined PASI score combining Erythema, Scaling and Skin thickness scores.* ***B****- Summary of the development of Erythema, Scaling and Skin thickness from day 1 of Imiquimod application to day 5 (culling day) in WT (black) and ICCR $^{-/-}$ (red) skin. 1= no symptom, 3= maximum severity.*

3.5.3 Bone Marrow and Blood Subpopulations- Imiquimod

Bone marrow, blood and affected skin were also collected and analysed via flow cytometry to assess the cellular composition of these organs during inflammation. After exposure to Imiquimod, Ly6C++ inflammatory monocytes were found elevated in both CCR2$^{-/-}$ and iCCR$^{-/-}$ bone marrow (WT: 15.3%, CCR2$^{-/-}$: 19.3%, iCCR$^{-/-}$: 19.3%, Figure 3-10A, 3-10B), in line with previous studies showing an accumulation of inflammatory monocytes in the bone marrow after CCR2 deletion during inflammation[292][74]. Similarly to resting blood, circulating inflammatory monocytes are reduced in Imiquimod treated iCCR$^{-/-}$ (Figure 3-11A), but during inflammation this defect becomes more severe. This time, while inflammatory monocytes constitute on average 10% of CD45+ cells in WT circulation, iCCR$^{-/-}$ Ly6C++ monocytes are found at significantly lower levels (4%) than in CCR2$^{-/-}$ circulation (6%) (Figure 3-11 B), suggesting an additional role of CCR1 and CCR5 in recruitment of inflammatory monocytes during Imiquimod-induced inflammation.

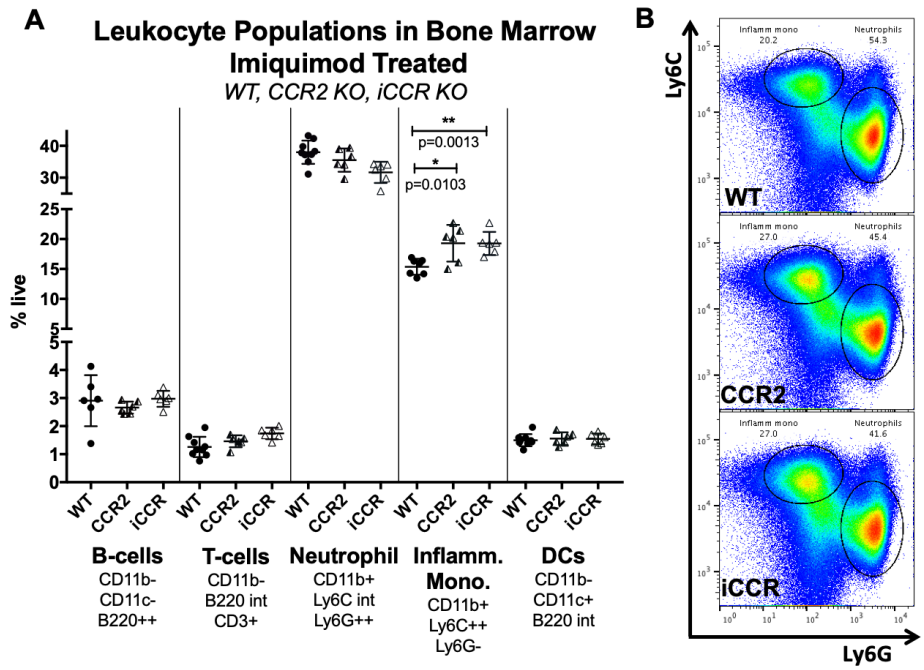

***Figure 3-10- Bone Marrow leukocyte subpopulation in Imiquimod Treated Mice. A** – Bone marrow leukocyte subpopulations in Inflamed bone marrow from WT, CCR2 and iCCR $^{-/-}$ mice. **B**- Representative FACS plots from WT, CCR2 and iCCR $^{-/-}$ bone marrow showing the distribution of inflammatory monocytes (Ly6C++ Ly6G-) and neutrophils (Ly6C int Ly6G++). Ordinary one-way ANOVA with Dunnett's multiple comparisons test was performed to determine statistical significance, with a p value of 0.05 determined as significant. Data representative of one experiment with n=5.*

Furthermore, alterations in circulating lymphoid subpopulations were observed for the first time in iCCR$^{-/-}$. As seen in Figure 3-11 C, both NK and CD8+ CD44+ T-cells appear elevated in iCCR$^{-/-}$ blood, and this difference does not seem to be attributable to CCR2, as CCR2$^{-/-}$ blood shows normal T-cell numbers (NK- WT: 8.8%, CCR2$^{-/-}$: 9.2%, iCCR$^{-/-}$: 17.8%. CD8mem- WT: 2.0%, CCR2$^{-/-}$: 3.2%, iCCR$^{-/-}$: 4.5%).

Activated T-cells upregulate CD44[293], and are known to express CCR3, CCR5[294] and NK cells CXCR4, but also CCR1, CCR2 and CCR5[295][296]. Lack of some iCCRs might therefore prevent these specific leukocyte subsets from reaching their final destination in the dermis, leading to an accumulation in the circulation.

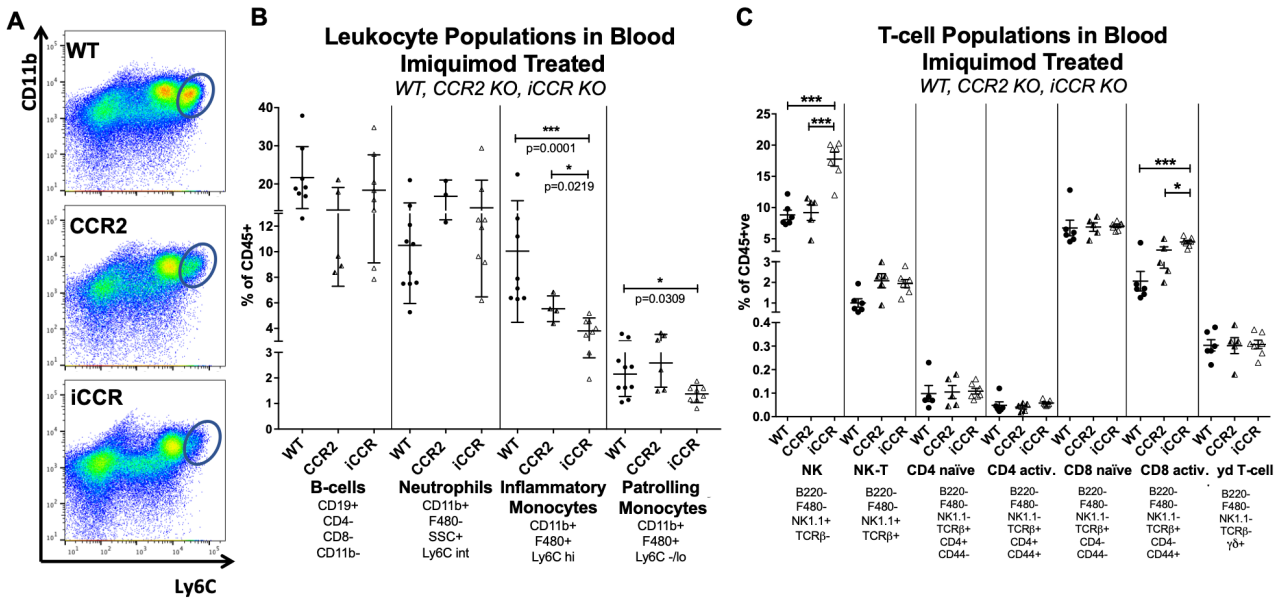

Figure 3-11- Blood leukocyte subpopulation in Imiquimod Treated Mice. A*- Representative FACS plots showing inflammatory monocytes (CD11b+ Ly6C++) in WT, CCR2 and iCCR $^{-/-}$ circulation. Ordinary one-way ANOVA with Dunnett's multiple comparisons test was performed to determine statistical significance, with a p value of 0.05 determined as significant.* ***B****- Blood Leukocyte subpopulations in inflamed blood from WT, CCR2 and iCCR $^{-/-}$ mice.* ***C****- Blood T-cell subpopulations in inflamed blood from WT, CCR2 and iCCR $^{-/-}$ circulation. Blood data representative of two experiments with n=3. T-cell data of one experiment with n=5.*

3.5.4 Skin Subpopulations and Resolution- Imiquimod

The site of cream application was also excised, digested and analysed via flow cytometry. The number of CD45+ cells in skin was significantly lower in iCCR$^{-/-}$ mice (Figure 3-12A), and further analysis showed that tissue macrophages, inflammatory monocytes and monocyte derived dendritic cells were all reduced (Figure 3-12B). Again, this was expected, as monocytes from the bloodstream can differentiate *in situ* into either tissue macrophages or monocyte derived dendritic cells under inflammatory conditions[297][259]. Further analysis in CCR1, CCR2, CCR3 and CCR5$^{-/-}$ skin performed by Dr. Dyer and Dr. Medina-Ruiz showed that the decrease observed in multiple iCCR$^{-/-}$ myeloid leukocyte subsets could be attributed to lack of progenitor monocytes in circulation caused by the deletion of CCR2 exclusively[271].

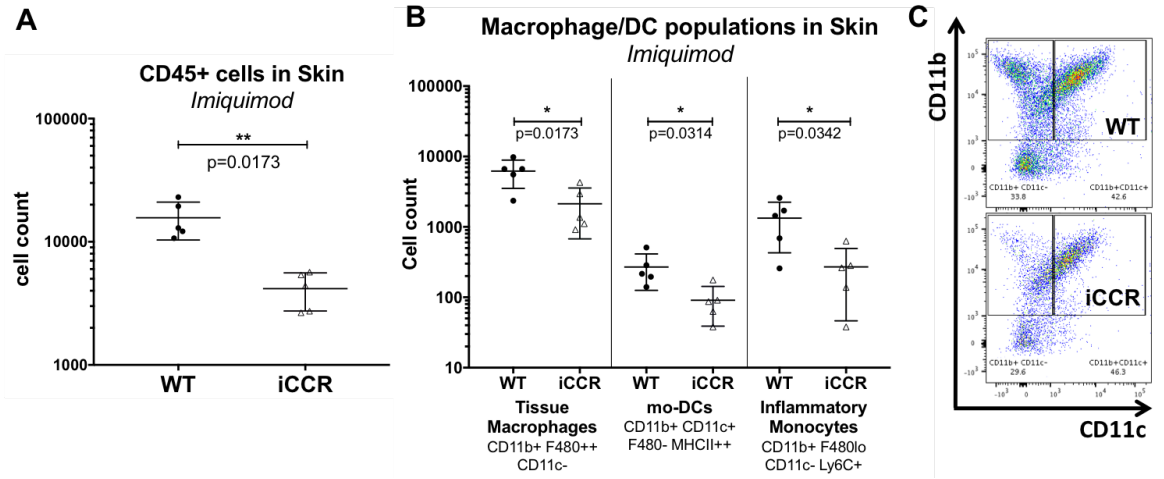

Figure 3-12- Skin Leukocyte Distribution after Imiquimod Induced Inflammation. ***A****- CD45+ cell counts in excised Imiquimod treated skin from WT and iCCR $^{-/-}$ mice.* ***B****- Graph summarising the number of tissue macrophages, monocyte derived DCs and inflammatory monocytes detected in excised inflamed skin samples.* ***C****- Representative FACS plots showing the decrease in CD11b+CD11c- (tissue macrophages and inflammatory monocytes) and CD11b+ CD11c+ (monocyte derived DCs) subpopulations in excised inflamed skin samples. Ordinary one-way ANOVA with Dunnett's multiple comparisons test was performed to determine statistical significance, with a p value of 0.05 determined as significant. Data representative of one experiment with n=5.*

While a reduction in inflammation was indeed observed, it was still unclear if iCCR$^{-/-}$ mice were genuinely incapable of mounting an efficient inflammatory response, or if inflammation was just delayed and leukocytes would eventually be able to reach the target organ using iCCR independent pathways. The ability of iCCR$^{-/-}$ mice to resolve inflammation was therefore also assessed.

Following imiquimod application, the mice were allowed to recover for 1 week, and their tissues were again analysed via flow cytometry to uncover any differences in resolution of inflammation. One week after inflammation, both WT and iCCR$^{-/-}$ tissues showed the same differences previously seen in resting tissues (Figure 3-13 A,B), suggesting that iCCR$^{-/-}$ mice can resolve effectively with no evidence of delayed onset inflammation.

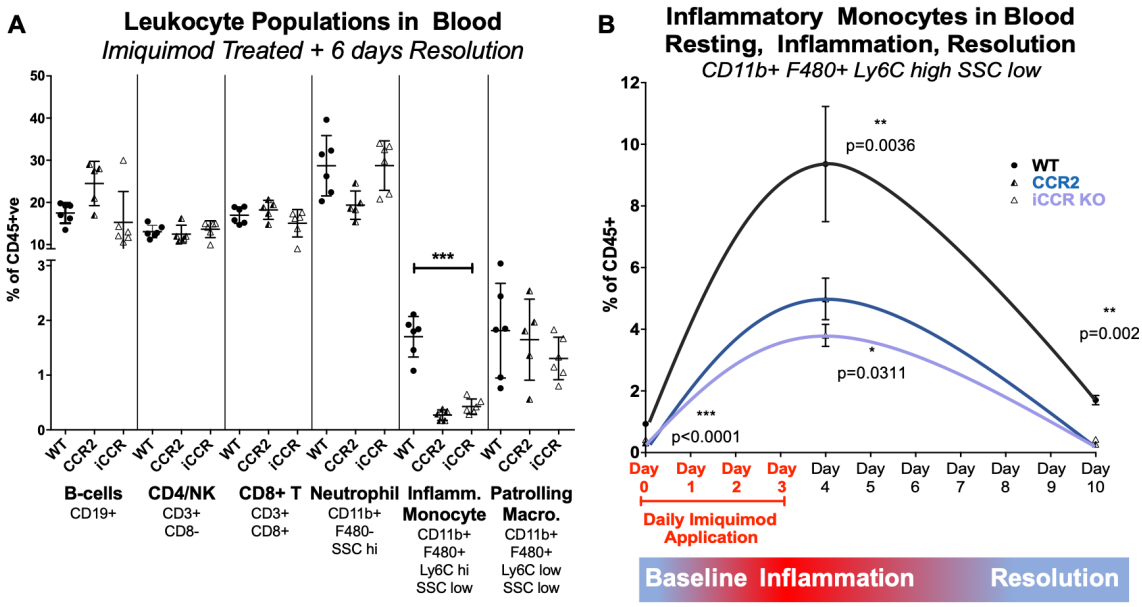

Figure 3-13- Resolution of Inflammation after Imiquimod- A*- Leukocyte Distribution in murine circulation six days after the final application of Imiquimod to the skin.* ***B****- Summary of the fluctuation of circulating inflammatory monocytes before, during and after inflammation (Ly6C++ F480 low) in WT (black), CCR2 $^{-/-}$ (dark blue), iCCR $^{-/-}$ (light blue). Ordinary one-way ANOVA with Dunnett's multiple comparisons test was performed to determine statistical significance, with a p value of 0.05 determined as significant. Data representative of one experiment with n=5.*

3.5.5 Discussion- Imiquimod Model

Collectively, the data generated so far showed that iCCR cluster deletion results in a dramatic reduction in the recruitment of myeloid cells both to resting tissues and to the site of inflammation, and a disruption activated T-cell and NK cell chemotaxis leading to an accumulation of these lymphoid subsets in the circulation. It was also obvious, however, that iCCR$^{-/-}$ mice are indeed capable of mounting an inflammatory response when treated with Imiquimod, as swelling and redness at the site of cream application was readily observed.

It was also surprising that most of the phenotypes observed were generally attributable to the deletion of a single receptor, CCR2, with little evidence of a combinatorial effect brought by the simultaneous deletion of CCR1 CCR3 and CCR5. Differentiated tissue macrophages have been shown to upregulate CCR1 and CCR5, and it has been suggested these two receptors contribute to the recruitment of monocytes into or within inflamed tissues[298][188]. Using this specific model of inflammation however, it appears that the contribution of CCR1 and CCR5 to the maintenance of tissue macrophages and monocyte derived dendritic cell populations in the skin is negligible.

The use of Imiquimod as an inflammation model however might offer an explanation for this discrepancy. Aldara cream has been shown to activate the immune response in TLR7 independent pathways: isosteric acid alone, one of the components of Aldara cream, has been shown to activate the inflammasome directly ultimately causing the release of the pro-inflammatory cytokine IL-1[299]. Other studies have also shown how the administration route of Aldara can have a dramatic impact on the development of skin inflammation. While the effects of Imiquimod ingestion on the development of dermatitis has never been tested before, Grine et al. tried to address the issue by placing small plastic "Elizabethan collars" on the mice to prevent them from licking their back skin. The group found that unintentional ingestion of Aldara can result in an inflammatory loop in which the intestine system enhances the inflammation of the skin via the release of high levels of type 1 interferons. Thus, while the topical treatment with Aldara does indeed induce dermatitis-like skin inflammation, the mode of action partially depends upon ingestion of the chemical, as preventing oral uptake blocked not only the systemic effects but also decreased the severity of local cutaneous inflammation[300].

Tissue macrophages have been shown to proliferate after stimulation with IFNy [301]–[303], suggesting that production of gut type 1 interferons caused by ingestion of Aldara cream might be activating and promoting the proliferation of skin tissue macrophages, masking the effects of CCR1 and CCR5 deletion in iCCR$^{-/-}$ skin. Thus, while the Aldara model is easy to perform, local proliferation of tissue-resident leukocytes may be confounding the contribution that recruited leukocytes bring to the development of inflammation. Furthermore, unintentional ingestion of Aldara cream by some mice might explain the high variability observed when assessing erythema, scaling and skin thickness. A new model of localised inflammation was therefore required, one in which the amount of inflammatory irritant can be controlled more effectively and where local tissue-resident leukocytes would only contribute minimally to the development of the immune response.

3.6 Effect of iCCR deletion on Synovial Inflammation – Air Pouch Model

3.6.1 Air Pouch Model- Overview

In the air pouch model, an air pouch is produced by the subcutaneous injection of sterile air under the dorsal skin of the mouse. The resulting cavity, providing a new localized environment in which to study cell trafficking and inflammatory responses. After repeated air injections, the pouch stabilises forming a thin protective membrane, which is essentially a new organ lacking any resident leukocyte populations. The stabilised cavity can be then be filled with inflammatory irritants that have known effects, such as the sea-weed extract carrageenan, and the membrane around the airpouch and the fluid inside can be analysed via flow cytometry to determine leukocyte recruitment. In addition, as the air pouch does not contain any resident leukocytes, this offers the opportunity to observe recruitment *de novo* without the confounding presence and contribution of resident cells.

Carrageenan extract has been routinely used for decades [246][304] to induce inflammation and to test the efficacy of novel anti-inflammatory agents [305][306]. Tumour Necrosis Factor a (TNFα) and vasodilator prostaglandin E2 can be detected as soon as 3 hours after Carrageenan administration in the air-pouch[307], rapidly followed by high levels of pro-inflammatory cytokines such as IL-1β and IL-6, iNOS (inducible nitric oxide synthase)[308] and vascular endothelial growth factor (VEGF) [309].

All these inflammatory mediators work together to rapidly recruit leukocytes from the circulation: vasodilation induced by prostaglandin E2 increases blood flow to the air pouch, VEGF secretion increases the expression of adhesion protein ICAM-1 on endothelial cells [310], aiding chemotaxis and trans-endothelial migration of leukocytes that have been activated by exposure to circulating TNFα, IL-1β and IL-6.

3.6.2 Bone Marrow and Blood Subpopulations – Air Pouch

Bone marrow leukocytes in the bone marrow 48 hour after carrageenan injection into the air-pouch showed a similar distribution observed in Imiquimod treated mice, with an accumulation of classical Ly6C++ inflammatory monocytes observed in both $CCR2^{-/-}$ and $iCCR^{-/-}$ mice (Figure 3-14 A, Representative FACS plots 3-14B). This accumulation however is surprisingly more severe in $CCR2^{-/-}$ (WT: 8.9%, $CCR2^{-/-}$: 15.9%, $iCCR^{-/-}$: 12.2%, Figure 3-14C).

Three different mechanisms might be at play here: lack of all iCCRs simultaneously might either be promoting iCCR independent migration by inflammatory monocytes causing some to escape the bone marrow regardless of CCR2 expression, or iCCR absence in bone marrow might be affecting proliferation rates and activation of inflammatory monocytes. In other words, while the absence of CCR2 does indeed trap Ly6C++ monocytes in the bone marrow, engagement of CCR1 and CCR5 might be providing survival and proliferation signals to the developing monocytes. In their absence, the inflammatory monocytes would be both trapped and proliferating more slowly, resulting in an accumulation of monocytes similar to $CCR2^{-/-}$, but in lower numbers.

Alternatively, this apparent accumulation in $CCR2^{-/-}$ bone marrow may be the results of a shift in percentage in the $iCCR^{-/-}$ due to the accumulation of other cells which are not accounted for. For example, mast cells are also reportedly present in the bone marrow[311] and express CCR3[118]. In $iCCR^{-/-}$ bone marrow, the presence of additional trapped leukocytes might skew the percentages, making inflammatory monocytes appear less frequent in terms of percentage when compared to $CCR2^{-/-}$ bone marrow.

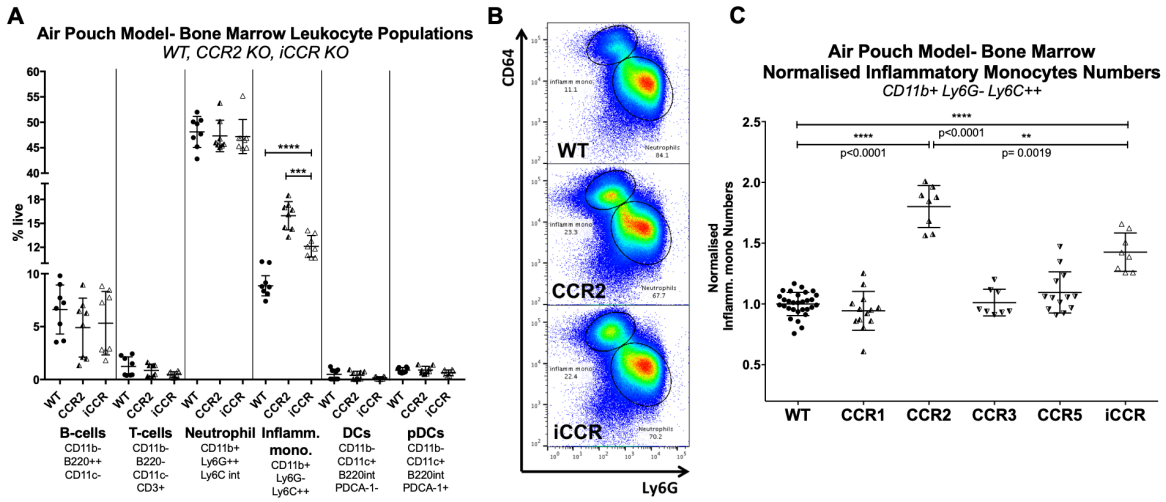

Figure 3-14- Air Pouch Bone Marrow Leukocyte Subpopulations- A*-Summary of the main leukocyte subpopulations present in iCCR $^{-/-}$, CCR2 $^{-/-}$ and WT BM after Imiquimod induced Inflammation.* ***B****- Representative FACS plots showing neutrophils (Ly6Cint Ly6G++) and inflammatory monocytes (Ly6Gint Ly6C++) in WT, CCR2 $^{-/-}$ and iCCR $^{-/-}$ BM.* ***C****- Graph comparing normalised Inflammatory monocyte (CD11b+ Ly6Gint Ly6C++) numbers in CCR1 $^{-/-}$, CCR2 $^{-/-}$, CCR3 $^{-/-}$, CCR5 $^{-/-}$ and iCCR $^{-/-}$ and their respective WT. Ordinary one-way ANOVA with Dunnett's multiple comparisons test was performed to determine statistical significance, with a p value of 0.05 determined as significant (n>3).*

The distribution of leukocytes in the circulation 48 hours after carrageenan administration into the air pouch showed a similar pattern to what has been observed in Imiquimod treated mice (Figure 3-15 A, Representative FACS plots 3-15B) but the defect was more severe.

iCCR deletion resulted in an approximate 90% reduction of the number of circulating inflammatory monocyte(WT: 2.1% iCCR: 0.28%), while the reduction observed in the circulation of Imiquimod treated mice was closer to 30% (WT: 5.5%, iCCR: 3.8%). A minor but significant difference in patrolling monocytes was also seen in CCR1$^{-/-}$ circulation (Figure 3-15C), but just like in Imiquimod treated mice, its biological relevance is unclear as the defect observed in iCCR$^{-/-}$ circulation can be wholly attributed to CCR2, with no evidence of an additive reduction brought by the absence of CCR1.

Similarly to inflamed skin in the Imiquimod model, the membrane around the pouch was removed, digested and its cellular content analysed, together with the cells in the air pouch fluid itself.

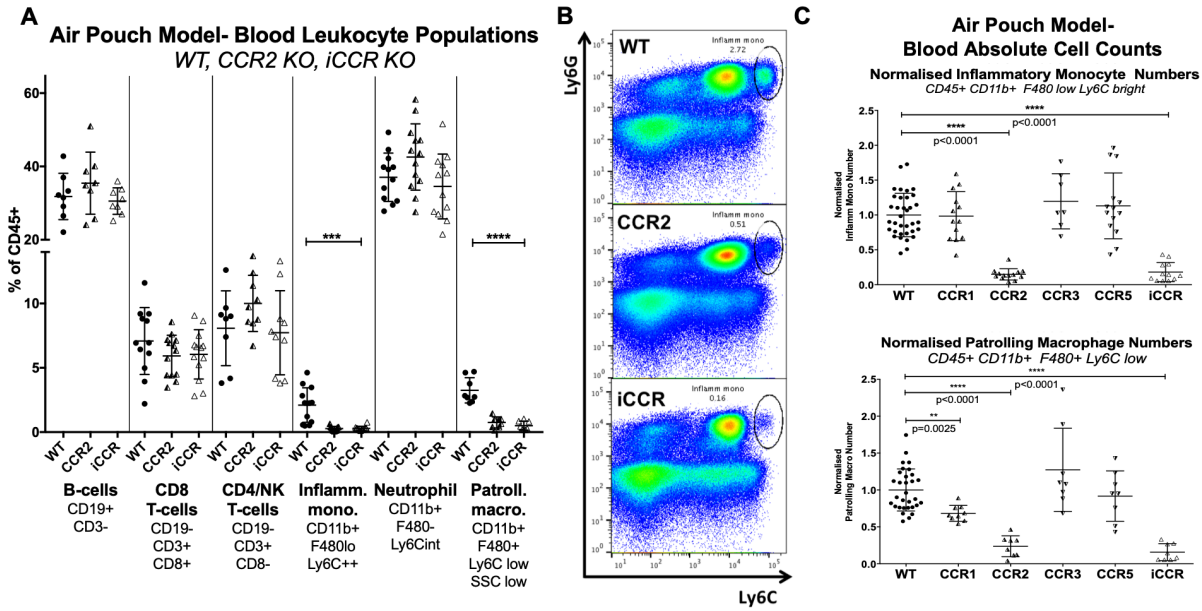

Figure 3-15 Air Pouch Blood Leukocyte Subpopulations- ***A****-Summary of the main leukocyte subpopulations present in iCCR $^{-/-}$, CCR2 $^{-/-}$ and WT BM after Imiquimod induced Inflammation.* ***B****-Representative FACS plots showing neutrophils (Ly6Cint Ly6G++) and inflammatory monocytes (Ly6Gint Ly6C++) in WT, CCR2 $^{-/-}$ and iCCR $^{-/-}$ BM.* ***C****- Graph comparing normalised Inflammatory monocyte (CD11b+ Ly6Gint Ly6C++) numbers in CCR1 $^{-/-}$, CCR2 $^{-/-}$, CCR3 $^{-/-}$, CCR5 $^{-/-}$ and iCCR $^{-/-}$ and their respective WT. Ordinary one-way ANOVA with Dunnett's multiple comparisons test was performed to determine statistical significance, with a p value of 0.05 determined as significant. (n>3)*

3.6.3 Membrane Subpopulations – Air Pouch Model

The cellular content of the air pouch membrane in iCCR$^{-/-}$ mice contained virtually no dendritic cells, macrophages or eosinophils. While the absence of dendritic cells was attributed to the lack of CCR2 exclusively (Figure 3-16 A,C) and the absence of eosinophil to CCR3 (Figure 3-16 C), macrophages in the air pouch (CD64+ F480+) were significantly more reduced in iCCR$^{-/-}$ membranes (99% reduction) compared to CCR2$^{-/-}$ (95% reduction compared to WT). This is evident in the representative FACS plots in Figure 3-16 B, showing a residual CD64+CD11b+ population in CCR2$^{-/-}$ samples, but a complete absence in iCCR$^{-/-}$ membranes. While the more profound defect in the iCCR$^{-/-}$ mice could be explained by a contribution of CCR1 and CCR5 to chemotaxis, the single knock-outs did not show any reduction in membrane macrophage numbers (Figure 3-16C), suggesting some level of redundancy by which CCR1 and CCR5 need to be deleted simultaneously to achieve a complete block of recruitment from circulation.

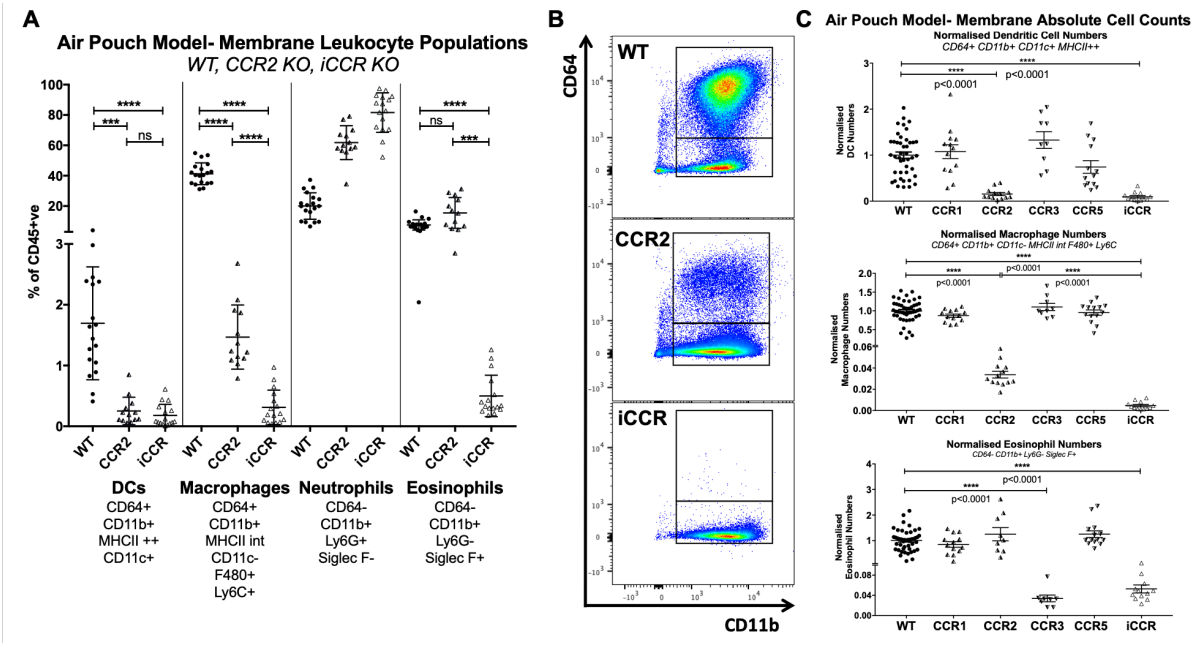

Figure 3-16 Air Pouch Membrane Leukocyte Subpopulations- ***A****- Summary of the main leukocyte populations in the membrane surrounding the air pouch in WT, CCR2 $^{-/-}$ and iCCR $^{-/-}$ mice.* ***B****- Representative FACs plots showing an absence of CD11b+ CD64+ cells in iCCR $^{-/-}$ membranes compared to CCR2 $^{-/-}$ and WT air pouch membranes.* ***C****- Graph comparing normalised Dendritic Cell (CD11b+ CD64+ CD11c+ MHCII ++), Macrophage (CD11b+ CD64+ CD11c- MHCII int F480 low Ly6C+) and eosinophil (CD64- CD11b+ Ly6G- Siglec F+) numbers in CCR1 $^{-/-}$, CCR2 $^{-/-}$, CCR3 $^{-/-}$, CCR5 $^{-/-}$ and iCCR $^{-/-}$ and their respective WT. Ordinary one-way ANOVA with Dunnett's multiple comparisons test was performed to determine statistical significance, with a p value of 0.05 determined as significant (n>3).*

What is also unclear is the nature of the residual population seen in CCR2$^{-/-}$ membranes but completely absent in the full knock-outs (Figure 3-16B). This monocyte/macrophage population either represents a phenotypically discrete CCR2 independent population dependent on CCR1 and CCR5, or could be the result of 'leaky' monocytes that have escaped the bone marrow and have managed to home to the membrane using CCR1 and CCR5 exclusively.

To address the issue, these cells were isolated from the air pouch of WT and CCR2-/- mice and their transcriptomic profile was compared[271]. The two populations differed significantly in gene expression, with 222 upregulated and 218 downregulated transcripts. This residual population in the CCR2$^{-/-}$ air pouch was characterised by high expression of CD209a and a range of transcripts involved in antigen presentation, suggesting that this population did not arise from 'leaky' monocytes but rather a specific monocyte subset which does not require CCR2 for bone marrow egress.

The transcriptomic data, particularly with respect to genes involved in antigen presentation, bear striking similarity to a novel DC-like CD209a+ monocyte subpopulation described by Menezes et al.[312]. According to their studies however, this monocytic population is reported to be substantially dependent on CCR2 for mobilisation from the bone marrow to peripheral blood. The data obtained so far might indicate that this CD209a population is in fact heterogenous, with one subset being dependent on CCR2 for recruitment to acutely inflamed sites, while the other one being CCR2 independent and at least capable of also using other iCCRs[271].

No difference in neutrophil recruitment was detected in CCR1$^{-/-}$, CCR3$^{-/-}$ and CCR5$^{-/-}$ mice, while in the iCCR$^{-/-}$ mice neutrophils made up more than 90% of the membrane cellular content. This apparent accumulation of neutrophil is not seen when the data are expressed as an absolute number, thus, the discrepancy is likely to be a consequence of a relative reduction in size of other cellular populations in iCCR and CCR2$^{-/-}$ air pouch membranes.

3.6.4 Fluid Subpopulations – Air Pouch

The air pouch fluid was finally analysed to determine which cells had successfully managed to cross the air pouch membrane and reach the carrageenan-filled cavity. The fluid represents the origin of the inflammatory stimulus, and inflammatory cells were expected to be fully differentiated by the time they reached this artificial niche. At this stage, monocytes recruited from the bone marrow should have fully differentiated into macrophages, and the impact of CCR1 and CCR5 deletion should be most evident. Analysis of the cellular components of the air pouch fluid revealed that the vast majority of cells in the cavity are neutrophils (~90%), with minimal contributions from DCs (~0.5%), macrophages (~5%) and eosinophils (~1%) (Figure 3-17 A). While expecting a more severe abrogation of myelomonocytic cell recruitment to the iCCR$^{-/-}$ air pouch compared to CCR2$^{-/-}$, it was surprising to discover that iCCR$^{-/-}$ recruitment of DCs (WT: 0.15%, CCR2$^{-/-}$: 0.01%, iCCR$^{-/-}$: 0.003%) and macrophages (WT: 1.52%, CCR2$^{-/-}$: 0.016%, iCCR$^{-/-}$: 0.016%) was identical to CCR2$^{-/-}$, with no evidence of any contribution of CCR1 and CCR5 (Figure 3- 17B, 17C).

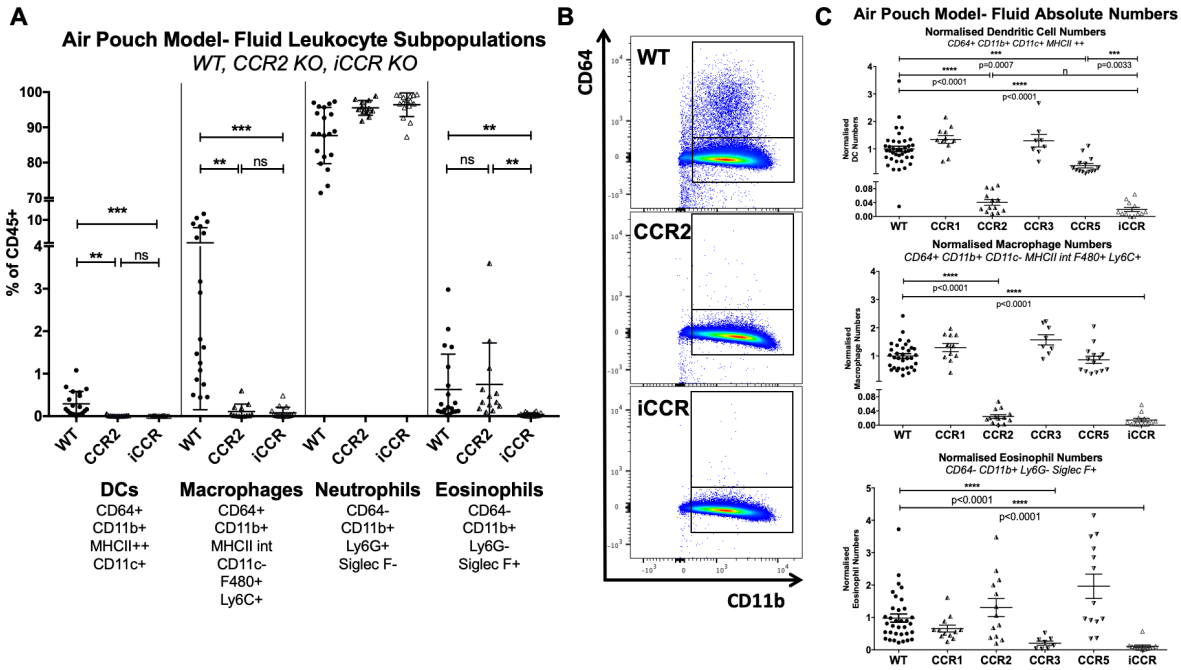

Figure 3-17 Air Pouch Fluid Subpopulations- ***A****- Summary of the main leukocyte populations in the fluid inside the air pouch of WT, CCR2 $^{-/-}$ and iCCR $^{-/-}$ mice.* ***B****- Representative FACs plots showing an absence of CD11b+ CD64+ cells in iCCR $^{-/-}$ and CCR2 $^{-/-}$ membranes compared to WT air pouch membranes.* ***C****- Graphs comparing normalised Dendritic Cell (CD11b+ CD64+ CD11c+ MHCII ++), Macrophage (CD11b+ CD64+ CD11c- MHCII int F480 low Ly6C+) and eosinophil (CD64-CD11b+ Ly6G- Siglec F+) numbers in CCR1 $^{-/-}$, CCR2 $^{-/-}$, CCR3 $^{-/-}$, CCR5 $^{-/-}$ and iCCR $^{-/-}$ and their respective WT. Ordinary one-way ANOVA with Dunnett's multiple comparisons test was performed to determine statistical significance, with a p value of 0.05 determined as significant (n>3)*

3.6.5 Discussion – Air Pouch Model

While the composition of bone marrow leukocyte subpopulations 48 hours after carrageenan injection into the air-pouch generally showed a similar distribution to that observed in Imiquimod treated mice (with an accumulation of 'classical' inflammatory monocytes in both iCCR$^{-/-}$ and CCR2$^{-/-}$ mice), the accumulation was surprisingly more severe in CCR2$^{-/-}$ (WT: 8.9%, CCR2$^{-/-}$: 15.9%, iCCR$^{-/-}$: 12.2%). Three different mechanisms were proposed to explain the phenotype: lack of all iCCRs simultaneously might either be promoting iCCR independent migration by inflammatory monocytes causing some to escape the bone marrow regardless of CCR2 expression, the iCCR absence in bone marrow might be affecting proliferation rates and activation of inflammatory monocytes, or simply a shift in percentage due to the accumulation of cells which were not accounted for.

While all mechanisms of action are plausible, CCR2 independent migration of inflammatory monocytes has only been observed in a handful of cases, all of which were seen in models

of severe systemic inflammation (such as La Crosse Encephalitis Virus infection [313] or 200μg dose of CpG1826 [314]) where other inflammatory stimuli, such as TLR9 engagement, were enough to override CCR2 mediated retention of monocytes ultimately mobilising them from the bone marrow.

It is thus unclear why and how deleting the iCCRs CCR1 and CCR5, whose expression is already minimal in bone marrow inflammatory monocytes, would cause the inflammatory monocytes to respond more potently to other inflammatory signals. On the other hand, studies have shown that soluble CCL5 (which binds to both CCR1 and CCR5) promotes macrophage survival through activation of the AKT and ERK signalling pathways, and its neutralisation *in vivo* reduced TNFα and IL-10 secretion after LPS administration[315]. Inflammatory monocytes in the bone marrow might be expressing both CCR1 and CCR5 transiently or at low levels during development, and their absence could potentially be affecting their proliferative rates resulting in lower numbers in iCCR$^{-/-}$ bone marrow compared to CCR2$^{-/-}$. Analysing bone marrow inflammatory monocytes from iCCR reporter mice would make it possible to confirm if CCR1 and CCR5 are indeed expressed at low levels in developing monocytes.

Finally, if the experiment were to be repeated, the addition of counting beads to the samples and an expanded flow cytometry panel would confirm If the accumulation of monocytes observed in CCR2$^{-/-}$ is genuine or an artefact due to the presence of unaccounted leukocyte subsets in iCCR$^{-/-}$ bone marrow.

Another surprising finding was the virtual absence of inflammatory monocytes and dendritic cells in the air-pouch fluid of both CCR2$^{-/-}$ and iCCR$^{-/-}$ mice. These data might suggest that the role of CCR2 goes beyond monocyte bone marrow egress and is actually vital in every step towards the inflammatory stimulus. For example, it could be argued that CCR2 engagement is providing the developing macrophage with a critical signalling event that enables migration through the air-pouch membrane, and without it, even if CCR1 and CCR5 are present, the cell is not capable of reaching the air pouch fluid. This would explain why the cellular component of the air pouch fluid in iCCR$^{-/-}$ mice is a phenocopy of CCR2$^{-/-}$ mice (Figure 3-17A, 17B). There is no evidence in the literature however of CCR2 mediating this 'critical signalling event', and it is also unclear how such a specific and important signal

would be provided by CCR2 exclusively, as CCR2 and CCR5 share several ligands and have overlapping signalling machinery[38].

More plausibly, 48 hours might have not been enough time for inflammatory monocytes from the bone marrow to egress, reach circulation, reach the membrane, differentiate and enter the fluid. This would also explain why no difference was observed in the air pouch fluid between CCR2$^{-/-}$ and iCCR$^{-/-}$, as impaired monocyte egress from the bone marrow in both strains would ultimately delay macrophage transmigration into the fluid until after 48 hours. Sampling the air pouch fluid at 72 or even 96 hours after carrageenan administration could address this hypothesis as it would allow more time for any defect brought on by the deletion of CCR1 and CCR5 to become more visible.

3.7 Overall Discussion

The main inflammatory chemokine receptors CCR1, CCR2 CCR3 and CCR5 have emerged as prominent players in the development of a wide range of inflammatory conditions. However, despite extensive research and attempts at pharmacological intervention, the combinatorial (and in some cases the individual), roles for these receptors in leukocyte recruitment remain unclear. The issue of iCCR redundancy versus specificity also remains unresolved.

While studies on single knock-outs have highlighted the importance of each receptor, what has emerged is that no single iCCR is completely responsible for the migration of a particular subset, suggesting that some compensatory mechanisms might be also regulating iCCR expression. Starting 'from scratch' by completely removing the iCCR locus provides a starting point to gradually develop a clearer picture of the roles of CCR1, CCR2 CCR3 and CCR5 in the development of the inflammatory response.

Homozygous mice with the iCCR deletion were born at expected Mendelian frequency from het/het crosses, were healthy and fertile. No differences in embryo size, foetal liver monocytes or cKIT+ haematopoietic progenitor cell numbers were observed, suggesting

that iCCRs are not essential for embryonic developmental and postnatal survival, and their deletion does not result in gross developmental abnormalities.

Immature B-cell numbers, T-cell precursor numbers, neutrophils and dendritic cells were not affected by the iCCR deletion. In contrast to other studies [73], no accumulation of inflammatory monocytes was observed in iCCR$^{-/-}$ or CCR2$^{-/-}$ bone marrow, suggesting that iCCRs are not involved in the maintenance of bone marrow leukocyte subpopulations under resting conditions. The only leukocytes affected in resting iCCR$^{-/-}$ bone marrow were eosinophils, which were elevated in the bone marrow and divided in two subpopulations (Siglec F+ and Siglec F++). As the Siglec F++ population is not present in other single knock outs, this would suggest that mature Siglec F++ eosinophils upregulate CCR3 expression and leave the bone marrow. This is in line with published literature[316][317].

iCCR$^{-/-}$ mouse circulation was characterised by reduced 'classical' Ly6C++ monocyte, 'non-classical' patrolling monocyte and eosinophil numbers, all of which were attributable to either CCR2 or CCR3, with no evidence of contribution from CCR1 or CCR5. Expectedly, deletion of iCCRs had no effect on resident populations which are generally thought of as iCCR independent, such as alveolar macrophages in the lung or red pulp macrophages in the spleen which distribute throughout these organs at birth and generally self-renew in resting conditions without contribution from the inflammatory monocytes recruited from the circulation.

At rest, the only evidence so far of a possible contribution of other iCCRs in directing leukocytes to different organs was found in the skin, where a decrease in skin tissue macrophages in iCCR$^{-/-}$ mice could not be completely explained by the absence of CCR2. It was still unclear, however, how much of this difference could be attributed to a combinatorial effect of the simultaneous deletion of CCR1, CCR2 and CCR5 on monocyte recruitment, or if the absence of CCR1 and CCR5 was affecting the proliferation of the newly recruited monocytes, further decreasing their overall numbers resulting in a phenotype that is more profound than in the CCR2$^{-/-}$ mice. The *in vitro* work on macrophages and monocyte derived DCs in the following chapter will address this issue.

Under inflammatory conditions, these initial defects in recruitment become more severe and affect more leukocyte subsets. While both the Imiquimod and the air pouch models

showed defects in recruitment of inflammatory monocytes, dendritic cells, eosinophils and macrophages, there was again little evidence of redundancy in receptor involvement, as most of the phenotypes were explained by an absence of either CCR2 or CCR3. Macrophages were the only leukocyte subset hinting at a possible combinatorial role for iCCRs, as the numbers in iCCR$^{-/-}$ air pouch membrane were lower than in the CCR2$^{-/-}$ mice.

Differences in lymphoid cells were not observed in the air pouch model. Conversely, after Imiquimod application to the skin, activated T-cells were found elevated in blood, potentially suggesting they were trapped in circulation. This could suggest that certain ligands can induce the upregulation of specific sets of iCCRs on a leukocyte, and changing the inflammatory conditions by using, for example, a model for bacterial or fungal infections, might reveal more profound phenotypes in more leukocyte subsets than was observed in iCCR$^{-/-}$ mice exposed to Imiquimod or carrageenan.

Overall, the data so far show that CCR2 is the dominant receptor for myelomonocytic cell recruitment to acutely inflamed sites and that, in its absence, migration is profoundly impaired with minimal evidence of other receptors 'taking over' and allowing efficient migration. What remains unclear at this stage is the contribution that CCR1 and CCR5 have on the development of the immune response. The roles these two receptors might have in myelomonocytic cell recruitment to acutely inflamed sites will be explored in more detail in the following chapters by analysing iCCR expression in recruited leukocytes from a novel iCCR reporter mouse model.

4 Results- *In vitro* Validation of iCCR Reporter Protein

4.1 Overview

While the data from iCCR$^{-/-}$ mice highlighted the importance of CCR2 and CCR3 in the recruitment of monocytes and eosinophils respectively, the specific role of inflammatory chemokine receptors CCR1 and CCR5 remained unclear. Indeed, every defect encountered in the resting iCCR$^{-/-}$ was attributed to the absence of either CCR2 or CCR3, with only a minor contribution by CCR1 and CCR5.

While CCR2 and CCR3 are expressed in resting inflammatory monocytes and eosinophils, CCR1 and CCR5 are generally thought to be upregulated only during differentiation and after activation with inflammatory mediators. Thus, for example, while CCR1 has been shown to aid trans-endothelial migration of inflammatory monocytes[298][318], those same cells are probably able to migrate just as efficiently using other receptors, and the absence of CCR1 would not stop chemotaxis but delay it slightly.

Similarly, while studies have described a wide range of phenotypes in CCR5$^{-/-}$ mice both at rest and under inflammatory conditions[79][319][320], humans with Δ32 mutation with a non-functional CCR5 are healthy, with minor phenotypes emerging only during severe infections or auto-immune disorders. Therefore, while CCR5's role in macrophage function has been well documented[321][322][323], the receptor itself might be dispensable or at least perform a function that overlaps with other receptors, allowing the leukocyte to function in its absence[324].

In other words, while the experiments on the iCCR$^{-/-}$ mouse model identified the pivotal roles of CCR2 and CCR3, it might not be the best model to uncover the role of CCR1 and CCR5 as these receptors are only upregulated during a specific temporal window in the life of a leukocyte and their effect might have gone undetected. Another model was therefore required to assess the temporal fluctuations of chemokine receptors on developing

leukocytes and uncover the potential additive roles of CCR1, CCR2 and CCR5 on leukocyte migration and function.

4.2 A novel iCCR Reporter Murine Strain

A reporter knock-in mouse defines an animal model in which fluorescent, bioluminescent proteins or biochemical tags are inserted into the genome. In this case, a murine stem cell was transfected with a BAC, a bacterial artificial chromosome, encoding the iCCR locus, but the genes for the iCCRs themselves have been replaced with genes encoding for fluorescent proteins (Figure 4-1).

Thus, mice generated from this transfected stem cell should express both the inflammatory chemokine receptors and specific fluorescent proteins under the same conditions, and allow for direct tracking of leukocyte migration and a direct visualisation of chemokine receptor expression in these cells without having to isolate them, stain them with antibodies or lyse them to infer iCCR expression via mRNA expression. This model also allows assessment in greater detail as to whether leukocytes express all iCCRs at the same time, if some chemokine receptors are preferentially expressed at different stages or if their expression is stochastic and follows random probability distribution.

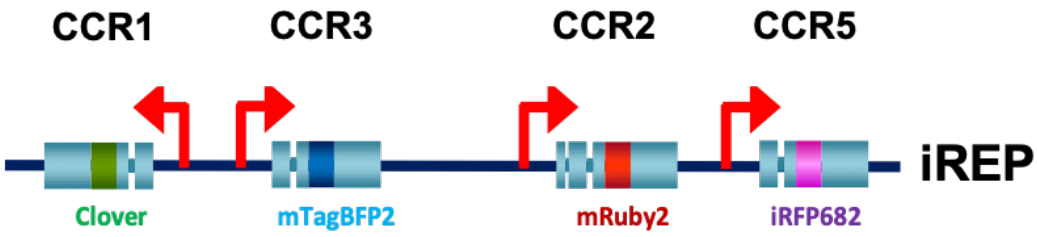

Figure 4-1- Generating a novel iCCR Reporter Strain (iREP)- *Schematic provided by Dr. Medina-Ruiz showing the structure of the BAC (Bacterial Artificial Chromosome) encoding for a modified iCCR locus expressing different fluorescent proteins instead of CCR1, CCR2, CCR3 or CCR5. Each florescent protein has a distinct emission spectrum which should be detectable by microscopy and flow cytometry. CCR1-Clover, CCR2-mRuby2, CCR3-mTagBFP2, CCR5-iRFP682*

4.3 BAC validation in iCCR expressing leukocytes *in vitro*

The early stage of this project was focused on determining if the BAC reporter proteins were detectable via flow cytometry and microscopy and to gain preliminary data to confirm whether the expression of fluorescent proteins correlates with iCCR expression. Macrophages were chosen as an ideal leukocyte model to track iCCR expression, as they express 3 of the 4 main iCCRs (CCR2, CCR1 and CCR5) *in vitro.*

For research purposes, macrophages can be obtained in three different ways. One method includes the isolation of primary macrophages or their precursors from bone marrow, but at this stage, live mice were not available as the strain was still being generated at Taconic. Another method includes culturing and analysing a macrophage lineage cell line. However, while this method gives yield to good cell numbers, the cells are removed from their normal state, are immortalised and are transcriptionally very different from their primary counterparts, thus not giving an accurate representation of what would happen *in vivo.* The third method involves culturing embryonic stem cells (ES cells) derived from the inner cell mass of blastocyst-stage mouse embryos and then differentiating the ES cells into macrophages[325].

This method generates a high number of differentiated macrophages with a similar lifespan to bone marrow macrophages. As a result, the macrophages obtained with this technique are transcriptionally closer to their *in vivo* counterpart than macrophages obtained using other methods[326]. Moreover, while primary cells are inherently difficult to genetically manipulate, ES cells are highly amenable to genetic manipulation, and can therefore be transfected with the same BAC found in the reporter (iREP) mice, ultimately resulting in the production of fully differentiated macrophages expressing the same fluorescent proteins under the endogenous iCCR promoters.

4.4 Generating ES-cell derived Macrophages

Generating ES-cell-derived macrophages requires up to three weeks of culture and involves growing ES cells under different growth factors and conditions [326]. Briefly, ES- cells were initially grown in media containing LIF (Leukaemia Inhibitory Factor) to maintain their undifferentiated state. Upon removal of LIF and addition of IL-3 and CSF-1, the ES-cells start differentiating into embryoid bodies which increase in size over the next 10 days. Over the next few weeks, these embryoid bodies release macrophage precursors into the media, which can then be collected and grown in CSF-1 containing media for a few days to obtain fully differentiated macrophages.

Initially, embryoid bodies were allowed to grow on petri dishes as described by Zhuang et al[326]. However, it was soon noticed that during the initial stages of differentiation, developing embryoid bodies are extremely 'sticky', causing many to fuse together and form large aggregates which ultimately resulted in low macrophage precursor yields. To avoid this issue, and to generate uniform embryoid bodies, the protocol was altered to include the 'hanging drop method' in the first days of ES-cell differentiation[327].

Briefly, ES-cells were collected and resuspended in IL-3+CSF-1 containing media, but instead of allowing differentiation and growth in petri dishes, the ES-cell suspension was carefully pipetted onto the lid of a petri dish in 27ul drops (approximately 1000 ES-cells/drop, 120-150 drops/petri dish). The drops 'hanging' from the inverted lid were then incubated for 4 days before collection and re-plating for an additional week in the same media. Differentiating ES-cells in individual drops allows each embryoid body to grow in isolation, enabling the formation of consistently uniform embryoid bodies that release high numbers of macrophage precursors after approximately 2 weeks of culture.

4.4.1 ES-cell-derived Macrophages are similar to their BM counterpart

At day 12 of the differentiation process, the media containing macrophage precursors was collected, spun down, and the pellet resuspended in CSF-1 containing media for 4-6 days to generate fully differentiated macrophages. These embryoid-body macrophages (Eb-macrophages) were then stimulated with inflammatory mediators, analysed and compared to bone-marrow-derived macrophages to assess whether ES-cell-derived macrophages can be used as an appropriate macrophage model for our studies.

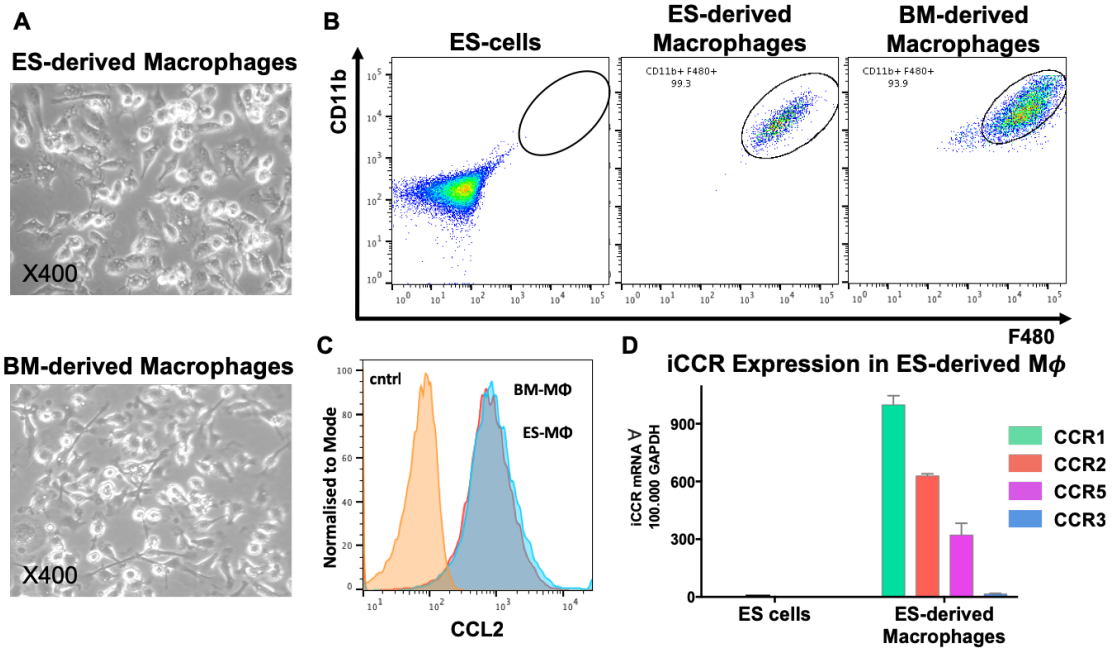

Figure 4-2 ES-derived macrophages are similar to BM derived macrophages- A- *x400 magnification representative bright field image showing the physical characteristics of macrophages grown from either embryonic stem cell (ES) or bone marrow precursors.* ***B-*** *Representative FACS plots showing CD11b+ F480+ cells in ES or BM derived macrophage cultures.* ***C-*** *Histograms showing expression of CCL2 in ES (red histogram) and BM derived macrophages (blue), unstained control in orange.* ***D-*** *qPCR data showing ES derived macrophages express macrophage-specific iCCRs CCR1, CCR2 and CCR5*

As seen in Figure 4-2 A, after 6 days of culture, differentiated embryoid body macrophages look morphologically similar to bone-marrow-derived macrophages. Flow cytometric analysis of those cells also show that >95% of cells in Embryoid body and bone marrow cultures express the macrophage markers CD11b and F480 (Figure 4-2B), and staining with fluorescent CCL2 (Figure 4-2C) showed that embryoid body and bone marrow macrophages express similar levels of CCR2. qPCR analysis on embryoid body macrophages showed that these cells also express CCR1 and CCR5, but not CCR3 (Figure 4-2D).

After overnight stimulation with 100ng/ml of LPS, embryoid body macrophages were shown to upregulate classical macrophage costimulatory molecules CD40 and CD86 (Fig. 4-3A), and secrete pro-inflammatory (Il-1β, IL-6 and TNFα) and anti-inflammatory cytokines (IL-10) at concentrations which were very similar to bone-marrow-derived macrophages cultured and stimulated in identical conditions (Figure 4- 3B). Overall, these results indicate that the differentiation of ES-cells results in the generation of *bona fide* macrophages which are phenotypically and morphologically very similar to bone-marrow-derived macrophages.

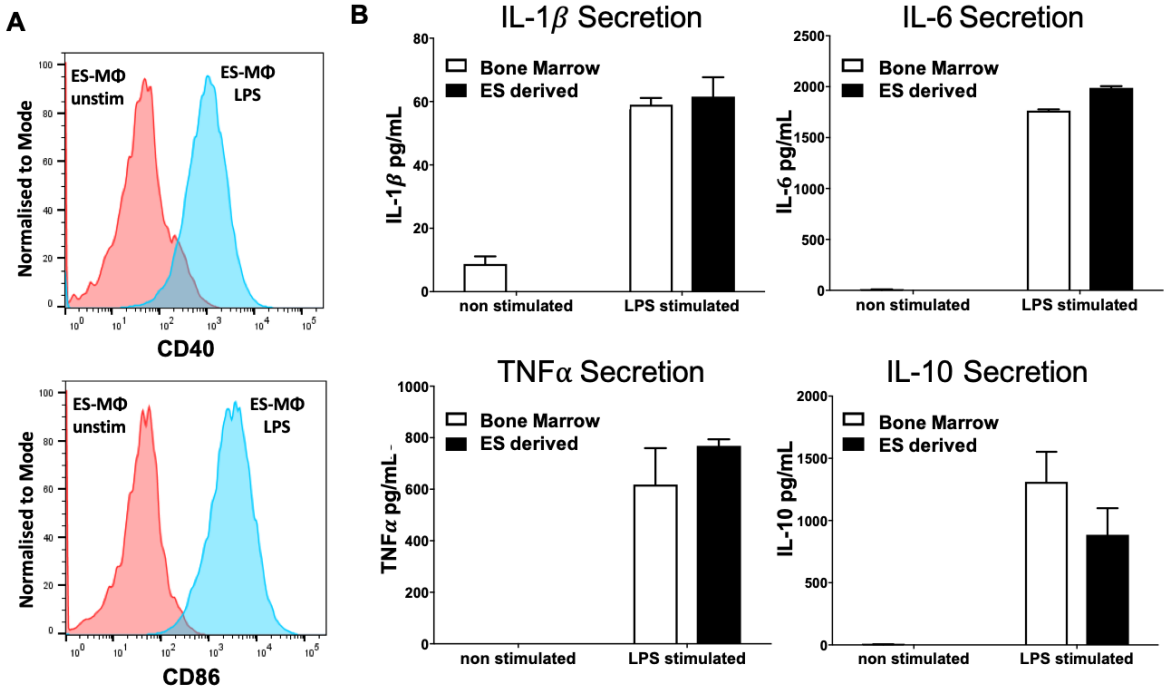

Figure 4-3- Costimulatory molecule expression and cytokine release by ES-derived macrophages- A- *Representative histograms showing ES-derived macrophages are capable of upregulating costimulatory molecules CD40 and CD86 after overnight exposure to 100ng/ml of LPS.* ***B-*** *Graphs summarising secretion of pro-inflammatory cytokines (IL-1β, IL-6 and TNFα) and anti-inflammatory IL-10 measured via ELISA in BM and ES-derived macrophages before and after exposure of LPS (100ng/ml) overnight. n=3*

4.5 Generating Macrophages from BAC-ES cells

After showing that the differentiation protocol successfully generates functional macrophages, the next stage focused on determining if the fluorescent reporter proteins that will be expressed in the iREP mouse strain are detectable via flow cytometry and if their pattern of expression in ES-derived macrophage recapitulates the fluctuating expression of CCR1, CCR2 and CCR5 of macrophages *in vivo*.

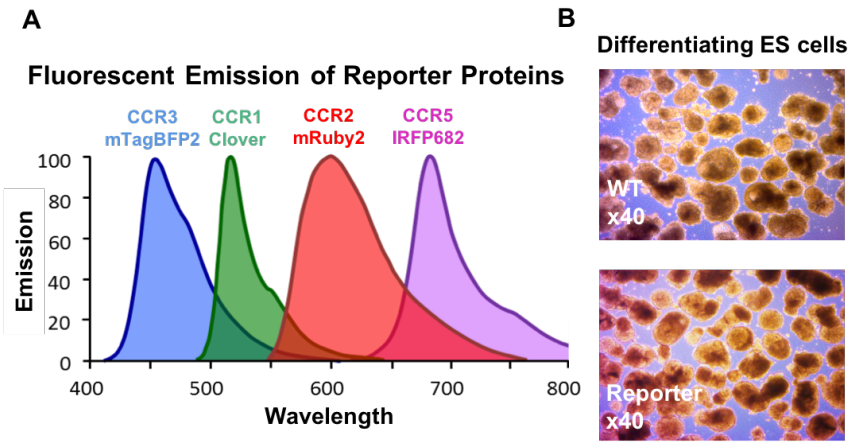

Figure 4-4 Emission of Fluorescent Reporter Proteins- A- *Histogram adapted from Dr. Medina-Ruiz showing the different emission spectra of each fluorescent reporter protein. Clover corresponds to CCR1 expression (green), mRuby2 to CCR2 (red), mTagBFP2 to CCR3 (blue), IRFP682 to CCR5 (purple).* ***B-*** *These representative images at x40 magnification show BAC insertion (reporter) has no effect on the development of embryoid bodies in culture.*

ES-cells transfected with the BAC encoding for iCCR-loci incorporating fluorescent reporter genes (Figure 4-4A) were differentiated into macrophages following the same hanging-drop method described previously. BAC insertion appeared to have no effect on the progress of differentiation, with transfected ES-cells growing into normal embryoid bodies undistinguishable from WT embryoid bodies (Figure 4-4B). Macrophage precursors from transfected and non-transfected embryoid bodies were then collected and differentiated into macrophages, stained for macrophage markers CD11b and F480 and analysed via flow cytometry at specific timepoints during differentiation or after exposure to inflammatory stimuli. As seen from the representative FACS plots in Figure 4-5, un-transfected embryoid-body derived macrophages did not express any of the fluorescent proteins and were not auto-fluorescent in any of the detection channels.

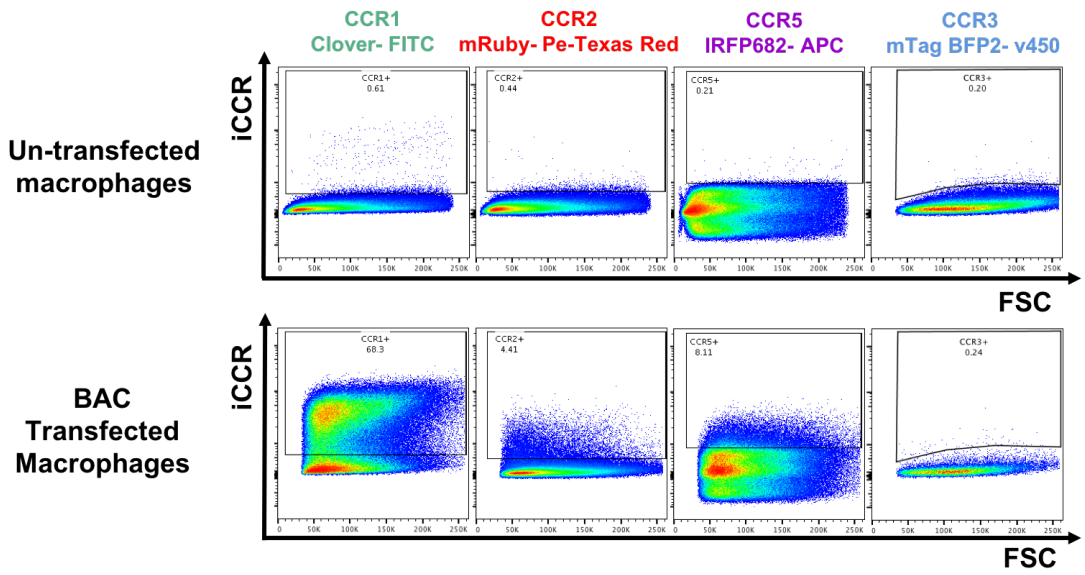

Figure 4-5- Reporter Expression in BAC-ES derived macrophages- *BAC-transfected macrophages express CCR1 (Clover), CCR2 (mRuby2) and CCR5 (IRFP682) but no CCR3 (mTagBFP2). WT ES-derived macrophages express none of the fluorescent proteins.*

4.6 iCCR Expression in ES-derived Reporter Macrophages

In contrast to WT differentiating macrophages, fluorescent reporter proteins could be detected from the first day of differentiation (Day 0) in reporter macrophages. As seen in Figure 4-6A, the macrophage precursors collected from developing embryoid bodies are mainly comprised of two populations based on expression of CD11b and F480. CD11b-F480- cells are considered 'uncommitted precursors', as these cells will eventually gain expression of CD11b+ F480+ after the first few days of culture. Almost 99% of cells in the uncommitted precursor stage express no florescent reporter proteins whatsoever, with a minor ~1% expressing CCR2 (measured as mRuby2 fluorescence, Figure 4-6 Bi).

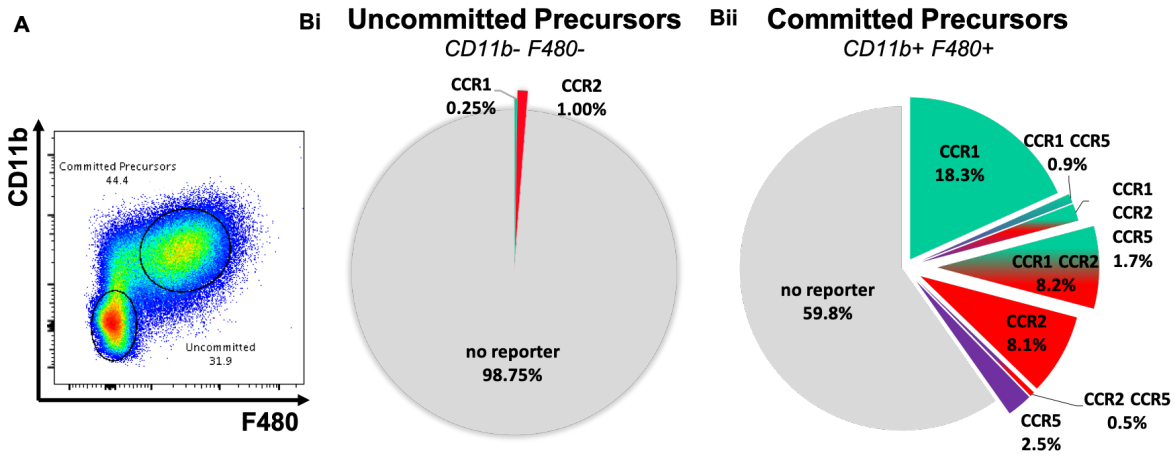

Figure 4-6 iCCR fluctuation in developing ES-derived macrophages- A- *Representative FACS plot showing CD11b+ and F480+ expression in ES-macrophage precursors collected ad day 10 of the ES differentiation process (day 0 in macrophage differentiation). The precursors can be split in two main populations: committed precursors are CD11b+F480+, uncommitted precursors are negative for both markers.* ***B-*** *iCCR expression on uncommitted (Bi) and committed precursors (Bii) (green= CCR1, red= CCR2, purple= CCR5, gradient colours indicate iCCR co-expression. n=4*

Fluorescent reporter protein expression was instead readily detected in precursor macrophages that already express CD11b and F480 (Fig. 4-5Bii). While the majority (~60%) of these 'committed precursors' expressed no fluorescent reporter protein, almost 30% expressed CCR1 (measured as Clover fluorescence), 20% expressed CCR2 and only a minor portion (~4%) expressed CCR5 (IRFP682 fluorescence). In these committed precursors, the dominant chemokine receptor pair was CCR1 and CCR2, with more than 8% of committed precursors co-expressing clover and mRuby2 fluorescent proteins.

As the precursors mature, expression of CD11b and F480 increases, the 'uncommitted precursor' population begins to differentiate and more that 95% of cells in culture are CD11b+ F480+ (Figure 4-7 A). After two days of culture (Day 2 Macrophages), differentiating macrophages markedly increased iCCR expression, with the majority now expressing at least one iCCR. More than 60% of cells at day 2 expressed CCR1, and the percentage of cells expressing CCR2 fell slightly, from 20% at the precursor stage to less than 10% after two days of culture (Figure 4-7 Bi). On the other hand, CCR5 expression doubled, from 4% to 9% at day 2 of differentiation. The dominant receptor pair was still CCR1 and CCR2, with 6% of cells co-expressing both mRuby2 and Clover fluorescent proteins.

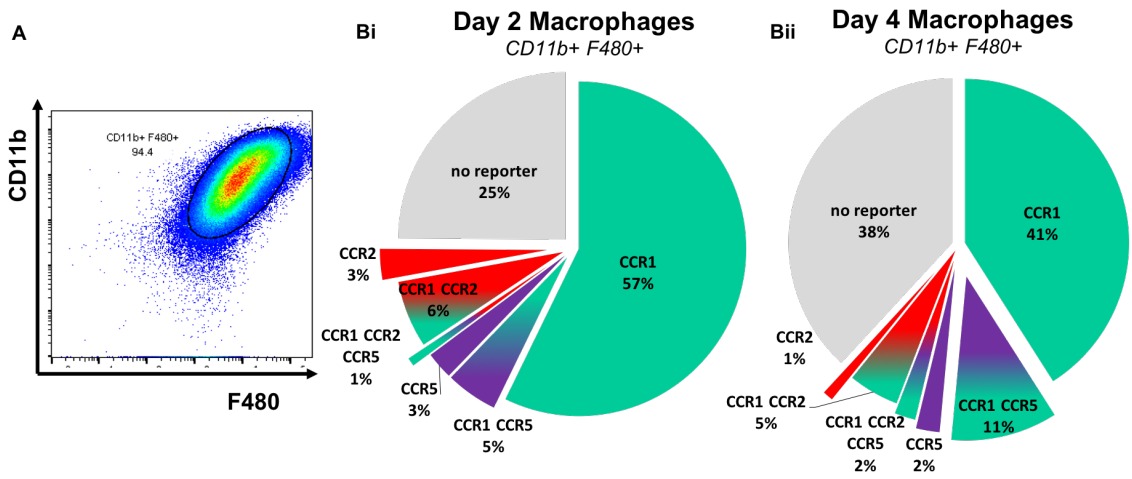

Figure 4-7 iCCR expression in differentiating macrophages (Day 2-4)- A- *representative FACS plot showing CD11b and F480 expression in Day 2 and Day 4 ES-derived macrophages.* ***Bi-*** *Pie chart showing iCCR expression in Day 2 macrophages (CD11b+ F480+).* ***Bii-*** *Pie chart summarising iCCR expression in Day 4 ES-derived macrophages. (green= CCR1, red= CCR2, purple= CCR5, gradient colours indicate iCCR co-expression. n=4*

As differentiation reached day 4, iCCR expression continued to change. The dominant receptor pair at this stage was CCR1 and CCR5, with ~11% of macrophages co-expressing both Clover and IRFP682. CCR2 expression dropped steadily as differentiation progressed, going from 20% at the precursor stage to 6% by day 4, while CCR5 increased from 4% to 15% by day 4 (Figure 4-7 Bii).

As macrophage reached the end of their life-span *in vitro* at day 7, iCCR expression generally decreased (Figure 4-8 A). By this stage, CCR2 expression was practically absent and the dominant receptor pair was CCR1 and CCR5. ICCR expression in these late-stage macrophages could be rescued by stimulating the cells with IFNy for 48 hours (Figure 4-8 Bi). Exposure to IFNy increased expression of all iCCRs, rescuing CCR2 and boosting CCR5 expression and tripling the number of macrophages co-expressing CCR1 and CCR5 from 4% to 12%. On the other hand, exposure to 100ng/ml of LPS decreased expression of all iCCRs, with more than 60% of macrophages negative for all reporter proteins after 48 hours (Figure 4-8 Bii).

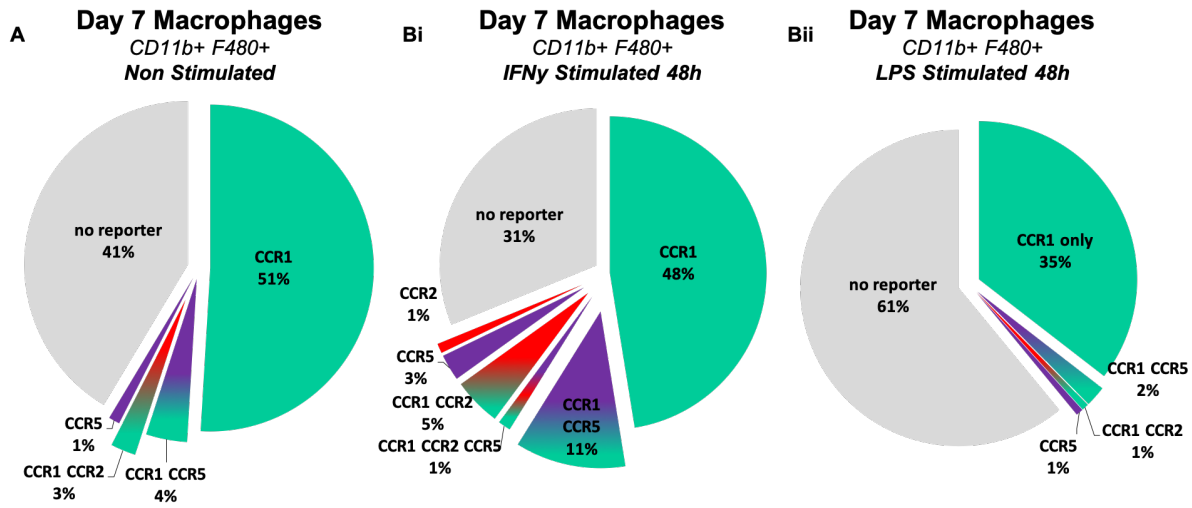

Figure 4-8- iCCR expression in Stimulated ES-derived macrophages. A- *Pie chart summarising iCCR expression in late stage macrophages (Day 7 of differentiation).* ***Bi-*** *Pie chart summarising iCCR expression in Day 7 macrophages stimulated at Day 5 with 100ng/ml of IFNy for 48 hours.* ***Bii-*** *Pie chart summarising iCCR expression in Day 7 macrophages stimulated on Day 5 with 100ng/ml of LPS for 48 hours.*

The macrophages were also stimulated with other inflammatory factors, such as TNFα, and iCCR expression was tracked from Day 0 to Day 7 at two-day intervals. As shown in Figure 4-9A, exposure to IFNy increased expression of all iCCRs (apart from CCR3, which was not expressed at any point). Conversely, TNFα and LPS decreased expression of CCR1, CCR2 and CCR5.

Inflammatory mediators were not the only factors affecting iCCR expression, as the time in culture also appeared to have an effect on the repertoire of iCCR expressed by macrophages.

As seen in Figure 4-9, expression of CCR2 peaked early in the differentiation pathway, with the highest levels detected in the 'committed precursor' stage and marked downregulation as maturation progressed. On the other hand, CCR1 expression was highest after 3-4 days of culture, and decreased as maturation progressed into the late stage or after addition of LPS or TNFα. Lastly, CCR5 expression peaked at the end of the macrophage's differentiation, reaching maximal levels at days 5-6. Thus, iCCR expression in BAC-transfected macrophages appears to fluctuate in 'waves', with CCR2 appearing early, followed by CCR1 and finally CCR5 (Figure 4-9B).

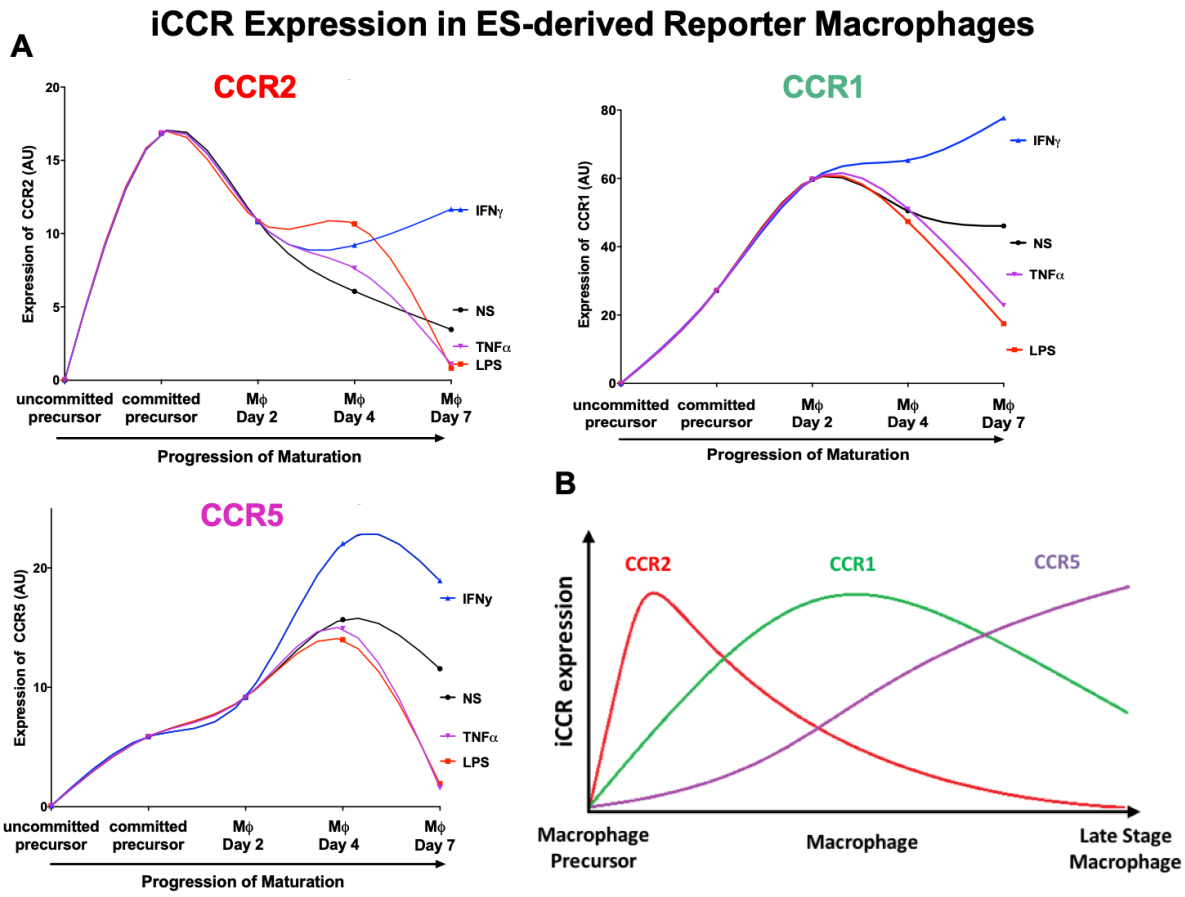

Figure 4-9 CCR2, CCR1 and CCR5 expression in Stimulated ES-derived Macrophages from day 0 to day 7. A- *CCR2, CCR1 and CCR5 expression as macrophages differentiate (Day 0-5) and after 48 hour stimulation (Day 5-7) with various inflammatory mediators (black: non-stimulated, blue: IFNy 100ng/ml, pink: TNF**α** 50ng/ml, red: LPS 100ng/ml)* ***B-*** *Summary of CCR2, CCR1 and CCR5 expression throughout macrophage differentiation, showing early CCR2 expression (red) and late CCR5 expression (purple). Data representative of one experiment with 3 different sets of pooled GGiREP bone marrow (n=3).*

4.7 Discussion

The results obtained show that the fluorescent proteins incorporated into the BAC are easily detectable by flow cytometry. In addition, the data suggest that the BAC is functioning correctly, as the main macrophage iCCRs were all detected in culture with the exception of CCR3 which was not expressed at any point.

When the data for the expression of each iCCR were overlaid (Figure 4-9B), it seemed evident that specific iCCRs are regulated at specific temporal stages, with CCR2 maximally expressed at the precursor stage and CCR5 peaking in expression at the latest stages of culture. This change in iCCR expression tied to macrophage maturation has already been reported both *in vitro* and *in vivo* [188][328][79].

4.7.1 Role of Inflammatory Mediators in Macrophage iCCR Expression

As shown in the previous chapter, CCR2 is pivotal for bone marrow egress of macrophage precursors into the circulation. Once the monocyte reaches the site of inflammation and differentiates into a macrophage, CCR2 expression is downregulated, presumably to restrict the 'reverse migration' back into the bloodstream and to promote retention of differentiated monocytes within the site of inflammation[328][329]. While the mechanisms of CCR2 downregulation can be varied, many studies have highlighted the importance of tissue specific factors in monocyte CCR2 regulation. In the aorta for example, oxidised LDL (low density lipoprotein) regulates PPARy signalling and markedly lowers CCR2 gene expression and mRNA stability, facilitating the accumulation of macrophages in atherosclerotic plaques [330]. Similarly, cytokines and reactive oxygen intermediates (ROI) frequently produced at the sites of acute inflammation can alter CCR2 expression, halving its mRNA half-life from 1.5 hours to 45 minutes [331]. Conversely, ROIs such as hydrogen peroxide have been shown to upregulate CCR5 expression in monocytes and increase their responsiveness to the CCR5 ligand CCL4 [332].

Microbial signals have also been shown to modulate iCCR expression. CCR2 in primary monocytes can be inhibited by bacterial LPS and other microbial agents [333], and although the exact mechanisms are still unclear, studies have suggested that this effect is mediated both directly and indirectly by LPS-induced production of inflammatory cytokines such as IL-1β and TNFα. Indeed, a simple injection of *Escherichia coli* LPS into human volunteers decreased the monocyte density (MFI) of CC chemokine receptor 2 (CCR2) [334], while exposing isolated monocytes to low doses of LPS (1ng/ml) can dramatically reduce transcript levels of CCR1 , CCR2 and CCR5 in as little as four hours [333].

Similarly to LPS, TNFα can also modulate iCCR expression in monocytes. This effect was first described in 1999, when it was observed that TNFα could reduce HIV-1 replication in human peripheral blood monocytes (PBMCs) and alveolar macrophages [335]. Further analysis via flow cytometry revealed that TNFα-treated PBMCs show decreased CCR5 expression on their surface, making them refractory to HIV-1 infection as CCR5 is required for viral entry [335]. While the mechanism of action of TNFα on CCR5 expression is still

unclear, a 2 hour pre-treatment of *in vitro* macrophages with TNFα is enough to decrease HIV-1 viral entry via CCR5 by 75% [336]. The *in vitro* data generated from BAC-transfected macrophages have shown a similar trend, with both LPS and TNFα downregulating expression of CCR1, CCR2 and CCR5.

4.7.2 Role of Differentiation in Macrophage iCCR Expression

iCCR expression also fluctuates according to age and state of cell differentiation. Several studies using human monocytes cultured *in vitro* have shown a dramatic decrease in CCR2 expression both on the surface and at the mRNA level as cells differentiate into macrophages [188] [337]. Specifically, CCR2 was shown to be expressed on the cell surface in day 0 monocytes, with a marked decrease after 24 hours and undetectable expression after 7 days of culture. At the same time, the decrease in CCR2 was accompanied by an up-regulation of CCR1 and CCR5 expression, which coincided with an increased responsiveness of monocyte-derived macrophages to CCL3 (chemokine binding to CCR1 and CCR5) [337].

Elegant studies *in vitro* have tried to uncover the specific roles of CCR1 and CCR5 in monocyte chemotaxis. Administration of CCR1 and CCR5 antagonists to monocytes in laminar flow assays that simulate the shear flow experienced by circulating leukocytes have shown blocking CCR1 but not CCR5 resulted in a significant inhibition of CCL5-induced arrest of these cells in flow [298].

Monocytes express more CCR1 than CCR5[298][188], while fully differentiated macrophages generally express more CCR5 than CCR1[111]. This suggests a role for CCR5 in events following arrest, such as affecting activity[321] or survival of emigrated cells[338] or to promote the morphological changes required by the cell to initiate trans-endothelial migration[298]. Therefore, this iCCR fluctuation on the surface of monocytes might reflect the cell's function over a specific temporal window, with CCR2 required for bone marrow egress into the circulation, CCR1 for arrest under shear flow conditions and CCR5 for trans-endothelial migration and 'spreading' at the site of inflammation.

While the process of monocyte migration described is probably an oversimplification, the findings on the BAC-transfected macrophages seem to support this current model. CCR2

was expressed at the very beginning of differentiation, and gradually decreased until undetectable by day 7, hinting at its role in early monocyte precursor migration from the bone marrow. CCR1 expression peaked at around days 3-4, possibly reflecting its role in vascular adhesion, while CCR5 was expressed towards the late stages of differentiation, supporting the notion that CCR5 has a role in modulating the activity and survival of late stage macrophages.

CCR5 expression was still relatively low however, with only 15% of differentiated macrophages expressing this receptor, compared with ~50% expressing CCR1. This apparent mis-match in receptor expression might be an effect of the nature of the BAC transfection process. Due to the BAC's relatively large size, integration in ES cells did not happen in a single event, as suggested by qPCR analysis on these cells by Dr. Medina-Ruiz (Figure 4-10 A,B).

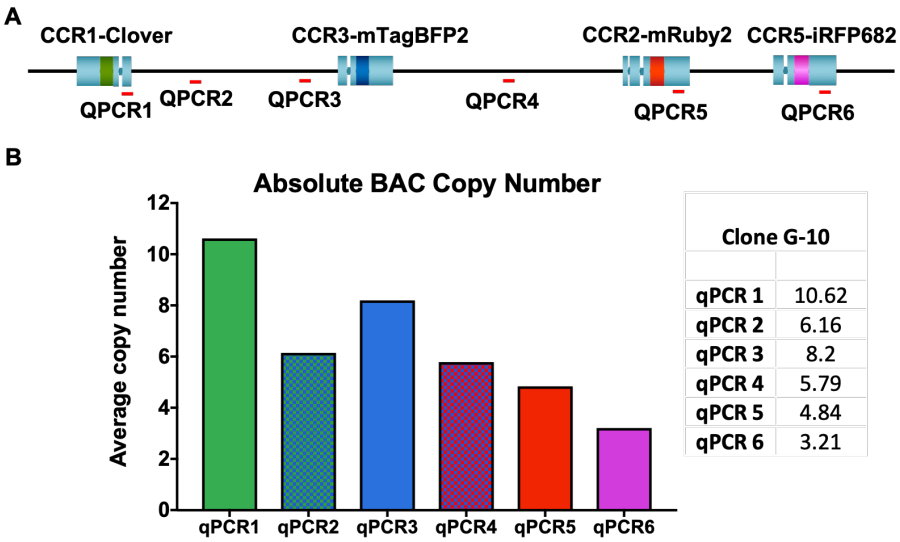

Figure 4-10- BAC copy number in transfected ES cells- A- *Diagram showing primer localisation to detect various BAC fragments. Some primers are specific for the genes encoding for the fluorescent reporter proteins (like QPCR1, QPCR5 and QPCR6). Other primers were designed to recognise regions in between the reporter proteins (such as QPCR2, QPCR3 and QPCR4). This specific primer design allows to determine how many BAC fragments are present in the genome.* ***B-*** *Bar graph showing the average BAC copy number for each fragment detected by BAC-specific primers. Bar graphs with multiple colours represent regions of the BAC in between two fluorescent proteins (for example, primer qPCR2 binds a region between qPCR1 (clover, green) and qPCR3 (mTagBFP2, blue), so the bar is both green and blue.*

Her findings show that the BAC has integrated itself in multiple fragments in different parts of the genome. Specifically, qPCR data using primers designed to detect particular sections of the BAC showed that these BAC-transfected ES cells possess 10 copies of CCR1-Clover, 5 copies of CCR2-mRuby2 and 3 copies of CCR5-IRFP682 (Figure 4-10 B).

Therefore, the overexpression of fluorescent reporter protein Clover (CCR1) in differentiated macrophages might be the result of a high number of integrations of Clover expressing BAC fragments. Similarly, fewer integrations of IRFP682 BAC fragments (corresponding to CCR5) compared to Clover might result in apparently low expression of CCR5. The location of the genome where the integrations took place probably also has an impact, with BAC copies integrated in highly transcribed areas producing larger amounts of fluorescent reporter proteins.

Regardless of the levels of expression, CCR1, CCR2 and CCR5 levels in ES-derived macrophages fluctuate with age and when exposed to inflammatory mediators in a way that is similar to what has been described in other studies, suggesting that the BAC itself is working properly and murine cells are capable of expressing detectable levels of fluorescent reporter proteins.

5 Results- iCCR Reporter Strain (iREP) *in vivo*

5.1 Overview

Once the BAC encoding for 4 iCCR reporter proteins had been validated *in vitro*, experiments were performed to determine if BAC expression could also be detected in the novel murine iCCR reporter strain and if it carried any negative burden on mouse fitness.

One of the concerns was a potential effect on leukocyte chemotaxis by a process known as 'squelching'[339]. As the fluorescent proteins (and each iCCR) share the same promoters, the presence of several BAC copies in the genome might make the transcription factors binding to these promoters less available for iCCR expression, ultimately making chemotaxis less effective.

Before analysing fluorescent reporter expression, iREP mice were analysed to determine if BAC expression had any effect on leukocyte subpopulations in various tissues at rest and if it also had any impact on the recruitment of leukocytes to the site of inflammation.

5.2 BAC Integration

The iCCR Reporter strain (iREP) was generated by Taconic via micro-injection of the BAC (designed and made by Dr. Medina-Ruiz) into developing murine embryos. In this way, two different founder lines containing several copies of the BAC were obtained. Extensive qPCR analysis showed the BAC integrated itself in multiple copies in a single area of the murine genome. The first founder line was found to possess 3-5 BAC copies located in the same area of chromosome 3. Analysis on the second founder line revealed that it contained 5-7 BAC copies, all integrated in the same area on chromosome 16. Further sequencing revealed that the BAC integrations in both founder lines happened in a section of the genome containing non-coding DNA, indicating that no genes were disrupted during insertion.

Both founder lines were caged separately, kept under specific pathogen free conditions and breeding was initially done using HETxHET crosses. Preliminary data had shown detectable fluorescence in BAC hemizygote mice, suggesting that BAC expression in a single chromosome is enough to produce detectable levels of fluorescent reporter proteins. Hemizygotes for the BAC were used for all the experiments in this chapter, as they possessed enough reporter proteins for detection while still expressing an unaffected endogenous locus, minimising any potential negative effects caused by BAC integration. Homozygote littermates were used for breeding purposes, while WT littermates were used as negative controls.

5.3 Effect of BAC Integration on Leukocyte Subsets

Adult 8-10 week BAC-hemizygote mice were culled, and their tissues were analysed to detect any effect of BAC expression on leukocyte subpopulations in various tissues under resting conditions.

As seen from Figure 5-1, BAC integration and fluorescent reporter expression had no significant impact on the composition of leukocyte subpopulations in the bone marrow (Figure 5-1A) and blood (Figure 5-1B) of resting reporter mice when compared to their WT littermates. In the bone marrow of all strains analysed, neutrophils were the most prominent leukocyte subset (WT: 37.1% , 3-5 BAC: 33.1%, 5-7 BAC: 36.5%), followed by B-cells (WT: 11.9% , 3-5 BAC: 12.1%, 5-7 BAC: 13.0%), inflammatory monocytes (WT: 7.8% , 3-5 BAC: 7.1%, 5-7 BAC: 6.9%) and eosinophils (WT: 4.2% , 3-5 BAC: 4.5%, 5-7 BAC: 4.5%). In circulation, the most prominent leukocyte subset was B-cells (WT: 52.2%, 3-5 BAC: 45.5%, 5-7 BAC: 52.9%), followed by neutrophils (WT: 8.8%, 3-5 BAC: 8.5%, 5-7 BAC: 12.1%), inflammatory monocytes (WT: 1.9%, 3-5 BAC: 1.9%, 5-7 BAC: 2.0%) and eosinophils (WT: 0.9%, 3-5 BAC: 1.0%, 5-7 BAC: 0.8%).

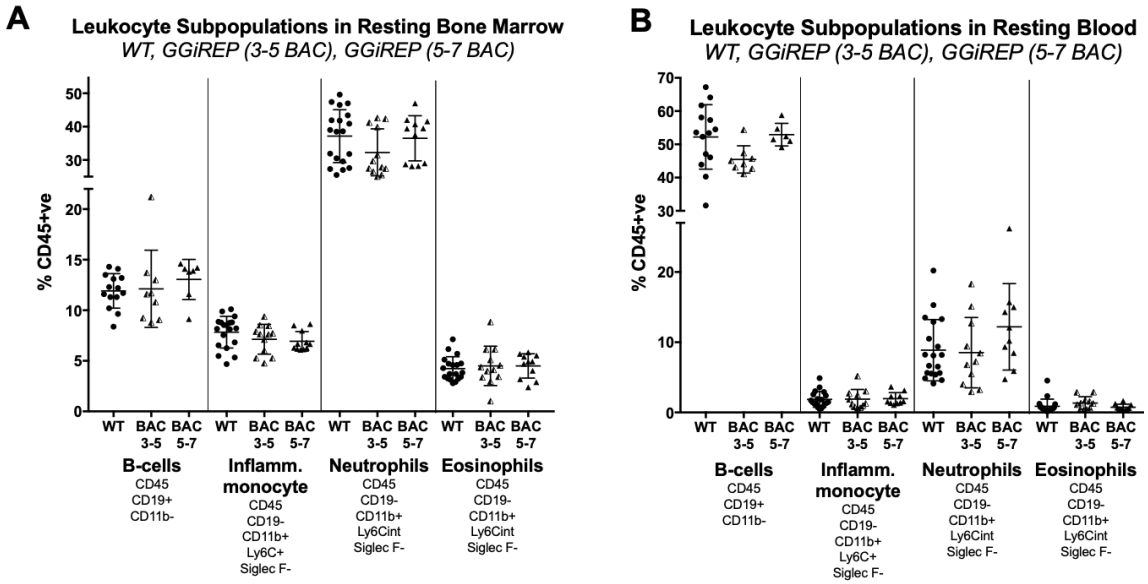

Figure 5-1 Leukocyte Composition of Resting iREP bone marrow and blood- A*- Graph summarising the prevalence of 4 main leukocyte populations in WT, iREPs 3-5 BAC copies and iREPs 5-7 copies bone marrow.* ***B****- Graph summarising the prevalence of 4 main leukocyte populations in WT, iREPs 3-5 BAC copies and iREPs 5-7 copies circulation. Both graphs summarise values expressed as % of CD45+ cells. 3-5 BAC data is an average of 2 experiments with n=3, 5-7 BAC data from 3 experiments with n>3.*

Similarly, no defect in leukocyte recruitment was detected under inflammatory conditions, with B-cell, Inflammatory monocyte, neutrophil and eosinophils numbers identical between reporter positive and reporter negative mice in inflamed bone marrow, blood, or air pouch membrane (Figure 5-2A,B,C). The number of BAC copies also did not appear to have an effect, with no differences observed in neutrophil, B-cells, inflammatory monocytes, eosinophils or macrophage numbers in inflamed tissues of 3-5 and 5-7 BAC mice (Figure 5-2).

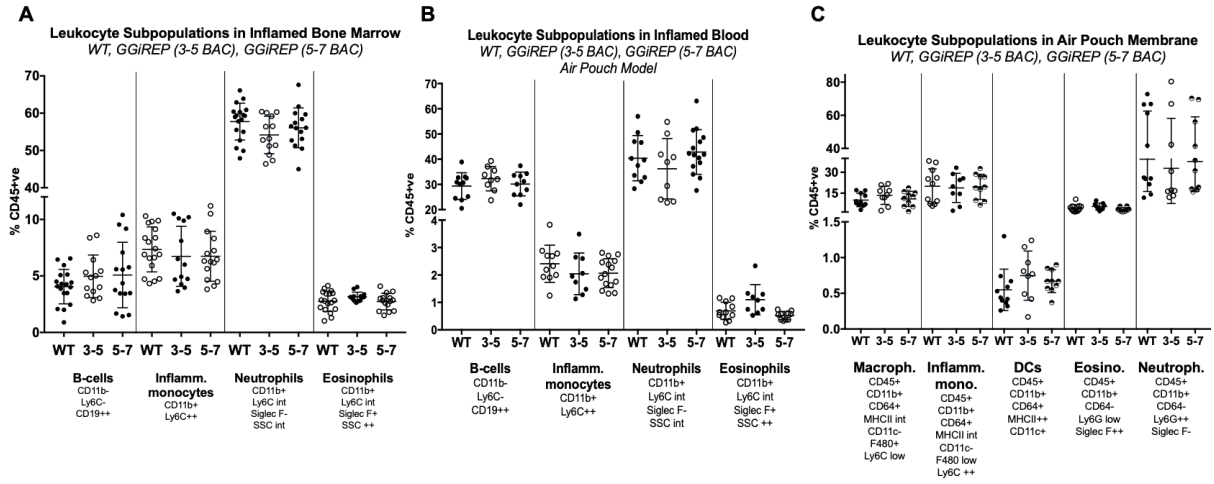

Figure 5-2- Leukocyte Composition of Inflamed iREP tissues- A*- Graph summarising the prevalence of B-cells, Inflammatory monocytes, neutrophils and eosinophils in inflamed bone marrow from WT, iREP 3-5 and iREP 5-7 BAC mice.* ***B****- Graph summarising the prevalence of B-cells, Inflammatory monocytes, neutrophils and eosinophils in inflamed circulation from WT, iREP 3-5 and iREP 5-7 BAC mice.* ***C****- Graph summarising the prevalence of B-cells, Inflammatory monocytes, neutrophils, dendritic cells and eosinophils on the air pouch membrane from WT, iREP 3-5 and iREP 5-7 BAC mice. Data is representative of 3 experiments, with n=3.*

Next, each individual leukocyte subpopulation was analysed to detect possible fluorescent protein expression.

5.4 Resting iREP Tissues

5.4.1 iREP Bone Marrow

As seen in Figure 5-3, fluorescent proteins were detected in several leukocyte subsets in iREP resting bone marrow. B-cells (CD45+ CD19++) were found to express fluorescent protein Clover (corresponding to CCR1), inflammatory monocytes were brightly positive for mRuby2 (corresponding to CCR2) and most eosinophils were positive for mTagBFP (corresponding to CCR3). Fluorescent protein IRFP682, corresponding to CCR5, was not detected in any bone marrow leukocyte subset. Furthermore, there was little evidence of iCCR co-expression, as each cell-type seemed to be expressing a single fluorescent protein at any given time. B-cells and eosinophils exclusively expressed CCR1 and CCR3 respectively, and <98% of inflammatory monocytes express mRuby2 (CCR2), with only ~3% also expressing Clover (CCR1).

As expected, neutrophils did not express any of the reporter proteins, although some neutrophils (~0.5%) appeared positive for CCR2 and CCR3. These CCR2+ and CCR3+ neutrophils are, in all probability, contaminating inflammatory monocytes and eosinophils, as no neutrophil specific antibody was added due to the limited number of colours available for detection on the flow cytometers. As a result, neutrophils were isolated via their physical characteristics (side scatter) and negative staining for other markers, and this gating probably includes a small fraction of other cell types.

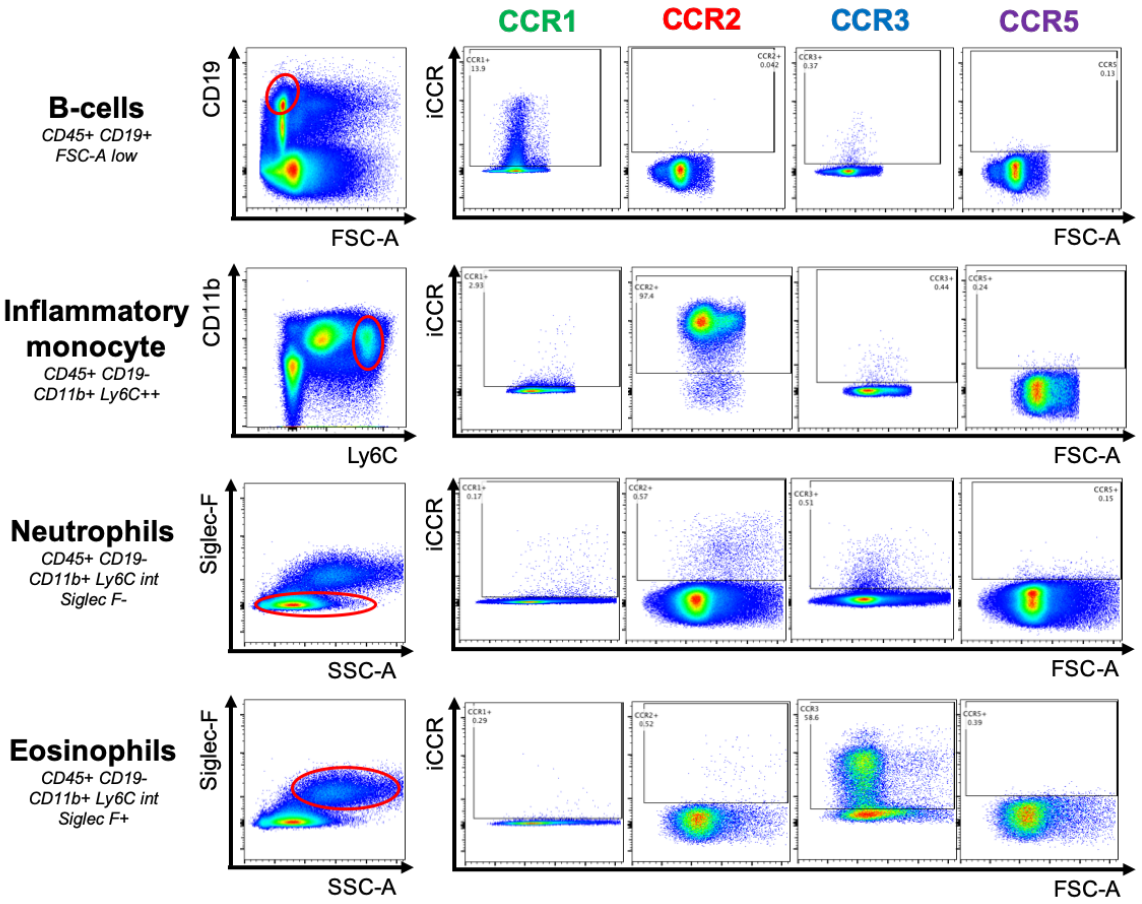

Figure 5-3 iCCR Expression on Bone Marrow Leukocytes. *The panel shows fluorescent reporter protein expression in B-cells, inflammatory monocytes, neutrophils and eosinophils. The markers and gating strategy used to identify these leukocytes are shown in the first FACS plot beside the name of each subset. The representative FACS plots that follow show leukocyte fluorescent reporter protein expression in each of the 4 main detection channels (CCR1= Clover= Green, CCR2=mRuby2=red, CCR3=mTagBFP=blue, CCR5=IRFP682=purple). Positive reporter expression was determined using non-fluorescent WT leukocytes*

While high CCR2 expression in inflammatory monocytes and CCR3 expression in eosinophils was expected and well documented[98][340], CCR1 expression in ~20% of bone marrow B-cells was somewhat surprising. While CCR1 expression has been described in B-cells only recently [341] [107], its function has not been fully investigated, as B-cells generally use other chemokine receptors for chemotaxis[342] and are thought to express CCR1 only under specific conditions of inflammation [343]. Thus, expression of CCR1 in unchallenged immature bone marrow B-cells is a novel finding.

While no difference in recruitment to inflamed sites was recorded between WT, 3-5 and 5-7 BAC mice, expression of fluorescent proteins was generally higher in leukocytes extracted from 5-7 BAC animals. As seen from Figure 5-4A, the number of CCR1+ B-cells in the bone marrow increased from 14% to 26% if 5-7 BAC copies are present. While the number of bone marrow inflammatory monocytes positive for CCR2 does not change between 3-5 or

5-7 BAC mice (97% vs 98%), a higher BAC copy number correlates with increased CCR2 MFI (9400 vs 1100) (Figure 5-4B). As a high BAC number had no effect on recruitment but facilitated iCCR detection, the remaining experiments focused on 5-7 BAC mice.

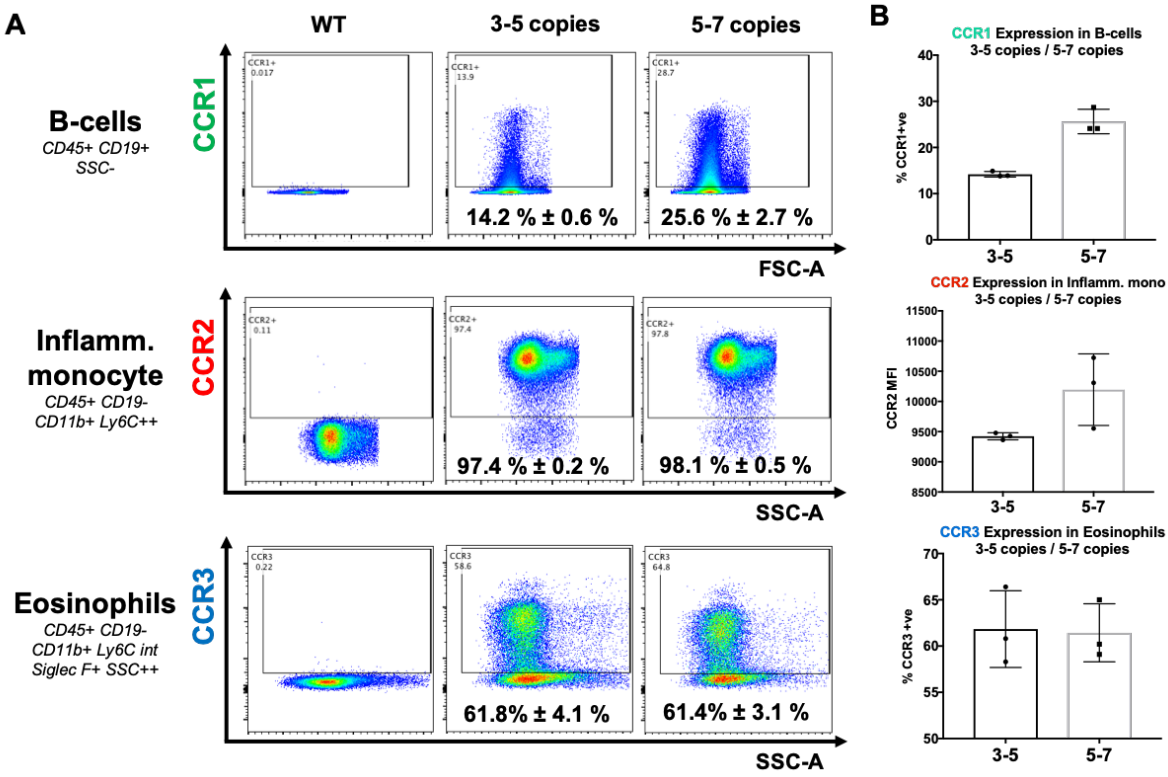

Figure 5-4 iCCR Expression in Bone Marrow Leukocytes with different BAC number. A*- Representative FACS plots showing increased fluorescent reporter protein expression in leukocytes expressing more BAC copies. The gating identifying positive reporter protein expression was drawn using non-fluorescent WT leukocytes.* ***B****- Bar graph summarising the change in iCCR expression in B-cells, inflammatory monocytes and eosinophils from the resting bone marrow of iREPs with 3-5 BAC and 5-7 BAC copies.*

5.4.2 iREP Blood

Next, circulating leukocytes from iREP mice were also analysed for fluorescent reporter protein expression. As seen in figure 5-5, CCR1 expression was detected in B-cells, CCR2 expression and marginal CCR1 expression in inflammatory monocytes and CCR3 in eosinophils. Almost 70% of patrolling vascular macrophages (Ly6Clow F480+), which derive from Ly6C+ inflammatory monocytes [344], also expressed CCR2. CCR5 was not detected in any leukocyte subset, and neutrophils were negative for all fluorescent reporter proteins.

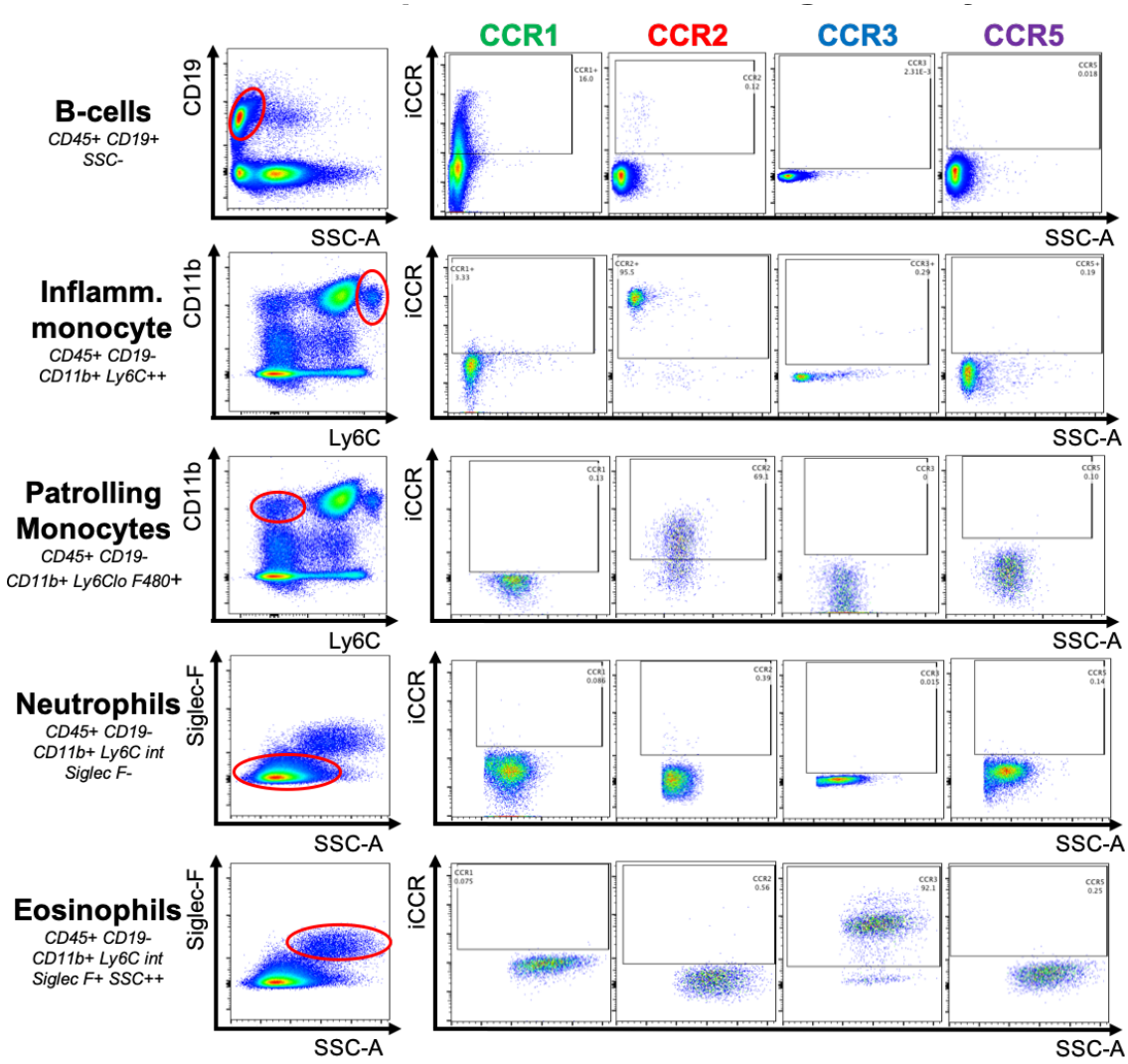

Figure 5-5 iCCR Expression on Circulating Leukocytes. *The panel shows fluorescent reporter protein expression in B-cells, inflammatory monocytes, vasculature patrolling macrophages, neutrophils and eosinophils. The markers and gating strategy used to identify these leukocytes are shown in the first FACS plot beside the name of each subset. The representative FACS plots that follow show leukocyte fluorescent reporter protein expression in each of the 4 main detection channels (CCR1= Clover= Green, CCR2=mRuby2=red, CCR3=mTagBFP=blue, CCR5=IRFP682=purple). Positive reporter expression was determined using non-fluorescent WT leukocytes*

5.4.3 Monitoring iCCR fluctuation

iCCR expression fluctuates according to the level of differentiation and activation of a leukocyte. This is the case with inflammatory monocytes, which express high levels of CCR2 in the bone marrow, higher levels of CCR2 while in the blood, and then decrease its expression during terminal differentiation into macrophages.

More than 90% of inflammatory monocytes in reporter mice were CCR2+, while 70% of patrolling monocytes in the circulation expressed this receptor. This finding raised the

possibility that fluorescent reporter expression could be used to track monocytes at different stages of differentiation. Bone marrow, blood, and lung samples were collected from 5-7 BAC reporter mice and CCR2 expression was analysed in all the monocyte and macrophage populations in these organs. Figure 5-6 summarises the average mean fluorescent intensity of mRuby2 (CCR2) on each of these cell subtypes. 'Classical' bone marrow inflammatory monocytes express high levels of CCR2, which peaks as the monocytes reach the circulation. As the monocytes differentiate into 'non-classical' patrolling monocytes or move into distal tissues, CCR2 expression decreases. Interstitial macrophages in the lung, which derive from inflammatory monocytes, showed only marginal CCR2 expression. Alveolar macrophages, which have a distinct origin and develop from CCR2 independent foetal monocytes [345] expressed no detectable levels of CCR2, and no CCR1, CCR3 or CCR5.

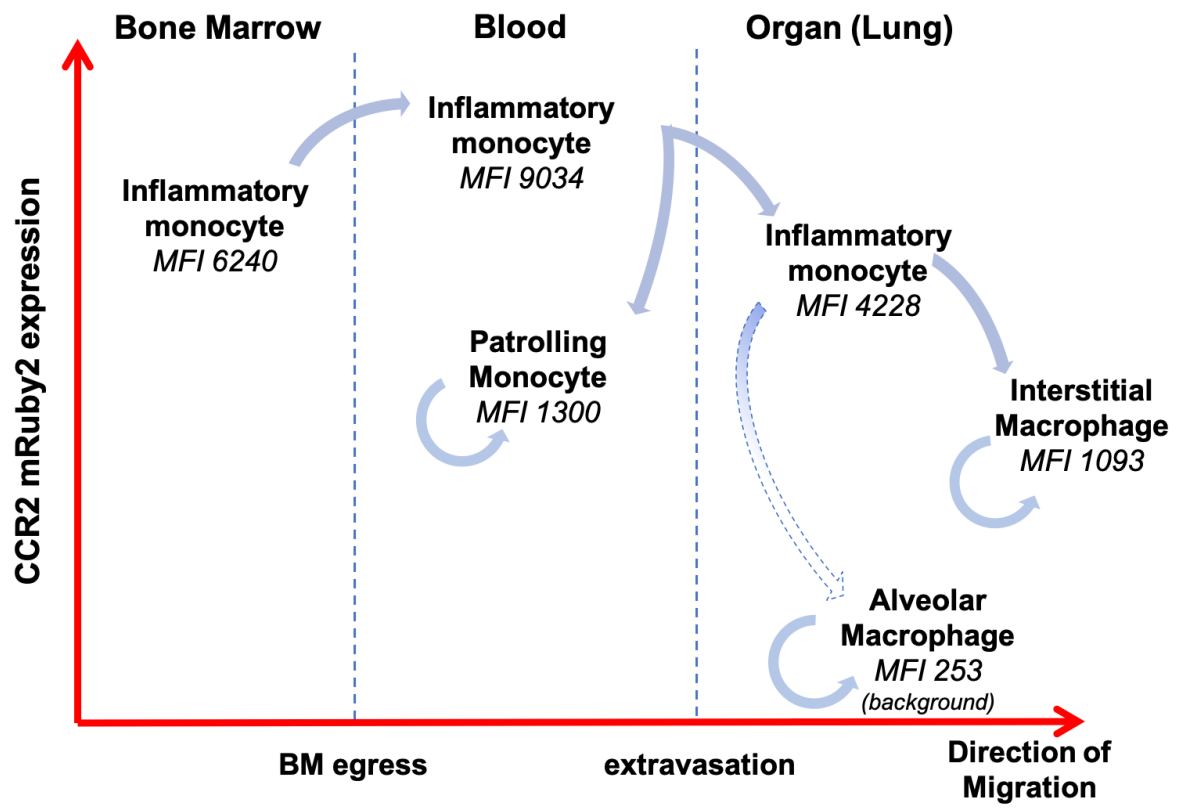

Figure 5-6 CCR2 expression on different monocyte/macrophage subsets from iREP bone marrow, blood and lung. *The different tissues were analysed on the same day on the same flow cytometer with identical settings. The MFIs for each subset represent an average from 3 3-5 BAC copy iREP mice. Solid arrows show the direction of migration. Circular arrows indicate the leukocyte in question has the ability to self-renew. The dotted arrow shows potential contribution of inflammatory monocytes to maintain the alveolar macrophage population in cases of severe inflammation.*

5.4.4 Preliminary Conclusions on Resting iREP mice

Collectively, the data gathered from resting iREP tissues show that fluorescent reporter proteins can be reliably detected in leukocytes and that their pattern of expression correlates with the correct iCCR, as almost the totality of inflammatory monocytes express CCR2 and marginal levels of CCR1, and eosinophils CCR3, consistent with the literature. CCR1 expression in resting bone marrow and circulating B-cells is a novel finding, and it would be interesting to explore the role this inflammatory chemokine receptor has in a leukocyte that generally relies on other receptors for chemotaxis.

The data on CCR2 expression in 'classical' inflammatory monocytes compared to lung resident macrophages show that this murine model also allows for direct visualisation of the activation and maturation state of a leukocyte by assessing the intensity of the fluorescence of the reporter protein.

Under resting conditions, those leukocyte subsets expressing iCCRs expressed only a single iCCR, with the exception of inflammatory monocytes which marginally (~3%) co-expressed CCR1 with CCR2. Not only does this indicate that iCCR expression under resting conditions is tightly regulated, but it also means that it is possible to identify leukocytes in the absence of lineage specific markers. In other words, an mTagBFP positive leukocyte in the circulation could only be an eosinophil, making the iREP strain ideal for microscopy or other imaging techniques where staining tissues to identify cells is impractical.

The number of BAC copies had no effect on leukocyte recruitment or leukocyte subpopulation distribution, but the mean fluorescent intensity for reporter proteins was higher in leukocytes with 5-7 BAC copies, compared to leukocytes with 3-5 BAC copies. Having several BAC copies also increased the number of leukocytes positive for a specific iCCR, with 17% of B-cells in 3-5 BAC mice expressing CCR1 compared to 30% in 5-7 BAC mice. As such, further experiments on iCCR upregulation during inflammation focused on 5-7 BAC mice, as it was hypothesised that 5-7 BAC copies would make small fluctuations in iCCR expression easier to detect.

5.4.5 Resting iREP Data Discussion

Fluorescent reporter protein expression in adult tissues revealed that iCCR-expressing resting leukocytes generally express only a single iCCR, with the exception of 'classical' inflammatory monocytes in which ~3% co-express CCR1 with CCR2.

This was somewhat surprising, as several studies have reported simultaneous expression of multiple iCCRs in certain leukocytes. A group looking at patterns of CCR2 and CCR5 expression in mouse peripheral blood leukocytes found that 2-15% of circulating T-cells express CCR2, and 10% of CD4 T-cells, 40% of CD8 T-cells and most NK cells expressed CCR5 [79].

To detect these receptors, the authors generated in house monoclonal antibodies by immunising rats with CHO cells stably transfected with murine CCR2 or CCR5. The specificity of the antibodies was determined on CHO cells overexpressing these two receptors, and while the data shows that the antibodies do not cross react with each other, the authors did not test these antibodies with CHO cells expressing other chemokine receptors. As mentioned in previous chapters, high structural homology between related chemokine receptors makes detection through antibodies prone to high levels of cross reactivity and false positives. It is therefore possible that some of the CCR2 and CCR5 expression detected in T-cells could be the result of cross-reactivity of the antibody with other T-cell chemokine receptors.

Another group looking at iCCR expression on leukocytes also found evidence of co-expression of CCR3 and CCR1 in RNA transcripts from resting eosinophils [340]. Transcription however does not necessarily result in expression of functioning protein on the surface, as chemokine receptors such as CCR5 can be stored in endosomes for later use[346] or can be recycled like CCR2 and CCR3[44] so expression can be immediate without *de novo* protein synthesis. Other receptors, such as CCR1, are not recycled and are continuously reformed [347], so CCR1 might appear more highly expressed than other receptors at the transcript level without necessarily being more evident on the surface.

Evidence from the literature of iCCR co-expression in resting leukocytes is vague, and reporter leukocytes from iREP mice showed no expression of iCCR in T-cells, no co-expression of iCCRs in eosinophils, and only minor co-expression of CCR1 and CCR2 in inflammatory monocytes.

Regardless of the method of detection however, strong iCCR upregulation has been well documented in leukocytes exposed to a variety of inflammatory stimuli, from macrophages in CNS lesions[348], inflammatory monocytes in atherosclerotic plaques[205], to T-cells and NK-cells during pulmonary inflammation [349] or viral infections[350].

During infection or acute inflammation, epithelial cells upregulate the secretion of a wide variety of inflammatory chemokines and cytokines. To respond to this wide variety of stimuli, recruited leukocytes have been shown to diversify their chemokine receptor expression to better respond to the local inflammatory conditions. Once engaged several times, a receptor becomes desensitised, gradually losing its ability to promote cell migration towards a specific target[71]. Expression of a second or a third receptor once the leukocyte has reached the inflamed site allows the cell to keep moving and follow other chemotactic cues. As mentioned previously, simultaneous expression of several receptors at the site of infection can also be considered a form of defence against pathogen-induced perturbation of chemotaxis[166], allowing the immune system to function even if the pathogen has managed to disable key signals. To assess the effect of inflammation on leukocyte iCCR expression (and potential co-expression), the air-pouch model was performed on iREP mice.

5.5 Inflamed iREP Tissues

5.5.1 Model of Inflammation

An air pouch was produced by the subcutaneous injection of sterile air into the back of the mouse, as described previously (see Methods). The resulting pouch mimics a body cavity, providing a new localized environment in which to study cell trafficking and inflammatory response. The benefits of the air pouch model have been described in detail earlier, but the main advantage in this particular case is that the air pouch is free of tissue resident leukocytes. As the main focus of this experiment was to assess any potential iCCR co-expression in recruited leukocytes, the absence of resident cells ensures that the data collected relates to recruited leukocytes only.

48 hours after the addition of carrageenan into the air pouch, iREP mice were culled. Bone marrow, blood, air pouch membrane and fluid were all collected, and fluorescent reporter protein expression on leukocytes was analysed via flow cytometry. The air pouch model was also simultaneously performed on non-fluorescent WT littermates, and their WT leukocytes were used as baseline to determine positive fluorescence in the iREPs.

This was important as inflammation causes some leukocytes to become auto-fluorescent at certain wavelengths. Once activated by an inflammatory stimulus, leukocytes will increase protein synthesis and vacuolisation, reflecting and diffracting light differently. This increase in cellular granularity is detected as genuine fluorescence by microscopes and flow cytometers, generating false positives[351]. WT leukocytes were therefore exposed to the same inflammatory conditions as the iREPs to determine a true negative baseline for reporter protein detection.

Bone marrow was extracted from iREP mice 48 hours after the administration of carrageenan, stained for the same markers and analysed with the same gating strategy used when fluorescent reporter expression under resting conditions was assessed. Specifically, fluorescent reporter protein expression was analysed in ‘classical’ inflammatory monocytes (CD11b+Ly6C++), eosinophils (CD11b+ SiglecF+) and B-cells (CD19++).

5.5.2 Inflammatory Monocytes

5.5.2.1 Bone Marrow

Similar to resting conditions, more than 95% of inflammatory monocytes expressed CCR2. CCR1 expression however increased more than 6-fold, from 3% at rest to 16% during inflammation (Figure 5-7 A,B). Analysis of iREPs with 3-5 copies also showed a similar increase in inflammatory monocytes co-expressing CCR1 and CCR2, but the increase was more modest (4-fold compare to resting), again showing that a higher BAC number correlates with higher levels of fluorescent protein detection.

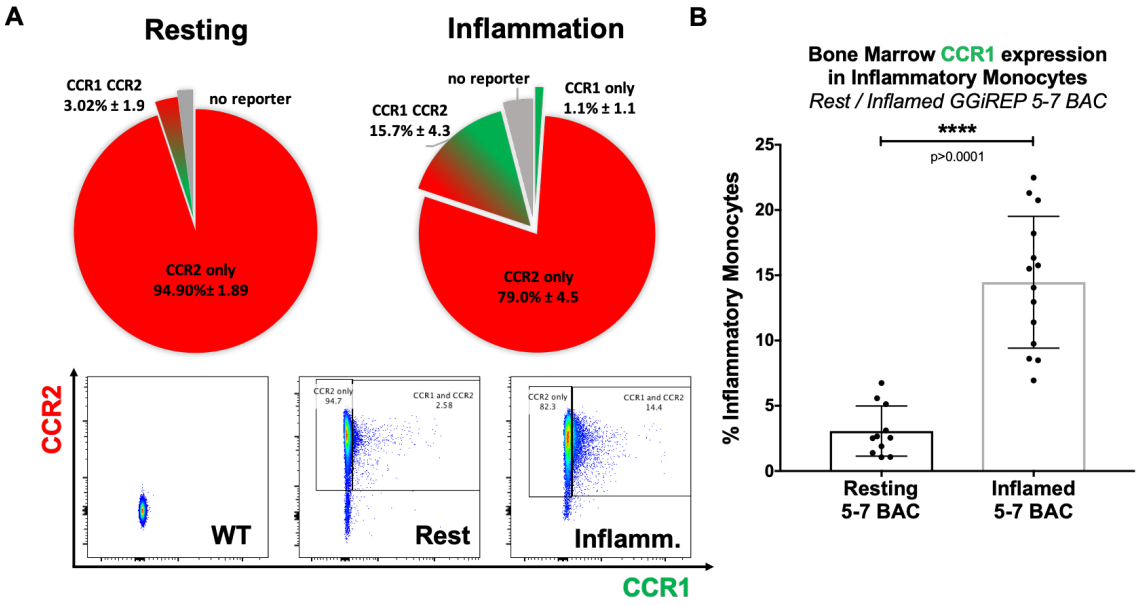

Figure 5-7 iCCR Expression on bone marrow inflammatory monocytes- A*- Pie charts and representative FACS plots showing the % of 5-7 BAC copy bone marrow inflammatory monocytes expressing CCR2 and CCR1 at rest and during inflammation. Expression of CCR2 alone is shown in red, co-expression of CCR2 and CCR1 in red/green shading, no fluorescent reporter protein expression is shown in grey. Positive reporter expression was determined using non-fluorescent WT inflammatory monocytes.* ***B****- CCR1 expression in bone marrow inflammatory monocytes with 3-5 or 5-7 BAC copies, both at rest and during inflammation. Resting data representative of 3 experiments with n>3, Inflammation data representative of 3 experiments with n=4.*

5.5.2.2 Blood

Inflammatory monocytes in the circulation showed a similar pattern to that seen in the bone marrow during inflammation. As seen in Figure 5-8 (A,B), circulating Ly6C++ leukocytes exposed to inflammation increased CCR1 expression 4-fold, from 2.5% at rest to 10.6%. The number of CCR1+ve monocytes however was higher in the bone marrow (16%) than in the circulation (10.6%). This finding was somewhat counter-intuitive, as CCR1

had previously been shown to facilitate firm adhesion of leukocytes to the blood vessels under shear flow[352], so CCR1 expression was expected to be highest while inflammatory monocytes are circulating in the blood.

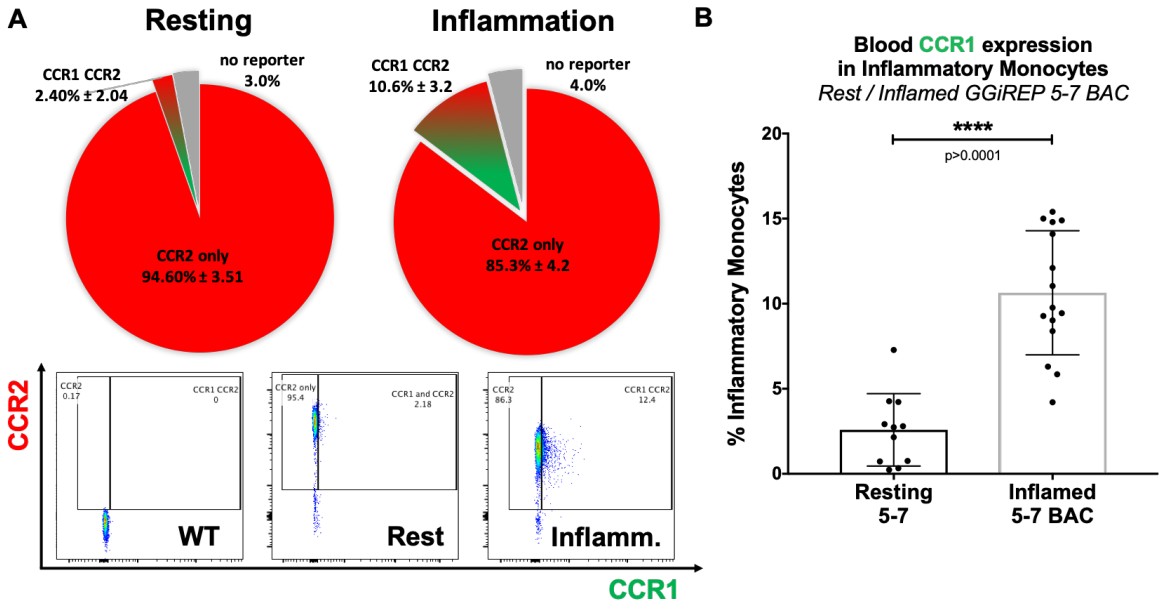

Figure 5-8 iCCR Expression on circulating inflammatory monocytes- A*- Pie charts and representative FACS plots showing the % of 5-7 BAC copy blood inflammatory monocytes expressing CCR2 and CCR1 at rest and during inflammation. Expression of CCR2 alone is shown in red, co-expression of CCR2 and CCR1 in red/green shading, no fluorescent reporter protein expression is shown in grey. Positive reporter expression was determined using non-fluorescent WT inflammatory monocytes.* ***B****- CCR1 expression in circulating inflammatory monocytes with 5-7 BAC copies, both at rest and during inflammation. Resting data representative of 3 experiments with n>3, Inflammation data representative of 3 experiments with n=4.*

As CCR1 expression was lower in circulating monocytes, this raises two possibilities. Either CCR1 is upregulated briefly during inflammatory monocyte bone marrow egress and downregulated rapidly while in circulation, or more simply, CCR1+ monocytes are 'stuck' along the inflamed vessel walls, leaving mainly CCR2+ monocytes in extracted blood. Data gathered previously from $CCR1^{-/-}$ and $iCCR^{-/-}$ mice showed no accumulation of inflammatory monocytes in the bone marrow, suggesting that CCR1 is dispensable for bone marrow egress.

If CCR1 is indeed required for inflammatory monocyte arrest under shear flow, then the air pouch membrane should be 'enriched' with CCR2+CCR1+ co-expressing monocytes, as the ones expressing CCR1 should have been able to cross the endothelial cells lining the blood vessels more easily.

5.5.2.3 Air Pouch Membrane

As inflammatory Ly6C+ monocytes from the circulation migrate towards the site of inflammation, increased exposure to inflammatory stimuli should promote their differentiation into F480+Ly6C low macrophages.

Analysis of the cellular content of the membrane surrounding the air pouch showed two populations of macrophages (CD11b+CD64+CD11c-), an F480++Ly6C low population and another Ly6C++ F480 low. These two populations are thought to represent the two opposites of monocyte/macrophage differentiation, with Ly6C++ F480- cell retaining their undifferentiated inflammatory monocyte phenotype, while Ly6C-F480++ cells represent fully differentiated macrophages. As seen in the FACS plots in figure 5-9A, the two Ly6C++ and F480++ populations are not clearly distinct but present themselves as two extremes of a 'smear', which contains monocytes/macrophages at different stages of differentiation.

CCR2 Expression

Unlike inflammatory monocytes in the circulation, CCR2 expression on monocyte/macrophages in the air pouch is not uniform but can be divided in two CCR2 expressing groups: CCR2 bright and CCR2 mid (FACS plots Figure 5-9 B,C). While the presence of CCR2 bright and CCR2 mid monocytes might suggest that these cells have different origins, the fact that the air pouch membrane is devoid of resident leukocytes and only one population of CCR2 bright monocytes was detected in the circulation suggests that the CCR2 mid population derives from the CCR2 bright, and not the result of another monocytic subset migrating from the periphery.

Supporting this hypothesis, CCR2 bright cells look phenotypically closer to circulating inflammatory monocytes, and represent almost 40% of Ly6C++ cells in the air pouch membrane (Fig. 5-9 B). In contrast, the vast majority (~85%) of F480++ cells express mid levels of CCR2 (Fig. 5-9 C). These finding suggest that inflammatory monocytes are CCR2 bright, and these cells differentiate into CCR2 mid F480++ macrophages.

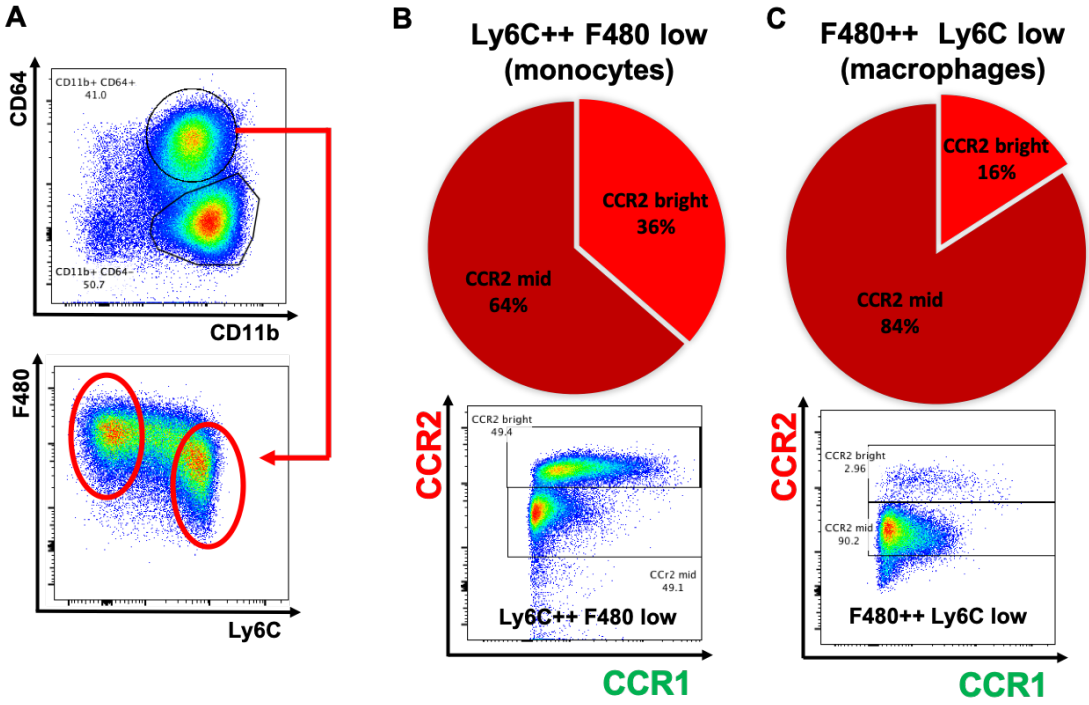

Figure 5-9- CCR2 Expression in Differentiating Monocytes- A*- Gating strategy showing CD64+CD11b+(CD11c-MHCII-) macrophages and their F480+ and Ly6C+ expression. F480++ were classified as macrophages, Ly6C++ as less differentiated monocytes.* ***B****- Pie chart showing the proportions of CCR2 bright and CCR2 mid Ly6C++F480low monocytes. The representative FACS plots shows CCR1 expression on CCR2 bright and CCR2 mid monocytes.* ***C****- Pie chart showing the proportions of CCR2 bright and CCR2 mid F480++Ly6C low macrophages. The representative FACS plots shows CCR1 expression on CCR2 bright and CCR2 mid macrophages.* ***D****- Graph comparing CCR1 expression on CCR2 bright and CCR2 mid monocytes. Graph representative of several experiments. n=4*

What is also of interest is how CCR2 expression is regulated as monocytes differentiate. As seen from the FACS plot in Figure 5-9A, staining for F480 and Ly6C expression presents itself as a smear, showing monocytes and macrophages at varying degrees of maturation. The FACS plots in Figure 5-9 B and C instead show that CCR2 bright and CCR2 mid populations present themselves as two distinct populations.

Downregulation of CCR2 expression during monocyte differentiation is well documented in the literature[188], and is believed to occur to avoid reverse migration back into circulation[328]. The data from iREP inflammatory monocytes suggest downregulation of CCR2 is more tightly controlled than previously thought, with two distinct expression ‘modes’: CCR2 bright for circulating monocytes and CCR2 mid for monocytes committing to differentiation

CCR1 expression

CCR1 expression was higher in CCR2 bright cells, compared to the CCR2 mid-monocytes (Figure 5-10 A). This difference in CCR1 co-expression on different monocyte/macrophage subsets can be explained by taking into consideration the differentiation state of these cells and the previous findings on iCCR expression on circulating inflammatory monocytes.

While only 10-15% of circulating inflammatory monocytes expressed CCR1, 24% of membrane inflammatory monocytes co-express CCR1 and CCR2 (Figure 5-10 B), indicating that the air pouch membrane is indeed 'enriched' for CCR1+CCR2+ monocytes and explaining the unexpected prevalence of CCR2+CCR1- monocytes extracted from blood.

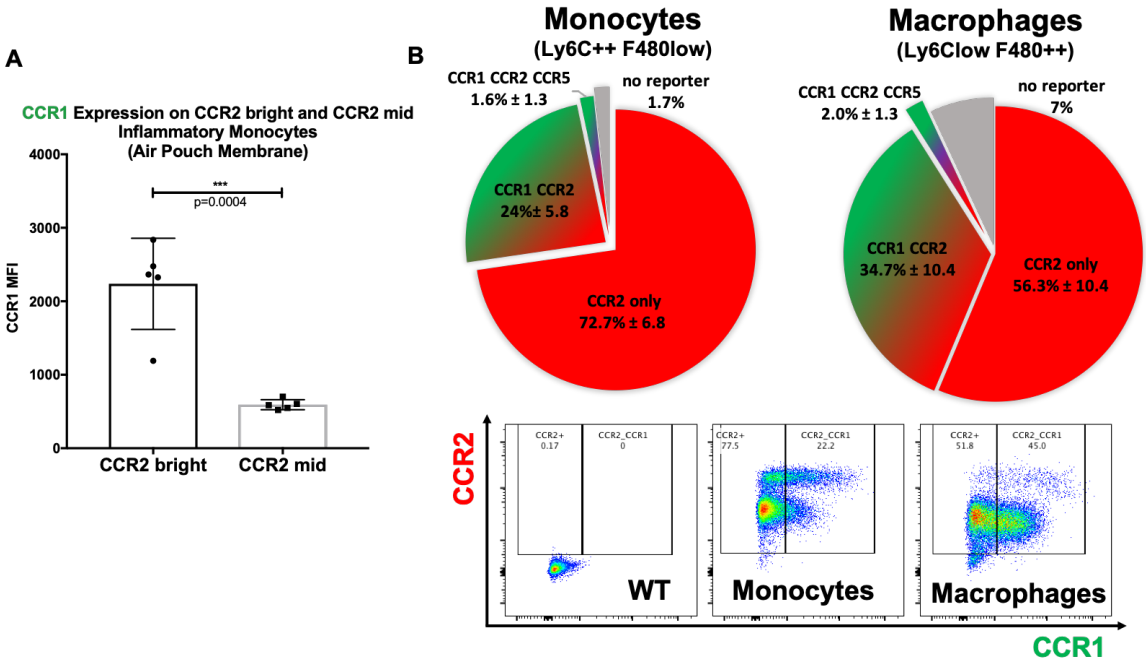

Figure 5-10- CCR1 expression on Monocytes and Macrophages in the Air Pouch Membrane. A- *CCR1 expression on CCR2 bright and CCR2 mid inflammatory monocytes in the Air Pouch Membrane.* ***B-*** *Pie charts (and representative FACS plots) summarising expression and co-expression of CCR1 (green) CCR2 (red) and CCR5 (purple) in Ly6C++F480low monocytes and F480++Ly6Clow macrophages. Data representative of 3 experiments with n=4.*

CCR1 expression, measured as mean fluorescent intensity, was highest in the CCR2 bright monocytes (~2200 MFI compared to 750 in CCR2 mid). On the other hand, while lower in MFI, more macrophages expressed CCR1 than inflammatory monocytes (35% against 24%) (Figure 5-10 B). This increase in CCR1 expressing macrophages in the air pouch membrane could either be the result of *in situ* CCR1 upregulation as the monocyte differentiates, or, more simply, evidence that CCR1+ inflammatory monocytes differentiate into CCR1+ macrophages.

In other words, while both CCR2+ and CCR2+CCR1+ inflammatory monocytes are capable of migrating towards inflammation, the presence of CCR1 might strengthen the monocyte's adhesion to the vessel walls and promote migration through the air pouch membrane. Thus, while both CCR2+CCR1- and CCR2+CCR1+ inflammatory monocytes can be found on the air pouch membrane, co-expression of both receptors speeds up the migration and differentiation process, resulting in a higher proportion of macrophages expressing CCR1.

CCR5 expression and Air Pouch Fluid

CCR5 expression (IRFP682 fluorescence) was detected for the first time in both Ly6C++ monocytes and F480++ macrophages in the air pouch membrane. 1.6% of inflammatory monocytes were triple positive for CCR1 CCR2 and CCR5, increasing slightly to 2% in macrophages (Figure 5-10 B). While the levels of CCR5 detected were still rather low, analysis of the cellular content of the air pouch fluid revealed further upregulation of this receptor. While Ly6C++ monocytes made up the majority (~60%) of the CD11b+CD64+CD11c- leukocytes in the air pouch membrane, the air pouch fluid contained a majority of F480++ macrophages, showing that effective migration into the site of inflammation correlates with differentiation state (Figure 5-11A). Analysis of iCCR expression of fluid F480+ macrophages revealed that 54.5% co-expressed CCR1 and CCR2 (increased from 34.7% in the membrane), and a six-fold increase in macrophages co-expressing CCR1 CCR2 and CCR5 (from 2% to 13%) (Figure 5-11 B), although this was still considered unexpectedly low.

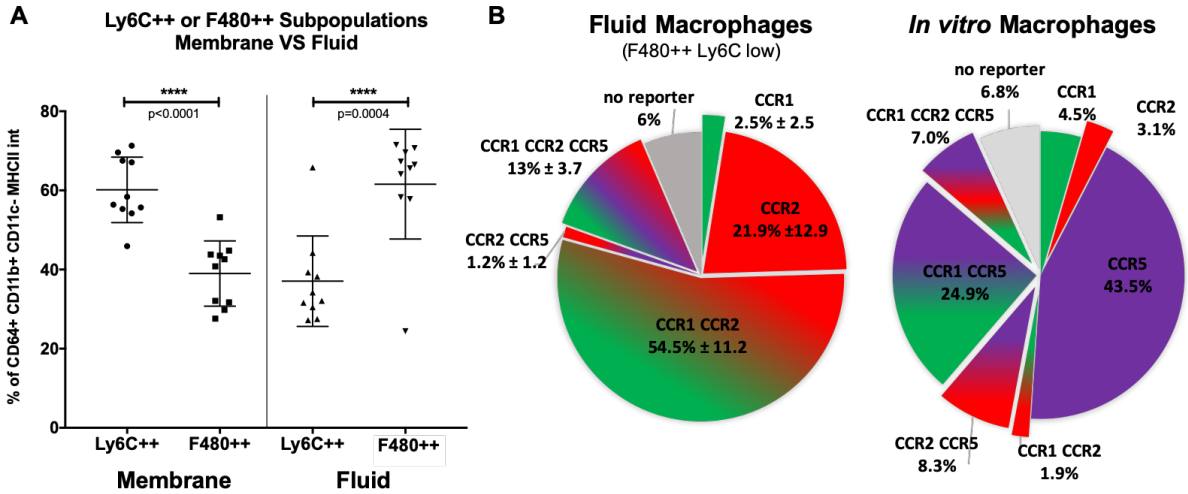

Figure 5-11 CCR5 Expression on Fluid Macrophages. A- *Scatter plot graph showing distribution of macrophages and monocytes in the membrane and the fluid of the air pouch.* ***B-*** *Pie charts summarising iCCR expression on F480++Ly6Clow macrophages in the air pouch fluid compared to bone marrow derived F480++ macrophages. Air pouch Data representative of 3 experiments with n=4. In vitro data representative of one experiment with 3 different sets of pooled iREP bone marrow (n=3).*

The short duration of the model (48 hours after carrageenan injection) might explain this, as there might not have been enough time for a monocyte recruited from the bone marrow to migrate successfully and differentiate sufficiently for CCR5 expression to be prevalent. To test if iREP macrophages were capable of expressing CCR5, bone marrow derived macrophages were grown as described previously, stained for CD11b and F480, and analysed for iCCR expression via flow cytometry. The *in vitro* pie chart in Figure 5-11 B shows that after 5 days of culture more than 85% of macrophages express CCR5, indicating that iREP macrophages are indeed capable of expressing CCR5 and that low expression *in vivo* is probably due to the temporal limitations of the air pouch model.

5.5.3 Eosinophils

5.5.3.1 Bone Marrow

Compared to inflammatory monocytes, inflammation appears to have the opposite effect on iCCR expression in eosinophils. Not only do CD11b+SiglecF++ cells not upregulate other iCCRs, but the number of bone marrow eosinophils positive for CCR3 (mTagBFP2) drops from 61.4% under resting conditions to 49.9% in the air pouch model (Figure 5-12 A,B).

The representative FACS plots offer a possible clue to explain this reduction. These images show a very small CCR3 bright population under resting conditions, which disappears completely under inflammatory conditions (Figure 5-12 A). This small population of CCR3 bright eosinophils looks similar to the population of mature eosinophils (Siglec F++) trapped in bone marrow of iCCR$^{-/-}$ and CCR3$^{-/-}$ mice (Figure 5-12 C) described in the previous chapter. A possible conclusion could be the following: under inflammatory conditions, rapidly maturing eosinophils increase CCR3 expression and leave the bone marrow, leaving behind a disproportionately high number of immature eosinophils (reporter negative) which are not yet equipped with the cellular machinery required for bone marrow egress. If high CCR3 expression is indeed a requirement for bone marrow egress, then almost the totality of eosinophils in the circulation should be CCR3+ve.

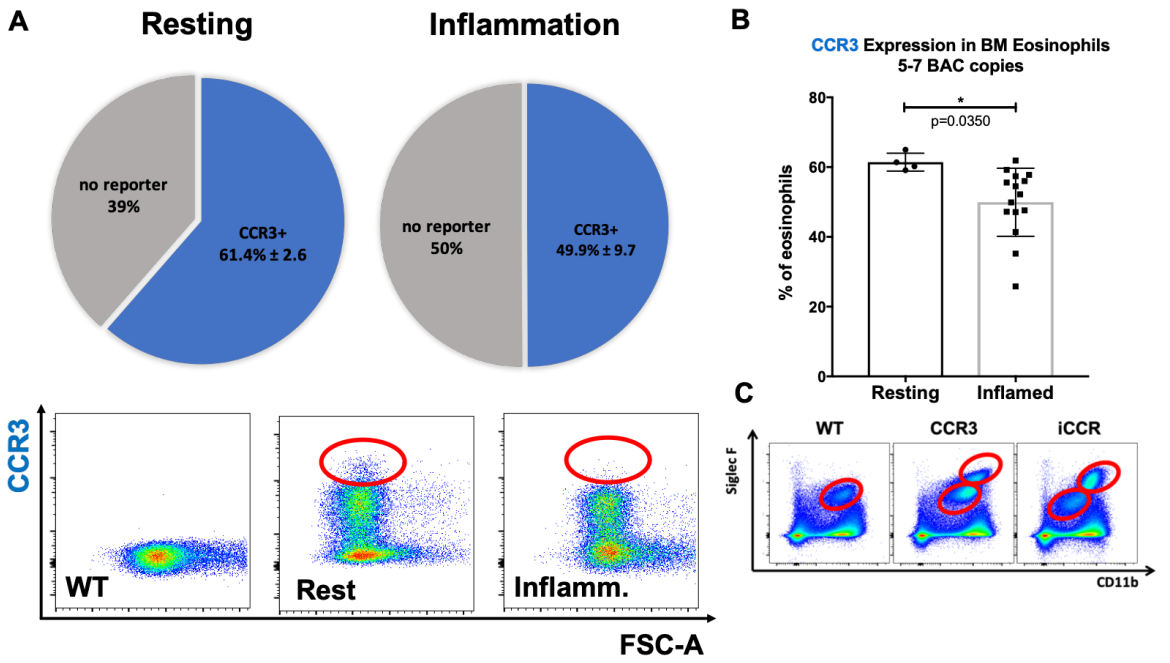

Figure 5-12 CCR3 Expression on Bone Marrow Eosinophils. A*- Pie charts summarising CCR3 (blue) expression in CD11b+Ly6G-SIglec F++ eosinophils at rest and during inflammation. Circled in red on the representative FACS plots the faint CCR3 bright eosinophil population at rest and during inflammation. Resting data representative of 1 experiment with n=3, Inflammation data representative of 3 experiments with n=4.* ***B****- Graph showing the decrease in CCR3 expression (MFI) in CCR3 positive eosinophils after inflammation.* ***C****- Representative FACS plots showing eosinophils in WT, CCR3 KO and iCCR KO bone marrow. 2 populations (Siglec F++ and Siglec F+) can be seen in the CCR3 and iCCR KO.*

5.5.3.2 Blood

As seen in Figure 5-13A. more than 90% of eosinophils express CCR3 in the circulation, suggesting that CCR3 is a necessary requirement for eosinophil bone marrow egress.

While the vast majority of eosinophils in the circulation express CCR3 (>90%), a small percentage (~5% at rest) expresses very high levels of the receptor, and was classified as CCR3 bright. While the number of CCR3+ eosinophils does not change before or after inflammation (91.3% and 92.7% respectively), inflammation reduces the numbers of CCR3 bright eosinophils in circulation, from 5% to 1.5% (Fig. 5-13B).

The mechanism of action could be similar to the one proposed for inflammatory monocytes, as CCR3 has been shown to promote eosinophil arrest on inflammatory endothelium in shear flow[353].

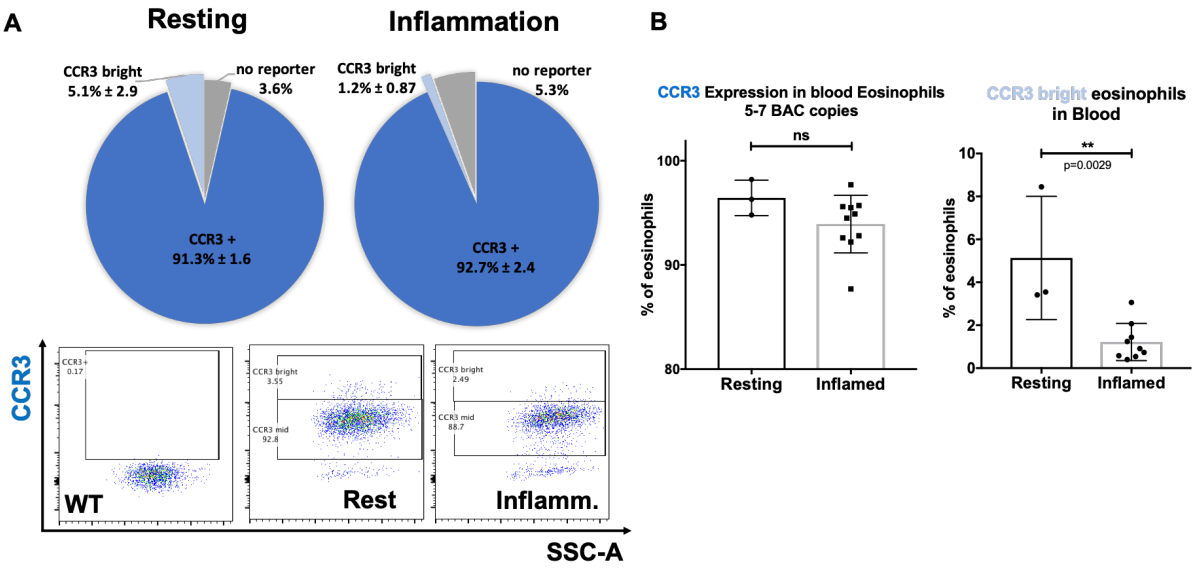

Figure 5-13 CCR3 Expression on Circulating Eosinophils. A- *Pie charts showing the proportions of CCR3 bright and CCR3 positive eosinophils in circulation at rest and during inflammation. Representative FACS plots shown below. B- Graphs summarising the change in distribution of CCR3 positive and CCR3 bright eosinophils before and after inflammation. Resting data representative of one experiment with n=3, Inflammation data representative of 3 experiments with n=4.*

CCR3 bright eosinophils are more mature, better capable of binding to the activated endothelium, and probably better equipped for trans-endothelial migration than CCR3 positive eosinophils. Similar to CCR1 expressing monocytes, CCR3 bright eosinophils might be 'stuck' along the inflamed vessel walls, leaving only CCR3-mid eosinophils in extracted blood. As a result, CCR3 bright eosinophils should be enriched in the air pouch membrane and fluid, as these cells should have been able to transmigrate from vasculature more efficiently.

5.5.3.3 Air Pouch Membrane and Fluid

Analysis of iCCR expression of air pouch membrane eosinophils revealed that 24% could be classified as CCR3 bright (Figure 5-14A), the highest concentration of CCR3 bright cells in any of the tissues analysed. Only 1.2% of eosinophils in circulation were CCR3 bright, suggesting that either CCR3 positive cells upregulate the receptor once at the site of inflammation, or that CCR3 bright eosinophils are better equipped for chemotaxis and migrate more effectively to inflamed areas than their CCR3-mid counterparts.

Analysis of CCR3 expression in air pouch fluid revealed a drop in CCR3 bright eosinophils, from 23.8% to 14.6% (Figure 5-14 B,C). If CCR3 expression was tied to proximity to the inflammatory stimulus, then a higher proportion of eosinophils should have been CCR3 bright, as the fluid contains the inflammatory stimulant. It is therefore more likely that CCR3 bright eosinophils are better equipped for chemotaxis and downregulate CCR3 expression once the site of inflammation is reached.

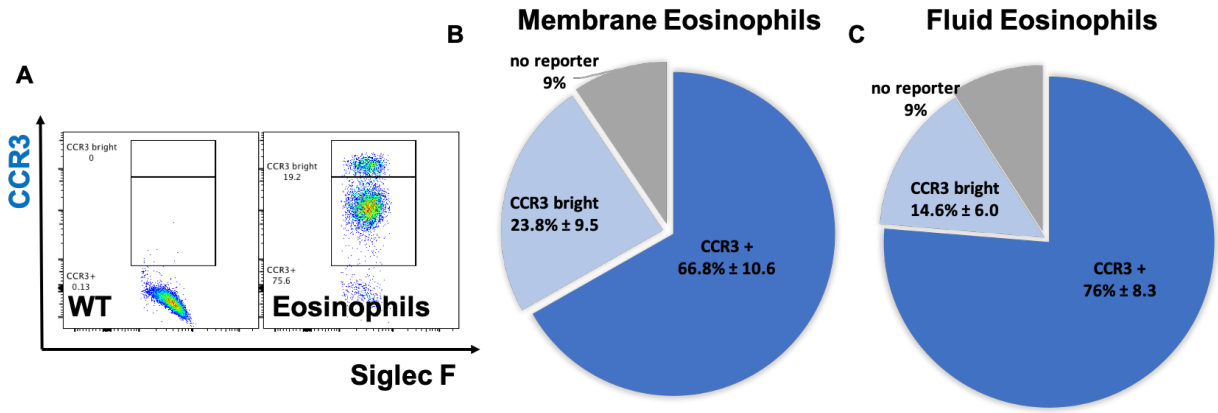

Figure 5-14 Distribution of CCR3 bright and CCR3 positive Eosinophils in the air pouch membrane. *A- Representative FACS plots showing two main CCR3 expressing populations of eosinophils on the air pouch membrane, CCR3 bright and CCR3 mid. B- Pie charts summarising the proportion of CCR3 bright and CCR3 positive eosinophils in the membrane and the fluid of the air pouch. Data representative of 3 experiments with n=4.*

The nature of CCR3 expression in eosinophils (bright and mid distinct populations) is reminiscent of CCR2 expression in inflammatory monocytes, where CCR2 bright inflammatory monocytes differentiate into macrophages and downregulate to CCR2 mid expression to avoid reverse migration back into circulation. The same mechanism could be at play here, where CCR3 bright eosinophils that have reached the site of inflammation downregulating to CCR3 mid expression to avoid reverse migration.

5.5.4 B-cells

5.5.4.1 Bone Marrow

Inflammation did not appear to have a major effect on B-cell iCCR expression. B-cell (CD19++) CCR1 expression increased modestly (6%) after inflammation from 25.3% to 31.8% (Figure 5-15 A,B), but no other iCCR was detected. The failure of B-cells to upregulate other iCCRs is not surprising, as B-cells in the bone marrow do not simply home to a site of inflammation, but migrate through the blood towards secondary lymphoid organs where they will receive a constant supply of antigen through circulating lymph and activate accordingly. As this process is dependent on CCR7, CXCR4, CXCR5 [354], upregulation of other iCCRs would be counterproductive and result in aberrant B-cell activation.

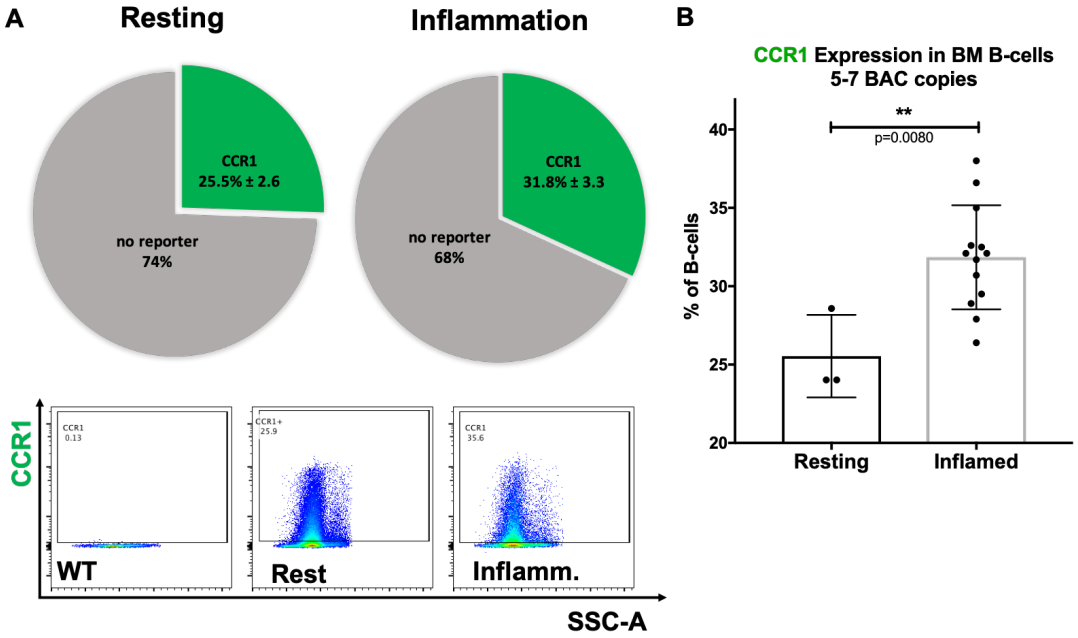

***Figure 5-15 CCR1 Expression on bone marrow B-cells. A**- Pie charts (and representative FACS plots) showing the proportion of B-cells expressing CCR1 (green) in resting and inflamed bone marrow. **B**- Graph showing the proportion of CCR3 expressing B-cells at rest and during inflammation. Resting data representative of one experiment with n=3, Inflammation data representative of 3 experiments with n=4.*

5.5.4.2 Blood

Circulating B-cells during air-pouch induced inflammation also increased CCR1 expression by 6% (Figure 5-16A). The proportion of CCR1+ B-cells in the bone marrow and in the blood is very similar (31.8% and 27.7%), and inflammation increases CCR1 expression in both tissues by the same amount (+6%) (Figure 5-16B). In addition, B-cells were not detected in

the air pouch membrane or fluid. The findings suggest that CCR1 expression in B-cells is not required for bone marrow egress, migration through blood or transendothelial migration. While the reasons as to why this receptor is expressed are still unclear, Dr. Medina-Ruiz detected very high levels of CCR1 in splenic B-cells, suggesting the expression of this receptor might have a role in B-cell positioning in the spleen or in other secondary lymphoid organs.

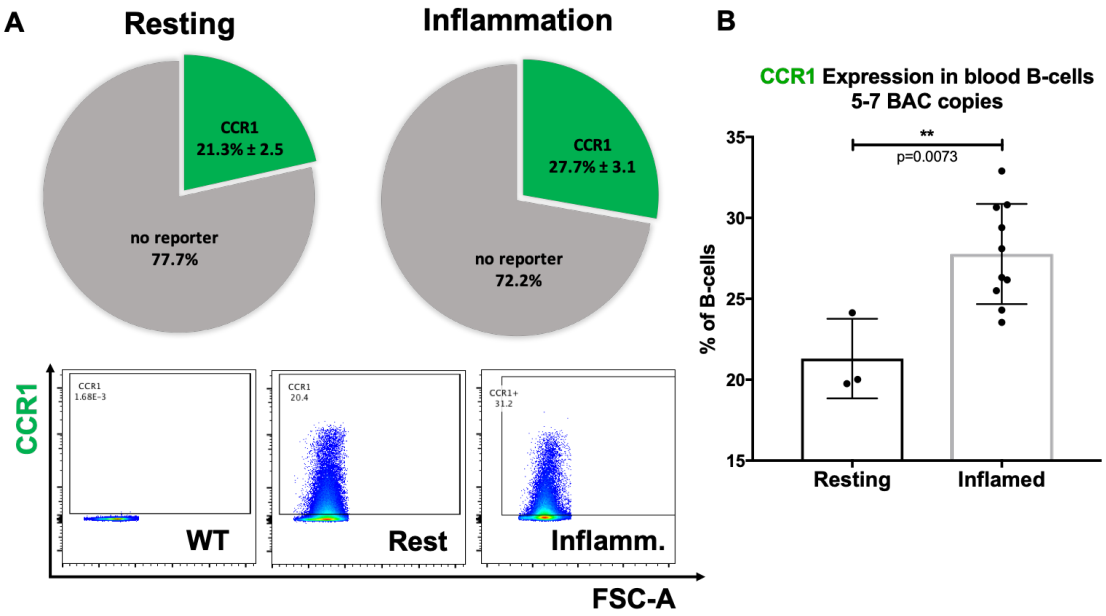

Figure 5-16 CCR1 Expression on circulating B-cells. A*- Pie charts (and representative FACS plots) showing the proportion of B-cells expressing CCR1 (green) in resting and inflamed blood.* ***B****- Graph showing the proportion of CCR3 expressing B-cells at rest and during inflammation. Resting data representative of one experiment with n=3, Inflammation data representative of 3 experiments with n=4.*

5.6 Overall Discussion

Studying the fluctuations of inflammatory chemokine receptor expression on the surface of leukocytes has, until now, relied heavily on mRNA analysis and antibody staining. Both methods of detection have their drawbacks: iCCR specific antibodies are prone to cross-reactivity and detection of false positives, and mRNA analysis relies on extensive procedures of leukocyte isolation before the RNA is extracted, which in itself could modify the activation state of the leukocyte and alter iCCR transcript detection. mRNA analysis also does not take into account the contribution that recycled chemokine receptors have on leukocyte activation.

This novel inflammatory chemokine receptor reporter (iREP) mouse strain has the potential to revolutionise our understanding of the role of inflammatory chemokine receptors CCR1, CCR2, CCR3 and CCR5 on the development of the immune response. The data gathered in this chapter show that this reporter strain allows for effective iCCR detection on a variety of leukocytes, and can be used to track changes in iCCR expression *in vivo* as leukocytes react to an inflammatory stimulant.

Analysis of iREP mouse fitness and tissue leukocyte content showed no difference between WT and iREP mice, indicating that BAC integration and fluorescent protein expression have no impact on mouse development or on the number of leukocytes in various tissues.

Fluorescent reporter proteins were detected in several leukocyte subsets, including 'classical' inflammatory monocytes (CCR2), eosinophils (CCR3), B-cells (CCR1), and 'non-classical' patrolling monocytes (CCR2). Under resting conditions, detection of fluorescent reporter proteins via flow cytometry revealed high CCR2 expression (>95%) in inflammatory monocytes and high CCR3 expression in eosinophils (>90% in circulation), in line with expectations. In the circulation, patrolling monocytes (which have been shown to derive from CCR2+ Ly6C+ inflammatory monocytes[344]) also expressed CCR2 (>65%).

The level of CCR2 expression on a monocyte/macrophage could also be used to determine the leukocyte's differentiation state or location in the body: CCR2 expression was highest in circulating 'classical' monocytes and decreased gradually as the monocytes developed into 'non-classical' patrolling monocytes or lung tissue macrophages. CCR2 was not expressed in alveolar macrophages, which have a distinct embryonic origin[345].

Numerous reports in the literature describe upregulation of chemokine receptors and iCCR co-expression on leukocytes during inflammation, so the air pouch model was performed on iREP mice to assess if it was possible to detect iCCR fluctuations in inflamed leukocytes.

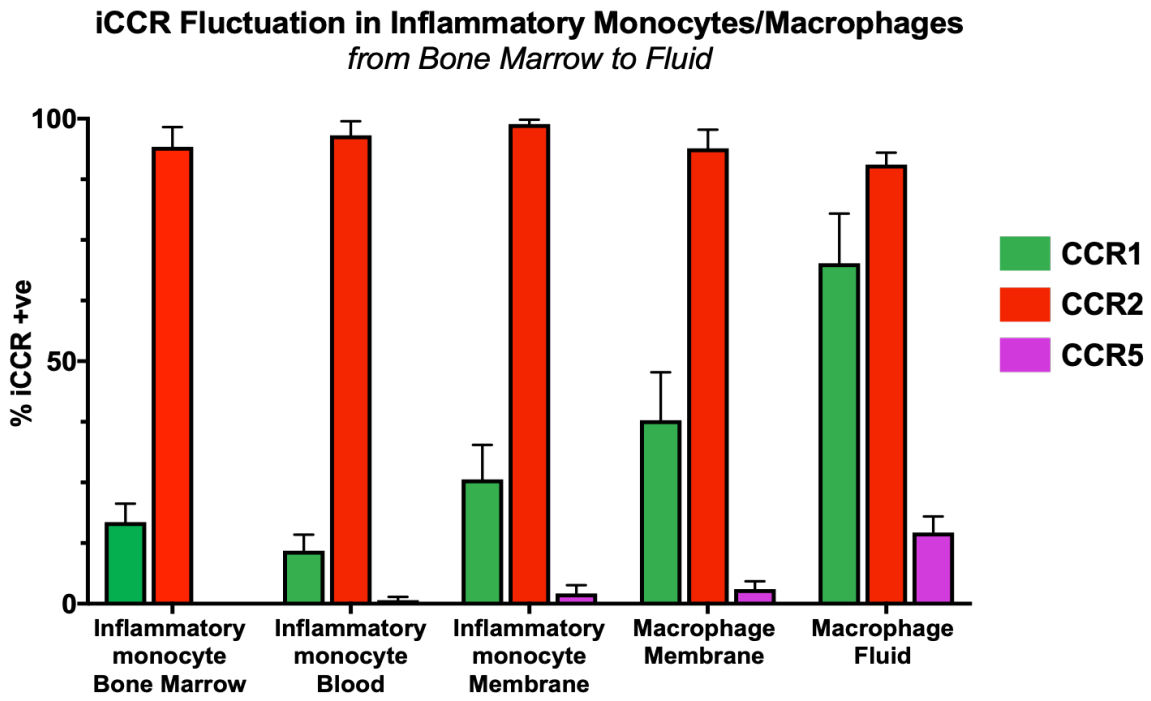

Figure 5-17- iCCR expression in Inflammatory Monocytes during migration and differentiation in vivo- *Graph summarising the expression of CCR1 (green), CCR2 (red) and CCR5 (purple) (expressed as percentage positive for each population) as inflammatory monocytes migrate from the bone marrow into the air pouch fluid, differentiating into macrophages. Inflammation data representative of 3 experiments with n=4.*

Not only was iCCR co-expression detected in inflamed inflammatory monocytes, but their distribution throughout various tissues on their way to the air pouch revealed that CCR1/CCR2 co-expressing monocytes are probably better able to chemotax than their CCR2+CCR1-negative counterpart, resulting in an accumulation of CCR1+ macrophages (>65% in the air pouch fluid). CCR5 was also detected in air pouch fluid macrophages, suggesting that this receptor is only expressed at the latest stages of differentiation (Figure 5-17).

A portion of circulating B-cells also expressed CCR1, but no B-cells were found in the air pouch membrane or fluid, suggesting that expression of CCR1 is not enough for migration to inflamed sites.

Unlike inflammatory monocytes, eosinophils in inflammation did not upregulate other iCCRs, but some appeared to upregulate their levels of CCR3, resulting in two populations of CCR3 bright and CCR3 mid eosinophils in the air pouch membrane. As CCR3 bright eosinophils only accounted for 1.2% of eosinophils in the circulation but made up 24% of eosinophils in the membrane, it was concluded that CCR3 bright eosinophils are preferentially recruited to the site of inflammation over CCR3 mid eosinophils.

Detection of the mean fluorescence intensity of reporter proteins in iREP leukocytes also revealed differential mechanism of CCR2 and CCR3 regulation compared to CCR1 and CCR5. As seen in Figure 5-18A, both eosinophils and inflammatory monocytes present themselves as two clearly separated populations, a bright and a mid. On the other hand, expression of CCR1 on B-cells and CCR1 and CCR5 on inflammatory monocytes appears as a 'smear', representing more of a spectrum of expression.

This difference may be attributable to the nature of CCR2 and CCR3 expression, as these were the only receptors found to be expressed constitutively at high levels under resting conditions. As these two receptors control basal migration of inflammatory monocytes and eosinophils respectively, their expression has to be tightly regulated. On the other hand, upregulation of CCR1 and CCR5 might be tied more closely to the individual activation status of the cell or the local inflammatory conditions, and thus appear as a spectrum of expression.

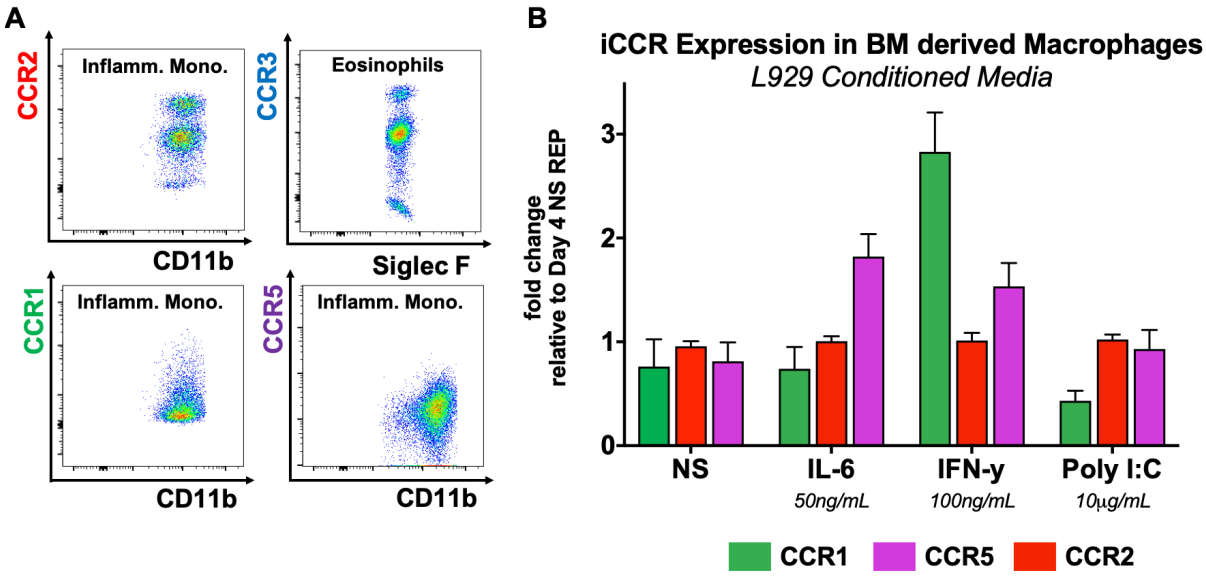

Figure 5-18 Patterns of iCCR expression in iREP leukocytes- A- *Representative FACS plots showing the pattern of expression of CCR2, CCR1 and CCR5 in inflammatory monocytes and CCR3 in eosinophils.* ***B-*** *Graph showing iCCR expression in iREP bone marrow derived macrophages stimulated overnight with IL-6 (50ng/ml), IFNy (100ng/ml) and Poly I:C (10μg/ml). Values expressed as fold change in percentage positive using non-stimulated iREP macrophages as baseline. Data representative of one experiment with 3 different sets of pooled iREP bone marrow (n=3).*

The findings on *in vitro* bone marrow macrophages summarised in Figure 5-18B support this hypothesis: CCR2 expression on macrophages stimulated with a variety of inflammatory stimulants does not change, probably because most macrophages in culture have a stable CCR2 mid expression. CCR1 and CCR5 expression on the other hand fluctuates depending on the inflammatory stimulant (3-fold increase in CCR1 expression after addition of IFNy in culture, 2-fold increase in CCR5 after IL-6).

Ultimately, all the data collected so far validates the iREP as a novel reporter mouse model for studying the main inflammatory CC-chemokine receptors, and lays the foundation for further future experiments that will dissect the roles of CCR1, CCR2, CCR3 and CCR5 with a wider range of inflammatory models.

6 Results- Role of iCCRs in Macrophage and DC function *in vitro*

6.1 Overview

In vivo analysis of iCCR$^{-/-}$ mice revealed a profound defect in recruitment of inflammatory monocytes from the bone marrow and a severe decline in numbers of macrophages and dendritic cells in most tissues analysed. While the role of 'classical' inflammatory monocytes in replenishing macrophages and dendritic cells both at rest[187][355][356] and during inflammation is well established[357][297][358][98], it is unclear if the defects seen in iCCR$^{-/-}$ tissues are exclusively caused by decreased migration of inflammatory monocytes to the site of inflammation, or if the reduction in macrophage and dendritic cell numbers is also exacerbated by the failure of the inflammatory monocytes to respond to local environmental stimulation and differentiate into effector cells.

In vivo work on iCCR Reporter (iREP) mice has shown that macrophages can express various combination of chemokine receptors, with CCR2+, CCR1+CCR2+ and CCR1 CCR2 and CCR5 triple positive populations all present simultaneously in the air pouch membrane. It was unclear however if these different subsets were phenotypically identical, or if the expression of particular chemokine receptors was able to alter differentiation and effector function of these cells.

Indeed, some groups have shown that engagement of inflammatory chemokine receptors is necessary for correct maturation and function of macrophages and dendritic cells. Chemokines have been shown to prolong macrophage survival[338] , while interaction between CCL2 and CCR2 has been shown to provide critical signals for correct dendritic cell maturation in the lung[102]. Furthermore, experiments performed by Dr. Hughes using Influenza virus as a model of inflammation on iCCR$^{-/-}$ mice, have shown an altered immune memory response, suggesting a potential role of CCR1, CCR2 CCR3 and CCR5 in correct maturation and development of the adaptive immune response.

To explore the potential effect of iCCR engagement and signalling on inflammatory monocyte differentiation, bone marrow inflammatory monocytes were collected and differentiated *in vitro* into either macrophages or dendritic cells. The differentiated leukocytes were then collected and analysed by flow cytometry, ELISA and PCR arrays at different stages of development to track changes in phenotype and effector function.

6.2 iCCR$^{-/-}$ Bone Marrow Derived Macrophages and DCs

Isolation and characterisation of resident macrophages from mouse tissues usually requires multiple steps of enzymatic digestion and purification, generally resulting in low yields of highly heterogenous cells[359]. An alternative method for generating high numbers of homogenous macrophages for functional assays involves the culture of bone marrow cells *in vitro* with appropriate growth factors.

Several early studies on macrophage differentiation have identified two main cytokines responsible for macrophage proliferation and survival: GM-CSF and CSF-1[360]. Mice deficient in CSF-1R expression exhibit a more significant phenotype than mice deficient in GM-CSF [361]. Indeed, studies using the *Csf1*$^{op/op}$ murine strain which possesses an inactivating mutation in the CSF-1 gene, have established the central role of CSF-1 in a wide range of developmental abnormalities, including skeletal, neurological, growth and fertility defects, primarily arising from the severe deficiency in tissue macrophages [186][362][363]. Furthermore, CSF-1 has non-redundant functions in development, as administration of GM-CSF is not able to correct the growth and skeletal deficiencies in CSF-1-deficient mice [364].

While these developmental defects are severe, CSF-1-deficient mice exhibit surprisingly few immunological defects. This is in contrast to granulocyte-macrophage colony-stimulating factor (GM-CSF), where GM-CSF deficient mice are superficially healthy and fertile, have normal levels of the major types of mature hematopoietic cells and their precursors in blood, marrow, and spleen[365], but profound defects in pulmonary physiology and inability to clear to local infections[366].

CSF-1 is constitutively expressed by many tissues and exhibits a mostly homeostatic expression pattern, suggesting that it controls macrophage phenotype and function under basal conditions[361]. Accordingly, CSF-1 regulates the numbers of various tissue macrophage and monocyte populations without altering their activation status or cytokine secretion profile. Conversely, expression of GM-CSF is rapidly upregulated during inflammation, and can stimulate monocytes directly, causing a polarisation towards a pro-inflammatory phenotype characterised by high secretion of IL-1, IL-12, IFNy, a high expression of antigen presentation molecules [367], and increased phagocytic rates [368], ultimately resulting in the differentiation of macrophages with dendritic cell (antigen-presenting) properties [369]. Thus, while colony-stimulating factor 1 (CSF-1) regulates the survival, proliferation, and differentiation of macrophages at rest, GM-CSF is responsible for shaping the monocyte/macrophage function during an inflammatory response [370].

As inflammatory chemokine receptors might be involved in both CSF-1 and GM-CSF maturation pathways, iCCR$^{-/-}$ bone marrow cells were grown in media supplemented with either GM-CSF or CSF-1, and the development of macrophages and dendritic cells was tracked for several days until terminal differentiation in an attempt to uncover any phenotypic difference between WT and iCCR$^{-/-}$ monocyte differentiation.

6.3 Effect of iCCR deletion on CSF-1 dependent monocyte- derived Macrophages

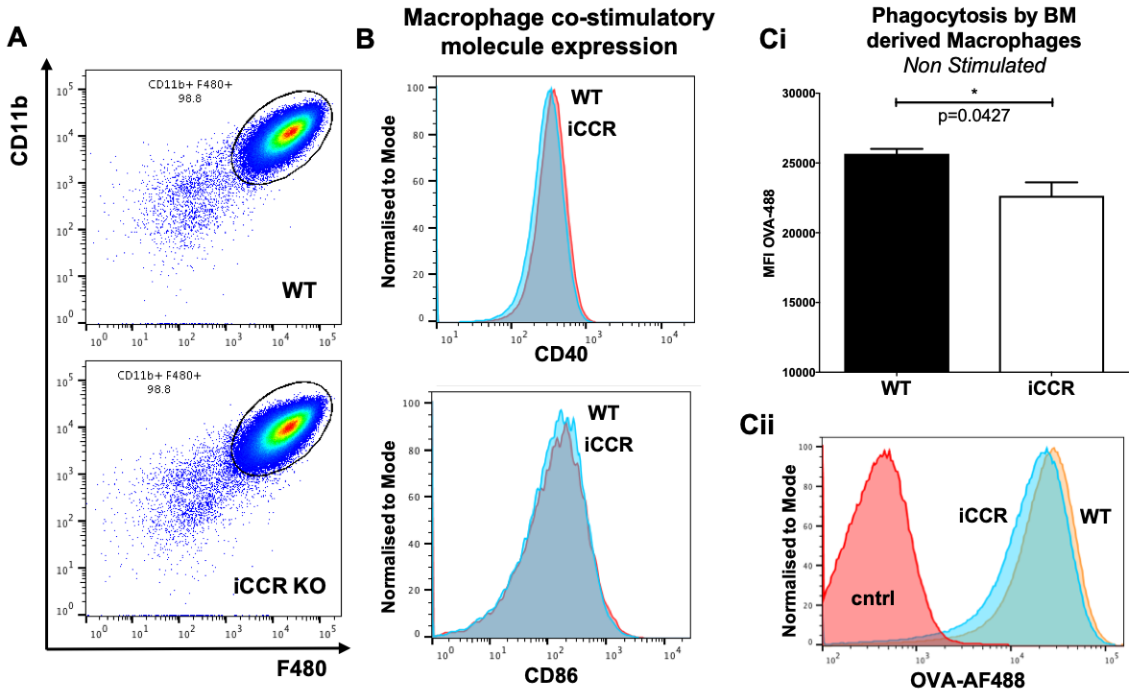

Figure 6-1 CSF-1 BM derived macrophages- A*- Representative FACS plots showing CD11b+F480+ cells in WT and iCCR cultures after 7 days of growth under CSF-1.* ***B****- Representative histograms showing expression of macrophage costimulatory molecules CD40 and CD86 (WT: blue, iCCR: red).* ***Ci****- Phagocytic Rates of WT/iCCR$^{-/-}$ macrophages, measured as incorporation of fluorescent ovalbumin. n=4, Student T-test was performed to determine statistical significance, with a p value of 0.05 determined as significant.* ***Cii****- Representative histograms showing a slight decrease in fluorescent OVA incorporation by iCCR$^{-/-}$ macrophages (blue) against WT macrophages (orange). 4'C control shown in red.*

After 1 week of culture with media containing CSF-1, both WT and iCCR$^{-/-}$ bone marrow cultures produced a uniform population (~99%) of CD11b+ F480+ cells (Figure 6-1A). Macrophages from both cultures express identical levels of the macrophage costimulatory molecules CD40 and CD86 under resting conditions (Figure 6-1B). The only difference observed was a minor decrease in phagocytosis in iCCR$^{-/-}$ macrophages after incubation with fluorescent ovalbumin (Figure 6-1Ci, 6-1Cii).

As no major differences were observed under resting conditions, macrophages in culture were stimulated overnight with LPS, and their co-stimulatory molecules, phagocytic rates and cytokine secretion profiles were analysed.

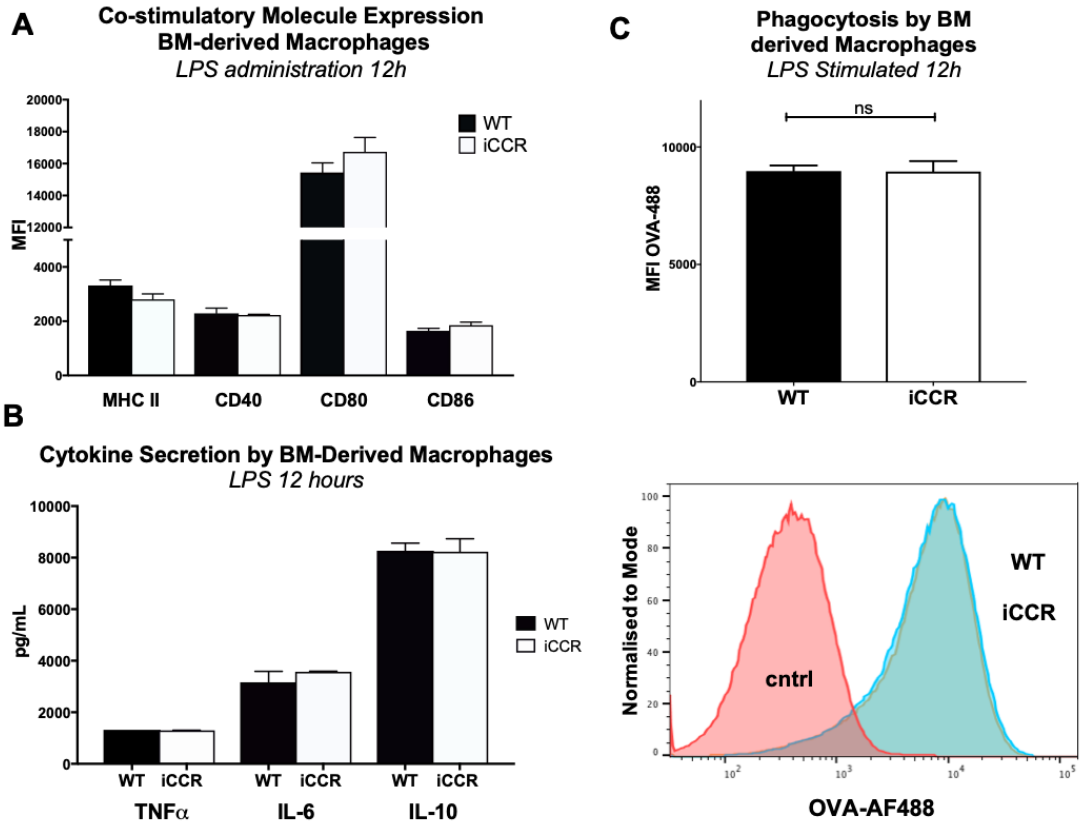

Figure 6-2- CSF-1 BM derived macrophages (LPS treated)- A- *Graph summarising costimulatory molecule expression (MHCII, CD40, CD80, CD86) in WT (black) and iCCR $^{-/-}$ (white) macrophages after stimulation with LPS overnight (n=3).* ***B-*** *Cytokine secretion (TNFα, IL-6 and IL-10) measured via ELISA by WT and iCCR$^{-/-}$ macrophages in 96 well plates (100.000 cells/well) after overnight LPS stimulation (n=4).* ***C-*** *Phagocytic Rates of WT/iCCR$^{-/-}$ macrophages, measured as incorporation of fluorescent ovalbumin with accompanying representative histograms (n=4).*

As seen in Figure 6-2, the absence of iCCRs had no effect on macrophage function after overnight LPS treatment: costimulatory molecule expression (Figure 6-2A) and pro and anti-inflammatory cytokine secretion (TNFα, IL-6 and IL-10) (Figure 6-2B) were identical between iCCR$^{-/-}$ and WT macrophages. Surprisingly, the small defect in phagocytosis observed at rest disappears after overnight LPS administration (seen as overlapping histograms in Figure 6-2C).

The absence of a phagocytosis defect under inflammatory conditions in iCCR$^{-/-}$ macrophages could be explained by a change in culture conditions after LPS administration. Under resting conditions, engagement of inflammatory chemokine receptors might be required for optimal phagocytosis. Under inflammation, macrophages can probably override the lack of iCCRs by engaging other receptors, such as cytokine receptors or costimulatory molecules, that ultimately promote phagocytosis in the absence of iCCR signalling.

As no major differences were observed in CSF-1 macrophages, bone marrow from iCCR$^{-/-}$ and WT mice was collected and grown in media supplemented with GM-CSF to obtain GM-CSF-derived macrophages and monocyte derived dendritic cells (mo-DCs). The phenotype of these cells was then assessed with various techniques to determine if iCCR deletion had any effect on proliferation rates or function.

6.4 Effect of iCCR deletion on monocyte-derived DCs

After 7 days of proliferation in GM-CSF, two main populations can be found in culture: in broad terms, non-adherent cells can be thought of as mature and immature monocyte derived DCs at different stages of maturation [371] although their nature is heavily debated [372][373] , while the adherent ones are generally discarded as "non-dendritic cells" or macrophages, even though they are very similar to DCs in surface marker expression and DC-related function[374] . Initially, the non-adherent fraction was collected after a week in GM-CSF containing media, and the proliferation, phagocytic rates and cytokine secretion of the mo-DCs were assessed before and after LPS administration. As shown in figure 6-3, iCCR$^{-/-}$ mo-DCs proliferated more slowly in culture (Figure 6-3A) and were less capable of phagocytosing antigen as measured via uptake of fluorescent OVA-488, both before (Figure 6-3 Bi,Bii) and after administration of LPS (Figure 6-3 Ci,Cii).

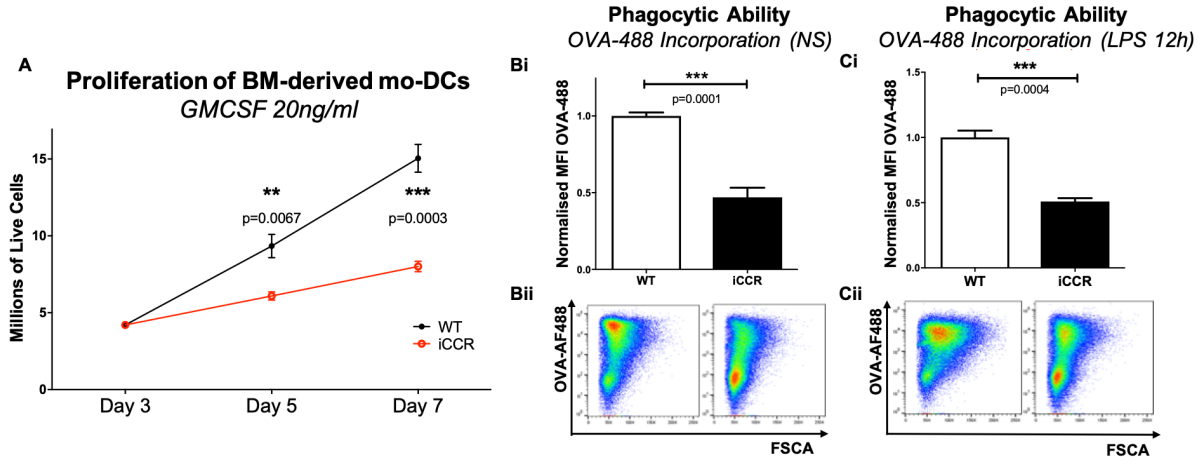

Figure 6-3 Proliferation Rates and Phagocytic Ability of iCCR$^{-/-}$ mo-DCs. ***A-*** *Cell number in GM-CSF cultures of WT/iCCR$^{-/-}$ bone marrow, monitored every 2 days after plating at 1million cells/ml in 10ml of IMDM media containing 20ng/ml of recombinant murine GM-CSF. Cell counts preformed automatically on the Luna Cell Counter.* ***Bi****- Graph showing phagocytic rates expressed as MFI for OVA-488 between WT/iCCR GM-CSF mo-DCs in non-stimulated cultures.* ***Bii****: Representative FACS plots showing decreased OVA-488 incorporation by WT/iCCR$^{-/-}$ DCs.* ***Ci Cii****- Phagocytic rates after overnight LPS administration. Student T-test was performed to determine statistical significance, with a p value of 0.05 determined as significant. Results elaborated from an average of at least 3 independent GM-CSF cultures for both WT and iCCR$^{-/-}$ samples.*

Cultured iCCR$^{-/-}$ mo-DCs stimulated with LPS for 12 hours secreted lower levels of IL-12p70 as measured by ELISA (a DC-specific cytokine capable of modulating T-cell development and function), but similar levels of anti-inflammatory IL-10 when compared to WT mo-DCs (Figures 6-4 A,B). Furthermore, as seen in Figure 6-4C, iCCR$^{-/-}$ mo-DCs show decreased expression of CCR7 (measured via binding of fluorescent CCL19[375]), the main chemokine receptor required for lymph node homing of activated DCs. The same iCCR$^{-/-}$ mo-DCs also seemed less capable of degrading antigen, as pHRODO fluorescence (pH sensitive fluorescent emission that increases in intensity with increasing acidity) is decreased both before and after LPS administration (Figure 6-4D).

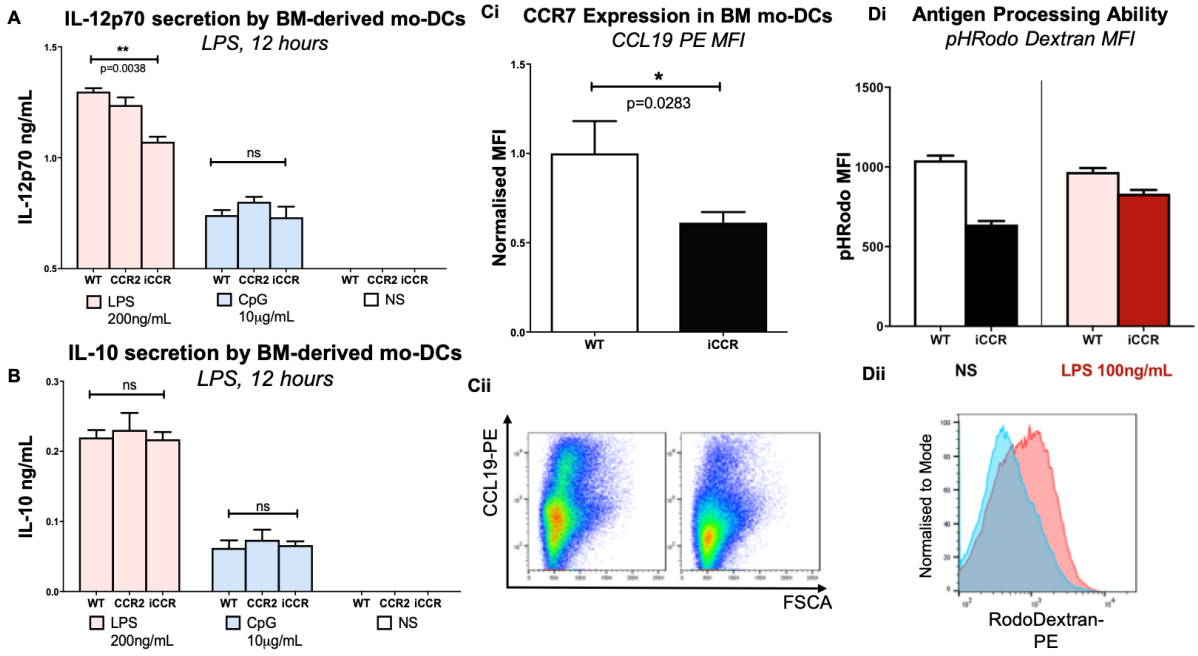

Figure 6-4 Cytokine Secretion and CCR7 Expression in iCCR$^{-/-}$ mo-DCs- A/B*- Graph showing cytokine secretion of bioactive IL-12p70 and IL-10 respectively, in both WT and iCCR cultures before and after administration of 200ng/ml of LPS for 12h, as measured via ELISA.* ***Ci****- Graphs summarizing expression of CCR7 in WT/iCCR GM-CSF-moDCs measured via flow cytometry by detecting fluorescently labelled CCL19 (after a 20 minute incubation at 4'C).* ***Cii****- Representative FACS plots showing decrease in CCL19 fluorescence in iCCR$^{-/-}$ cultures when compared to WT mo-DCs.* ***Di****- Graph showing decreased pH Rodo Dextran fluorescence after 20 minute incubation at 37'C (20μg/ml), indicating reduced ability to process antigen, in iCCR$^{-/-}$ mo-DCs.* ***Dii****- representative FACS histograms showing increased pH Rodo fluorescence in WT (red) and ICCR$^{-/-}$ (blue) mo-DCs. Student T-test was performed to determine statistical significance, with a p value of 0.05 determined as significant. Results elaborated from an average of at least 3 independent GM-CSF cultures for both WT and iCCR$^{-/-}$ samples.*

6.4.1 Effect of iCCR deletion on antigen presentation by mo-DCs

Dendritic cells are vital for the correct functioning of the immune system by linking the innate and adaptive branches of the immune response. Indeed, DCs are responsible for acquiring antigen at the site of infection, processing it and presenting on specialised molecules known as major histocompatibility complexes (MHCI and MHCII). The antigen-loaded dendritic cell then migrates to the closest draining lymph node using CCR7 by detecting gradients of CCL19 and CCL21 produced by lymphatic endothelial cells and lymph node stromal cells[16][376][15], and once in the lymph node, the DC is then capable of activating lymphocytes specific for the presented antigen, which then undergo clonal expansion[377][378] and antibody recombination[379][380] to produce cells and antibodies with high affinity against the invading pathogen.

Activating an antigen specific lymphocyte is a complex process, as aberrant activation can result in the activation of wrong lymphocytes or the generation of ineffective antibodies[381]–[383]. Currently, the 'three-signal hypothesis' is the accepted immunological model of how antigen specific lymphocytes can be activated by DCs [384]–[386]. The first signal involves the interaction between the DC's antigen loaded MHCII or MHCI with the lymphocyte's own antigen specific receptors (such as T-cell receptors, TCRs).

The second signal, also known as a co-stimulatory signal, is mediated by molecules collectively known as co-stimulatory molecules. Not only can engaged costimulatory molecules signal intracellularly, directly amplifying any activating signals[387], but by binding to each other (for example B7 on DCs binding to CD28 on T-cells) they can physically stabilise the immune synapse[388][389] and keep the MHC-peptide-TCR complex between the APC and T-cell in place for longer, resulting in prolonged signalling and complete cell activation. The third signal comes in the form of soluble factors, by which inflammatory cytokines such as IL-12 and IL-1 secreted by the DCs directly stimulate neighbouring T-cells, further amplifying activator signals and promoting T-cell proliferation[386][390].

The data shown so far suggest that iCCR$^{-/-}$ monocyte-derived DCs phagocytose antigen less efficiently (fluorescent ovalbumin uptake) and are unable to process it appropriately (pH Rodo dextran). Furthermore, LPS treated iCCR$^{-/-}$ mo-DCs show an altered cytokine secretion profile characterised by decreased production of IL-12p70. It was therefore hypothesised that iCCR$^{-/-}$ DCs would not be able to activate cognate T-cells effectively, as both signal one and signal three of the 'three-signal hypothesis' seem to be altered. To test the ability of iCCR$^{-/-}$ DCs to stimulate cognate lymphocyte, an antigen presentation assay was performed using ovalbumin as a model antigen.

After incubating the DCs with latex beads coated in ovalbumin (to mimic particulate antigen), the cells were then activated with LPS (which increases expression of MHC and costimulatory molecules on the DCs), fixed, and co-incubated with ovalbumin specific T-cells for 12 hours (OT-1 or OT-2). If all three signals are delivered correctly to the antigen specific T-cells, the T-cells should respond by rapidly increasing in number. To support this clonal expansion, correctly activated T-cells also secrete large amounts of IL-2, which can be detected by ELISA. Detecting IL-2 levels in media is thus an effective and established method to determine the extent of DC-dependent T-cell activation[247][391].

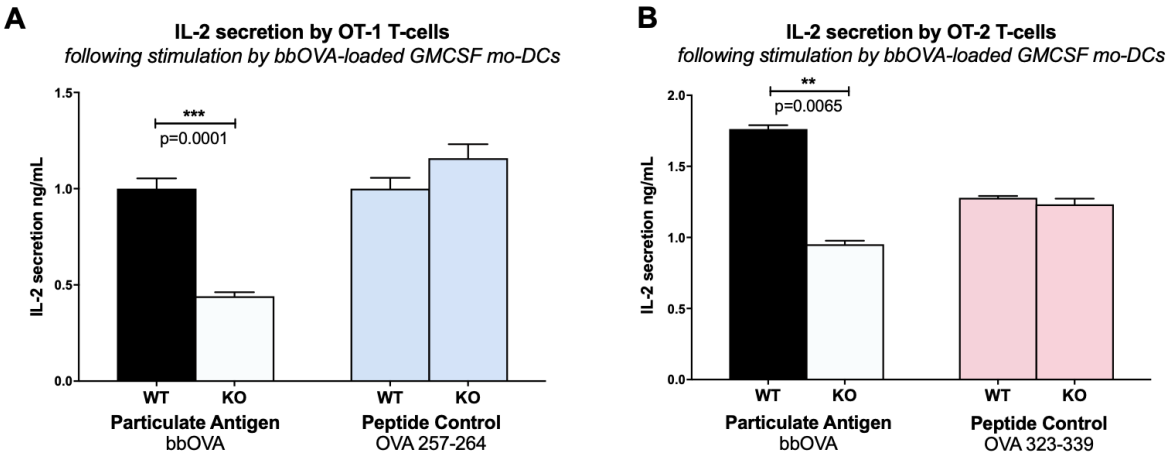

Figure 6-5- Analysis of antigen presentation by WT/iCCR mo-DCs. A*- IL-2 secretion by cognate cytotoxic T-cells after co-incubation with OVA-loaded DCs, indicating reduced antigen cross-presentation by iCCR$^{-/-}$ mo-DCs.* ***B****- IL-2 secretion by cognate T-helper cells after incubation with OVA-loaded DCs, indicating reduced antigen presentation by iCCR$^{-/-}$ mo-DCs. Student T-test was performed to determine statistical significance, with a p value of 0.05 determined as significant. Results elaborated from an average of at least 3 independent GM-CSF cultures for both WT and iCCR$^{-/-}$ samples.*

As seen in Figure 6-5, iCCR$^{-/-}$ DC-T-cell cultures produced half the amount of IL-2 compared to WT DC-T-cell cultures after 12 hours, suggesting that both antigen presentation and antigen cross-presentation are negatively affected in iCCR$^{-/-}$ DCs and that T-cells activated by iCCR$^{-/-}$ mo-DCs are less proliferative and probably less capable of performing their effector functions (direct cytotoxic killing by OT-1 CD8 T-cells or supporting antibody production by OT-2 CD4 helper T-cells).

Collectively, these results show that iCCR$^{-/-}$ mo-DCs proliferate more slowly, are less capable of phagocytosing and degrading antigen, are potentially less capable of migrating to the lymph node after antigenic challenge, show altered cytokine secretion profile characterised by decreased production of bioactive IL-12 and are less able to activate cognate T-cells. However, it is still unclear how the lack of iCCRs would mediate all these effects, so further analysis by flow cytometry of these 'mo-DCs' was performed to understand the nature of WT and iCCR mo-DCs.

6.4.2 Further characterisation of iCCR BM mo-DCs

Flow cytometric analysis of the suspension fraction in WT GM-CSF cultures revealed that this population is in fact composed of 4 subpopulations, each characterised by different expression of CD11c and MHC II (Figure 6-6A). As shown in figure 6-6C, iCCR mo-DC cultures appear to have an accumulation of CD11c-MHCII- cells, compared to WT cultures. At this stage however, it was still unclear what these 4 populations represent, and how the accumulation of CD11c- MHCII- in iCCR$^{-/-}$ cultures is causing the phenotypes observed so far. To determine the nature of each sub-population in the non-adherent fraction of GM-CSF cultures, WT 'mo-DCs' were cultured and analysed via flow cytometry to detect differences in co-stimulatory molecules and maturation markers. As shown in Figure 6-6A, WT mo-DC cultures can be divided into CD11c+ MHCII++, CD11c+ MHCII +, CD11c+ MHCII int and CD11c- MHCII- populations. Each population had a distinct phagocytic ability, as shown by the heatmap representing levels of incorporated OVA-488 in each cell (Figure 6-6B). CD11c+ MHCIIint and CD11c+ MHCII+ populations had the highest phagocytic rates, closely followed by CD11c+ MHCII++ cells. CD11c- MHCII- cells are instead non-phagocytic (Figure 6-6 D, E).

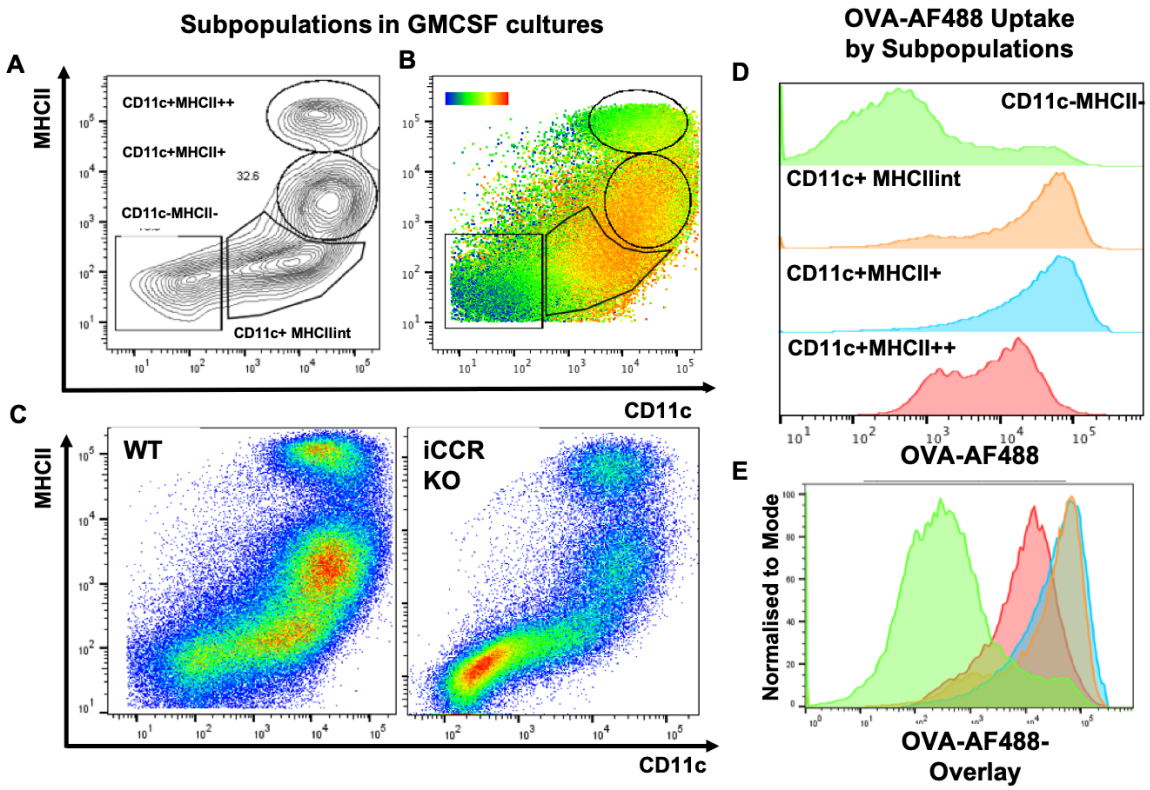

Figure 6-6 Subpopulations in WT and iCCR$^{-/-}$ mo-DC cultures. A- *Representative FACS contour plot showing the division of 4 subpopulations identified in WT GM-CSF cultures according to CD11c and MHCII levels (left).* ***B-*** *FACS heatmap showing OVA-488 fluorescence in different subpopulations, indicating varying levels of phagocytic ability. Orange: high fluorescence indicating high phagocytic ability Green/Blue: non-phagocytic.* ***C-*** *Representative FACS plots showing the distribution of cells in WT and iCCR$^{-/-}$ GM-CSF cultures along a CD11c and MHCII axis.* ***D, E-*** *Histograms and overlaid histograms showing the MFIs for OVA-488 for each CD11c and MHCII populations.*

6.4.3 The 4 sub-populations in mo-DC cultures have unique marker expression

These 4 sub-populations also expressed different levels of the lymph-node homing chemokine receptor CCR7. CD11c+ MHCII++ cells were CCR7+ve (>97%) and had the highest MFI as seen from heatmap representing levels of bound fluorescent CCL19 (Figure 6-7A, 6-7B). More than half of CD11c+ MHCII+ cells also expressed CCR7, while the MHCII int and MHCII- populations showed minimal CCR7 expression, both as percentage positive (Figure 6-7A) and as mean fluorescent intensity (Figure 6-7C).

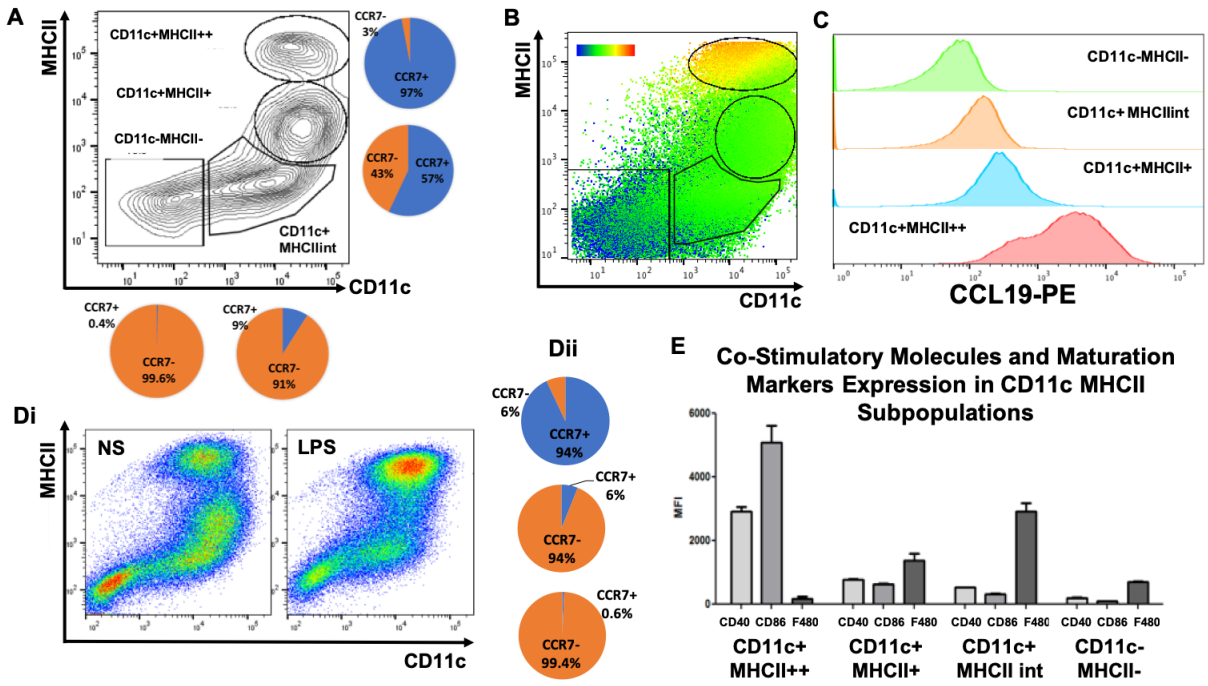

Figure 6-7 Phenotype of Subpopulations in WT and iCCR$^{-/-}$ mo-DC cultures. ***A****-Representative WT FACS plot of the four CD11c MHCII populations (shown previously) with associated pie charts showing % positive cells for bound fluorescent CCL19, indicating CCR7 expression in each population.* ***B****- FACS Heatmap showing Fluorescent intensity for CCL19 in each sub-population.* ***C****- FACS Histograms showing the MFIs for CCL19 for each CD11c and MHCII population.* ***Di****: Representative FACS plot showing the effect of overnight LPS stimulation on the composition of the 4 main subpopulations in WT GM-CSF cultures.* ***Dii****: Pie charts showing CCR7 expression of each of the 3 main sub-populations (CD11c+MHCII++, CD11c+MHCIIint, CD11c-MHCII-) in WT GM-CSF cultures after overnight LPS stimulation (100ng/ml).* ***E****: Levels of expression of main DC costimulatory molecules and maturation markers in each of the four CD11c and MHCII sub-populations identified in WT unstimulated cultures, measured as MFI via flow cytometry.*

Co-stimulatory molecule expression also varied greatly between sub-populations: CD11c+ MHCII++ cells were the highest expressers of costimulatory molecules CD40 and CD86, and the lowest expressers of the macrophage marker F480. On the other hand, F480 was the most highly expressed marker in CD11c+ MHCII int population, while on CD11c- MHCII- cells, costimulatory molecules and maturation markers were barely expressed (Figure 6-7E).

In WT cultures and after LPS administration, the CD11c+ MHCII+ population 'disappeared' as MHCII expression was upregulated, resulting in a larger proportion of CD11c+ MHCII++ cells in culture (Figure 6-7Di). In addition, all the LPS treated CD11c+ MHCII++ cells expressed CCR7 (>94%), while CCR7 expression never increased in CD11c+MHCII int and CD11c- MHCII- populations, even after LPS administration (Figure 6-7Dii).

With this information, it was now possible to classify these subpopulations:

CD11c+ MHCII++: mature DCs. Almost all are CCR7+, possess intermediate phagocytic ability and are the highest expressers of costimulatory molecules.

CD11c+ MHCII+: immature DCs. The majority are CCR7+, have very high phagocytic ability, lower expression of costimulatory molecules than DCs, but higher than CD11c+MHCII int. Upon LPS stimulation, this population disappears and shifts toward a 'mature' CD11c+ MHCII++ phenotype.

CD11c+ MHCII int: macrophages. CCR7-, very high phagocytic ability, low expression of costimulatory molecules and high expression of F480.

CD11c-MHCII-: monocytes. No CCR7 expression, very low phagocytic ability, costimulatory molecule and F480 expression.

6.4.4 Most cells in iCCR$^{-/-}$ cultures are undifferentiated monocytes

As seen in Figure 6-8 (Ai, Aii) the majority of cells (40%) after 7 days of culture of iCCR$^{-/-}$ bone marrow in GM-CSF were undifferentiated CD11c-MHCII- monocytes, double the number found in WT cultures (20%, Figure 6-8 (Bi,Bii). After LPS administration, the immature DCs (CD11c+MHCII+) in both WT and iCCR$^{-/-}$ cultures matured and became CD11c+MHCII++ (Figures 6-8 Ci,Di). In the iCCR$^{-/-}$ cultures however, a higher proportion of undifferentiated monocytes could still be observed (figure 6-Cii,Dii).

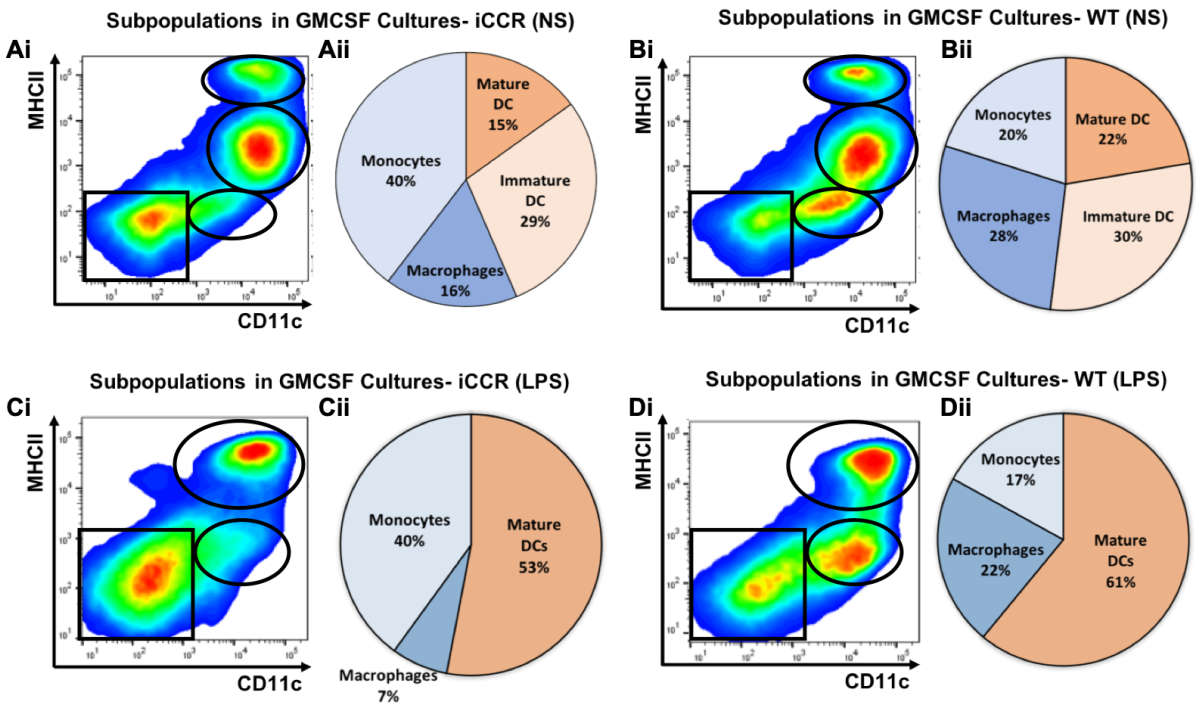

Figure 6-8 Subpopulations in WT and iCCR$^{-/-}$ mo-DC cultures before and after LPS. Ai- *Representative FACS plot of the 4 main subpopulations identified in iCCR$^{-/-}$ GM-CSF cultures (Mature DCs: CD11c+MHCII++, Immature DCs: CD11c+MHCII+, Macrophages: CD11c+MHCIIint, Monocytes (CD11c-MHCII-).* ***Aii:*** *Pie chart summarising the proportion of each sub-population in iCCR$^{-/-}$ cultures.* ***Bi-*** *Representative FACS plot of the 4 main subpopulations identified in WT GM-CSF cultures (Mature DCs: CD11c+MHCII++, Immature DCs: CD11c+MHCII+, Macrophages: CD11c+MHCIIint, Monocytes (CD11c-MHCII-).* ***Bii****- Pie chart summarising the proportion of each sub-population in WT cultures.* ***Ci-*** *Representative FACS plot representing the main subpopulations in iCCR$^{-/-}$ cultures after LPS administration overnight (Mature DCs: CD11c+MHCII++, Macrophages: CD11c+MHCIIint, Monocytes (CD11c-MHCII-).* ***Cii****- Pie chart summarising the proportion of each sub-population in LPS treated iCCR$^{-/-}$ cultures.* ***Di-*** *Representative FACS plot representing the main subpopulations in WT cultures after LPS administration overnight (Mature DCs: CD11c+MHCII++, Macrophages: CD11c+MHCIIint, Monocytes (CD11c-MHCII-).* ***Dii****- Pie chart summarising the proportion of each sub-population in LPS treated WT cultures. Results elaborated from an average of at least 3 independent GM-CSF cultures for both WT and iCCR$^{-/-}$ samples.*

Thus, the absence of CCR1 CCR2 CCR3 and CCR5 seems to affect differentiation and maturation of monocytes into dendritic cells, resulting in an accumulation in culture of undifferentiated CD11c-MHCII- cells. Some monocytes still managed to differentiate appropriately, albeit in lower numbers, and the ones that did look very similar to their WT counterparts. As seen in Figure 6-9, there was no difference in levels of costimulatory molecules or maturation markers between both WT and iCCR mature (Figure 6-9A) and immature DCs (Figure 6-9C), suggesting that if iCCR$^{-/-}$ monocytes do manage to differentiate, their phenotype will be very close to a WT mo-DCs. However, while costimulatory and maturation marker expression was identical, a slight decrease in phagocytic activity was still observed in both immature and mature iCCR$^{-/-}$ DCs (Figures 6-9B, 6-9D).

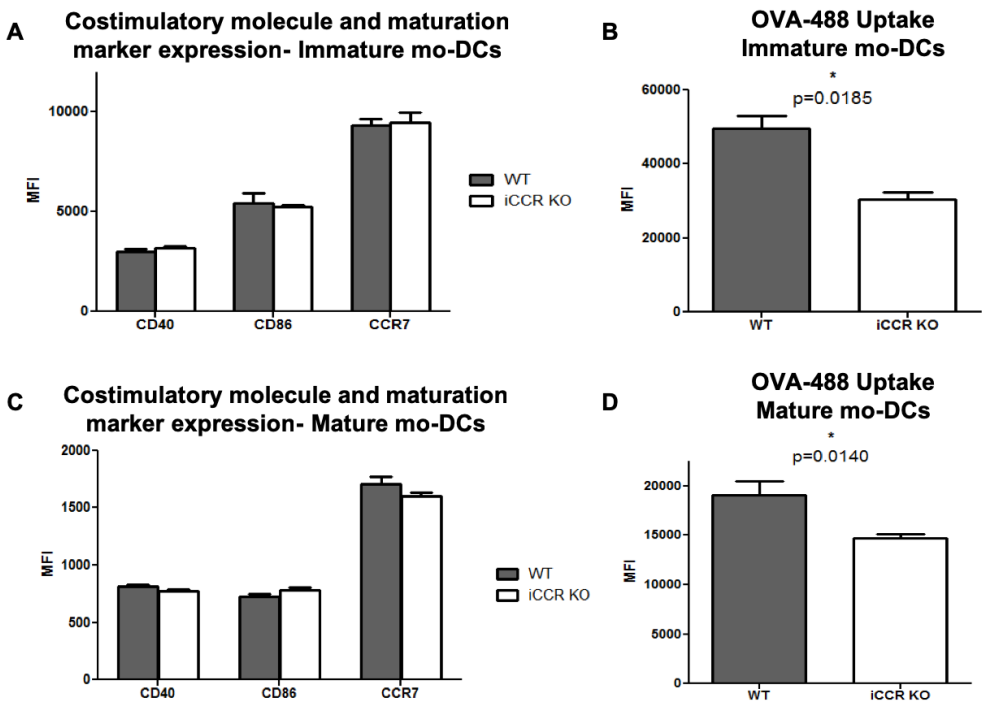

Figure 6-9 Costimulatory molecule expression on subpopulations in WT and iCCR$^{-/-}$ mo-DC cultures. A- *Graphs showing expression of major DC costimulatory molecules and maturation markers in WT and iCCR immature mo-DCs, measured as MFI via flow cytometry.* ***B-*** *Graph summarising the phagocytic rates of immature mo-DCs, measured as MFI of OVA-488 via flow cytometry.* ***C-*** *Graphs showing expression of major DC costimulatory molecules and maturation markers in WT and iCCR mature mo-DCs, measured as MFI via flow cytometry.* ***D-*** *Graph showing the phagocytic rates of mature mo-DCs, measured as MFI of OVA-488 via flow cytometry. Results elaborated from an average of at least 3 independent GM-CSF cultures for both WT and iCCR$^{-/-}$ samples.*

6.4.5 Accumulation of monocytes in culture explains the phenotypes observed

An increase of undifferentiated monocytes in iCCR$^{-/-}$ cultures explains most of the phenotypes observed so far:

Defect in Phagocytosis and Antigen Presentation: Undifferentiated monocytes are not able to phagocytose antigen and degrade it properly, resulting in overall decreased phagocytic rates, pH Rodo Dextran fluorescence and IL-2 secretion by T-cells after incubation with iCCR$^{-/-}$ cells.

Defect in CCR7 expression: reduced DC number and accumulation of monocytes explains the observed decrease in CCR7 MFI

Defect in IL-12p70 secretion but not IL-10: monocytes can secrete IL-10, but IL-12p70 can only be produced by antigen presenting cells by assembling the p35 and p40 subunits of IL-12 to form bioactive IL-12p70. Increased monocyte numbers result in lower IL-12p70 secretion, but unchanged IL-10.

6.5 Role of iCCR deletion in 'steady state' DCs

While GM-CSF is routinely used to generate *in vitro* mo-DCs from bone marrow monocytes [392], cultures can be very heterogenous and result in the formation of other cell types, namely macrophages and their precursors [393]. Several reports indicate that murine bone marrow-derived DCs (BM-DCs), human monocyte-derived DCs (MoDCs), and Langerhans cells (LCs) show considerable transcriptional overlap with macrophages [394]. Furthermore, it has also been shown that many of the proposed unique markers and functions of mo-DCs are, in fact, shared between different cell types [395].

Nonetheless, their similarity in phenotype, morphology, function and developmental origin have lead some groups to identify GM-CSF mo-DCs as the *in vitro* counterpart of Tip-DCs [373][396], dendritic cells characterised by the secretion of high levels of TNFα and iNOS, which emerge from inflammatory monocytes *in vivo* during inflammation[397].

However, GM-CSF is not the only way to generate bone marrow derived DCs *in vitro*. Both Flt3 ligand (Flt3L) and GM-CSF can stimulate the expansion of dendritic cells [398][399][400]. FLT3L was observed to increase DC numbers both *in vivo* and *in vitro* [401], and FLT3L receptor expression can be found on early DC precursors in murine bone marrow [402]. Unlike GM-CSF derived DCs, FLT3L DCs, take longer to mature, migrate more effectively and have a cytokine secretion profile which is more similar to *in vivo* 'steady-state' DCs [373].

To determine the effect of iCCR deletion on 'steady-state' DCs, iCCR$^{-/-}$ bone marrow was grown in FLT3L containing media for 10 days. In WT cultures, 3 main subpopulations can be identified on the basis of CD11c and MHCII: CD11c+ MHCII-, CD11c+ MHCII+, and CD11c+ MHCII++ (Figure 6-10A). All 3 subsets express CCR7, with CD11c+ MHCII++ cells expressing the highest levels, both as percentage (>92%, Figure 6-10B) and MFI (Figure 6-10C). The CD11c+ MHCII++ subset was identified as mature FLT3 DCs, while the CD11c+ MHCII+ population represented immature DCs, and the CD11c+ MHCII- cells represent undifferentiated DC precursors[399].

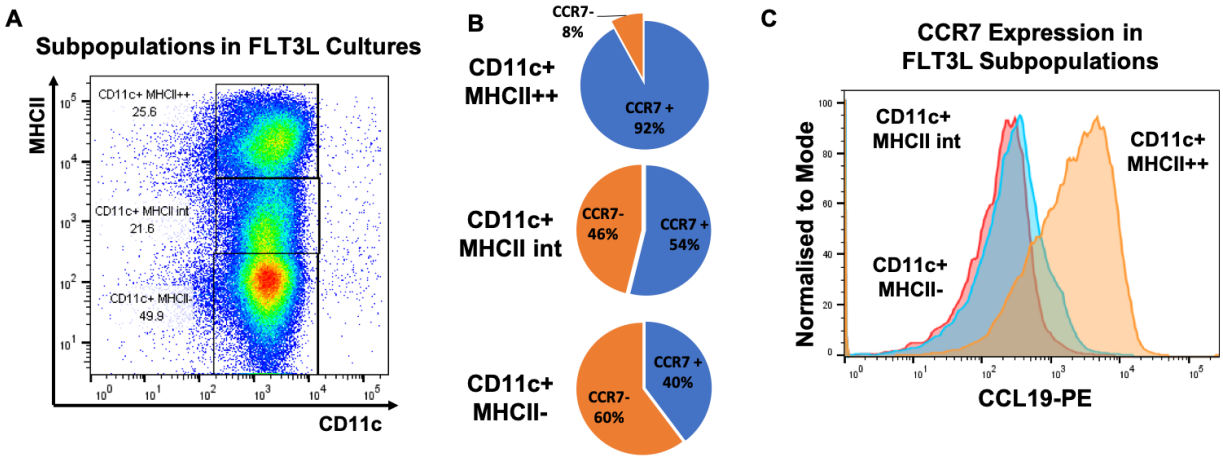

Figure 6-10 Subpopulations in WT FLT3 DC cultures- ***A****-Representative FACS plots showing 3 different subpopulations in WT FLT3 cultures identified by CD11c and MHCII expression.* ***B-*** *Pie charts showing expression of CCR7 on each subpopulation, measured as % positive for fluorescent CCL19.* ***C-*** *Overlaid FACS histograms showing MFI for fluorescent CCL19 in each CD11c MHCII sub-population in WT FLT3 cultures. Results elaborated from an average of at least 3 independent GM-CSF cultures for both WT and iCCR$^{-/-}$ sample*

Unlike GM-CSF derived mo-DC, FLT3L DCs in WT and iCCR$^{-/-}$ cultures were identical in terms of subpopulation percentages (Figure 6-11 A,B), both before and after LPS treatment (Figure 6-11 Aii,Bii).

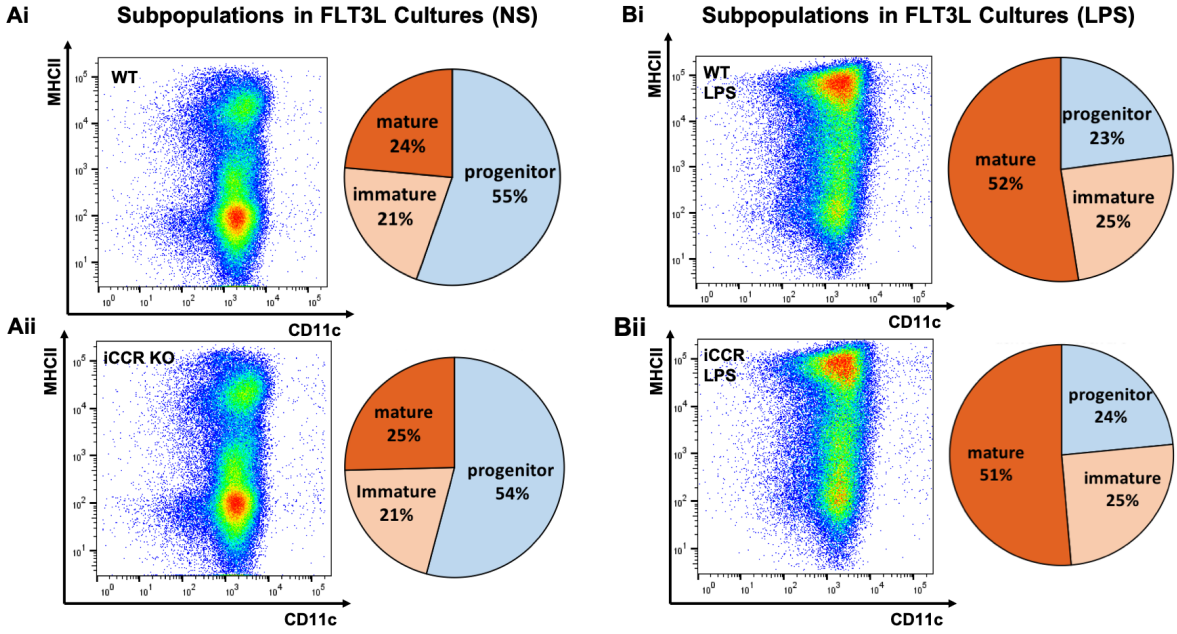

Figure 6-11 Subpopulations in FLT3L DC cultures before and after LPS– A*-Representative FACS plot and pie chart summarising the proportion of each CD11c MHCII sub-populations in WT and iCCR$^{-/-}$ FLT3L cultures.* ***B-*** *Representative FACS plot and pie chart summarising the proportion of each CD11c MHCII sub-populations in WT and iCCR$^{-/-}$ FLT3L cultures after exposure to LPS for 12h. Results elaborated from an average of at least 3 independent GM-CSF cultures for both WT and iCCR$^{-/-}$ samples.*

WT and iCCR$^{-/-}$ FLT3L DCs also express the same levels of costimulatory molecules CD40 and CD86 before and after LPS (Figure 6-12A), have similar phagocytic rates (Figure 6-12 B), and have identical secretion of both IL-10 and IL-12p70 (Figure 6-12C).

Thus, the previous findings of defective phagocytosis and maturation in iCCR$^{-/-}$ mo-DCs seem to be restricted to the 'inflammatory DC' subtype, with 'steady-state' DCs largely unaffected by iCCR deletion. However, it is still unclear how the absence of CCR1, CCR2, CCR3 and CCR5 cause the observed phenotype in GM-CSF-derived mo-DCs.

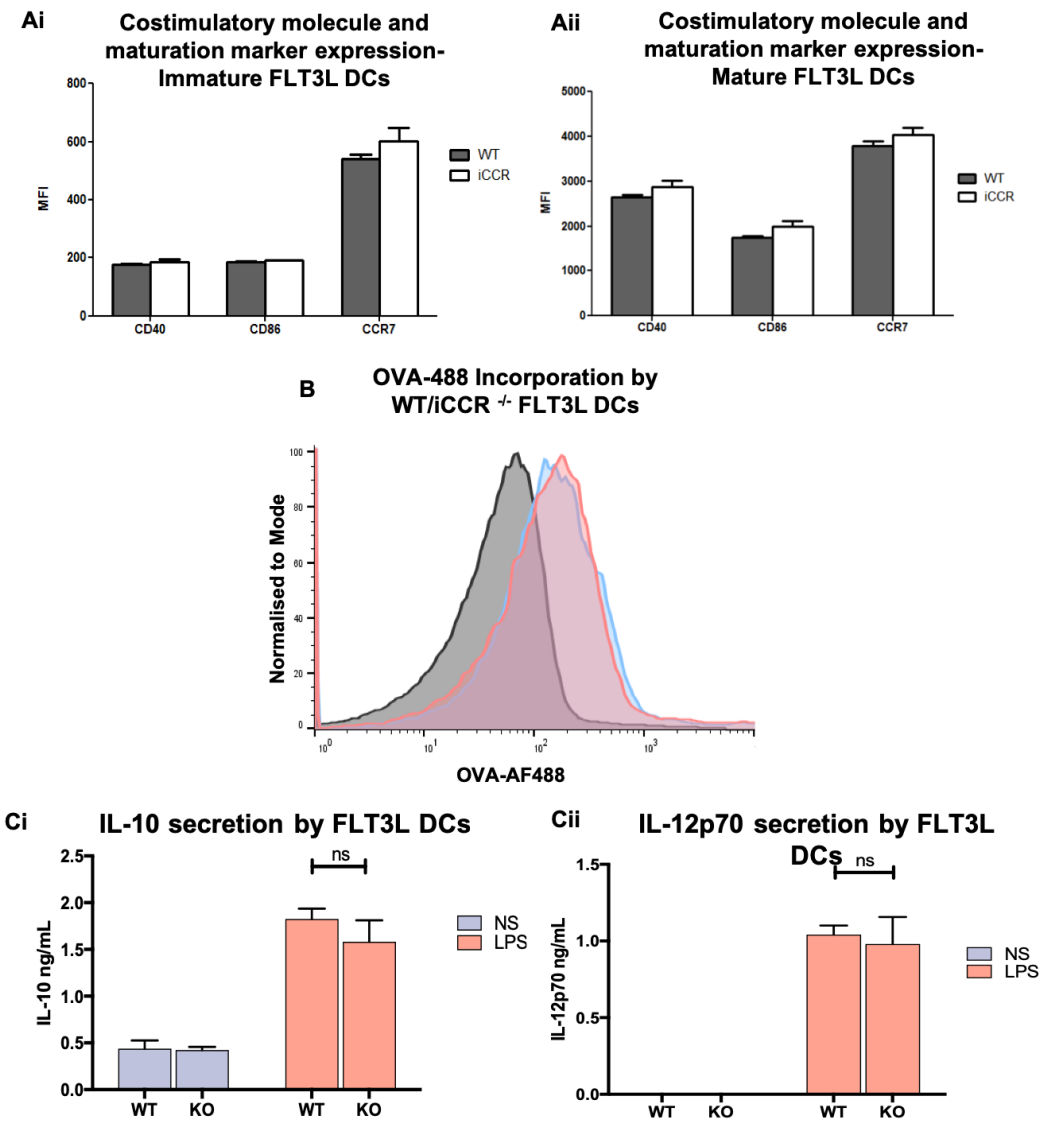

Figure 6-12- Costimulatory molecule expression and cytokine secretion by FLT3 DCs. A- *Graphs showing expression of major DC costimulatory molecules and maturation markers in WT and iCCR immature and mature FLT3 cultures, measured as MFI via flow cytometry.* ***B-*** *Overlaid histograms showing the phagocytic rates of mature FLT3 DCs, measured as MFI of OVA-488 via flow cytometry (Red: WT, Blue: iCCR$^{-/-}$).* ***C-*** *Graphs showing cytokine secretion of IL-10 and bioactive IL-12p70 respectively, in both WT and iCCR cultures before and after administration of 200ng/ml of LPS for 12h, as measured via ELISA. Results elaborated from an average of at least 3 independent GM-CSF cultures for both WT and iCCR$^{-/-}$ samples.*

6.6 Engagement of iCCRs in GM-CSF cultures

If iCCR deletion mediates the effect observed in GM-CSF mo-DCs, this means that CCR1, CCR2, CCR3 and CCR5 must be active in maturing WT cultures. While most cells can secrete chemokines under inflamed conditions, it is unclear which chemokines, if any, are present in resting GM-CSF cultures.

Surprisingly, ELISA for CCL2 (ligand for CCR2) and CCL5 (ligand binding to CCR1, CCR3 and CCR5) detected high levels of these chemokines in cultures, both at rest and after LPS administration. While there was no difference in CCL5 levels between WT and iCCR$^{-/-}$ supernatants (Figure 6-13A), CCL2 levels where twice as high at rest and remained elevated after LPS administration in iCCR$^{-/-}$ cultures (Figure 6-13 B,C).

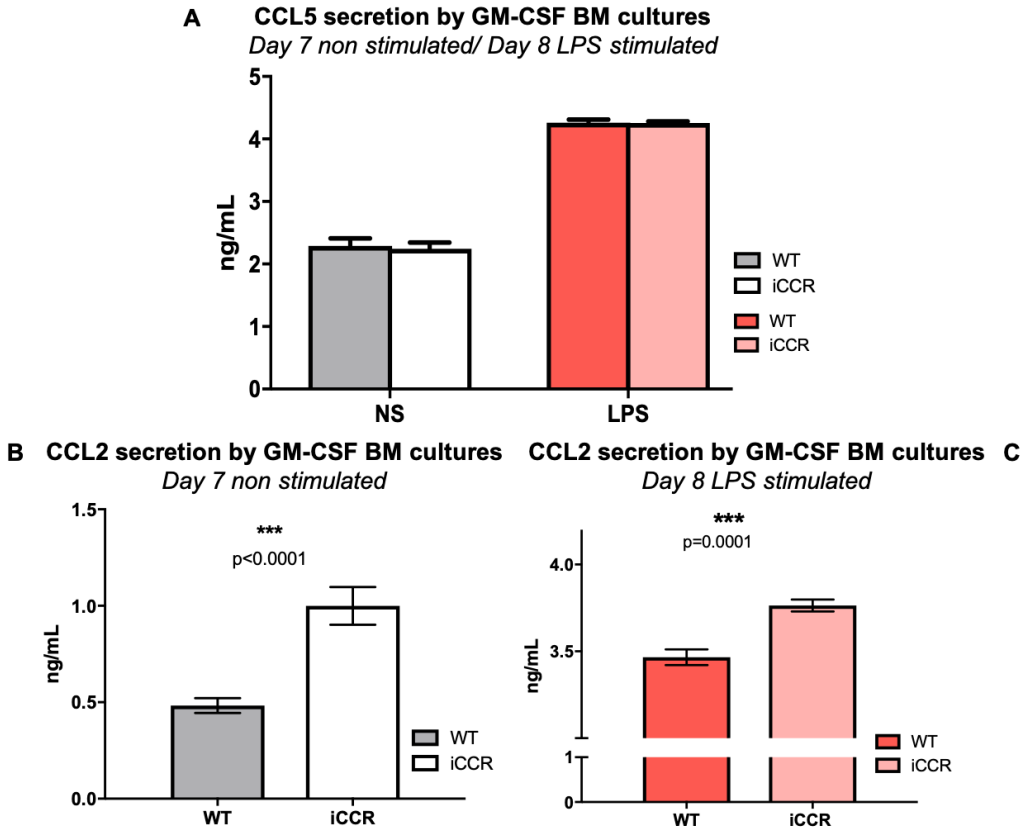

Figure 6-13 Chemokine secretion in GM-CSF cultures- ***A***- *Graphs showing cytokine secretion of CCL5 in both WT and iCCR cultures before and after administration of 100ng/ml of LPS for 12 hours, as measured via ELISA.* ***B,C***- *Graph showing cytokine secretion of CCL2 in both WT and iCCR cultures before and after administration of 100ng/ml of LPS for 12 hours, as measured via ELISA. Results elaborated from an average of at least 3 independent GM-CSF cultures for both WT and iCCR$^{-/-}$ samples. Student T-test was performed to determine statistical significance, with a p value of 0.05 determined as significant.*

Once engaged, CCR2 is rapidly internalised[403] and either recycled to the membrane or downregulated through lysosomal degradation[404]. The absence of CCR2 explains the elevated levels of CCL2 in culture, as the chemokine cannot bind to any receptor and is not internalised, thus accumulating in the supernatant.

Binding of CCL2 to its cognate receptor should be initiating a signalling cascade, and this signalling is completely absent in the iCCR$^{-/-}$ cultures. Thus, the absence of inflammatory CC-chemokine signalling in iCCR$^{-/-}$ cultures might explain the undifferentiated phenotype observed in iCCR mo-DCs.

6.6.1 Absence of iCCRs prevents formation of 'Proliferation Clusters' in GM-CSF cultures.

Together with decreased proliferation, GM-CSF iCCR$^{-/-}$ cultures also showed a complete absence of what were identified as 'proliferation clusters' (Figure 6-14), small clumps of differentiating cells that grow and increase in size as maturation progresses. To assess whether the absence of these proliferation clusters (which appear spontaneously in cultures by days 3 and 4) could be attributed to the absence of a single iCCR, bone marrow cells of each single iCCR knock-out strain were cultured in GM-CSF-containing media and the development of proliferation clusters was recorded between days 3 to 7.

Proliferation clusters were seen in all WT and single$^{-/-}$ GM-CSF cultures (figures 6-14 and 6-16A), suggesting that complete inhibition of cluster formation requires simultaneous inhibition of multiple chemokine receptors.

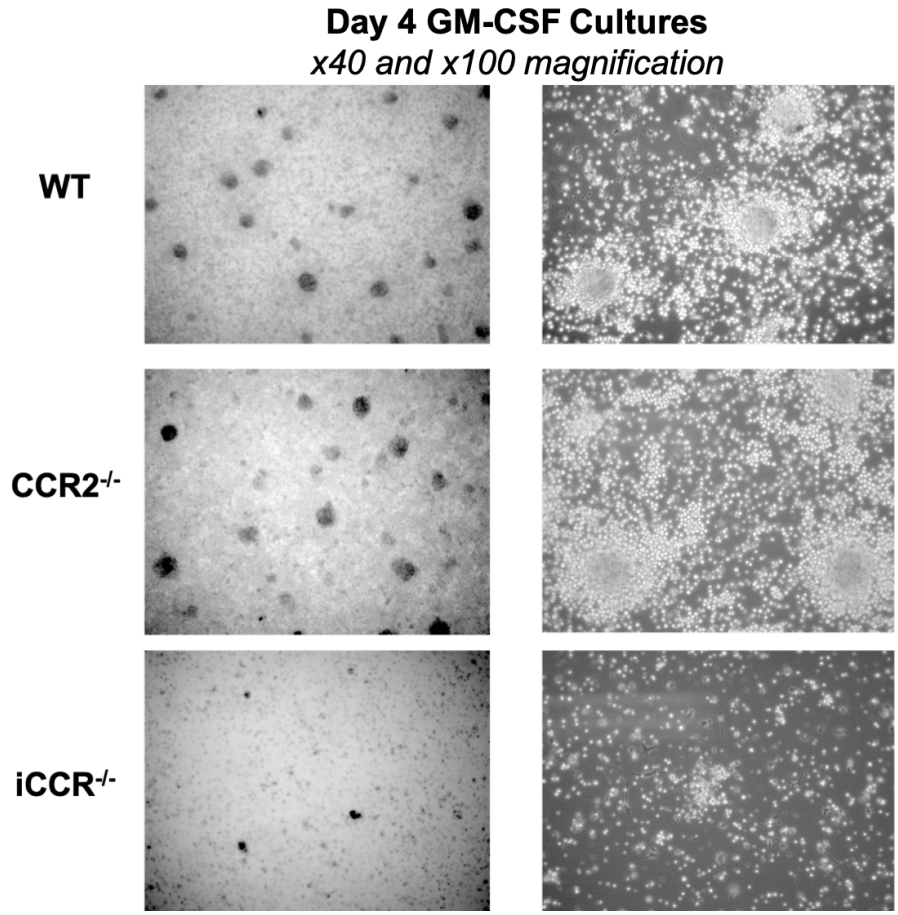

Figure 6-14- Proliferation Clusters in GM-CSF cultures- *Representative bright field images at x4 and x10 magnifications, showing proliferation clusters in GM-CSF WT, CCR2$^{-/-}$ and iCCR$^{-/-}$ mo-DC cultures.*

6.6.2 Inhibiting iCCRs in WT cells replicates the 'cluster-less' phenotype

To ensure that this 'cluster-less' phenotype was caused by the absence of iCCRs, WT cultures were allowed to grow in the presence of different combinations of chemokine receptor antagonists (CCR2 → BMS CCR2 22, CCR1/3 → UCB 35625, CCR5→ Maraviroc). Initially, WT cells were exposed for 48 hours to 3 different antagonists, both alone and in different combinations. Following this, cell numbers and cell viability were assessed to ensure that the antagonists and their combinations were not toxic to developing dendritic cells. 2uM was established as a safe dosage of chemokine receptor antagonist, and no decrease in cell number and viability was observed even with 3 antagonists delivered simultaneously (Figure 6-15 A,B).

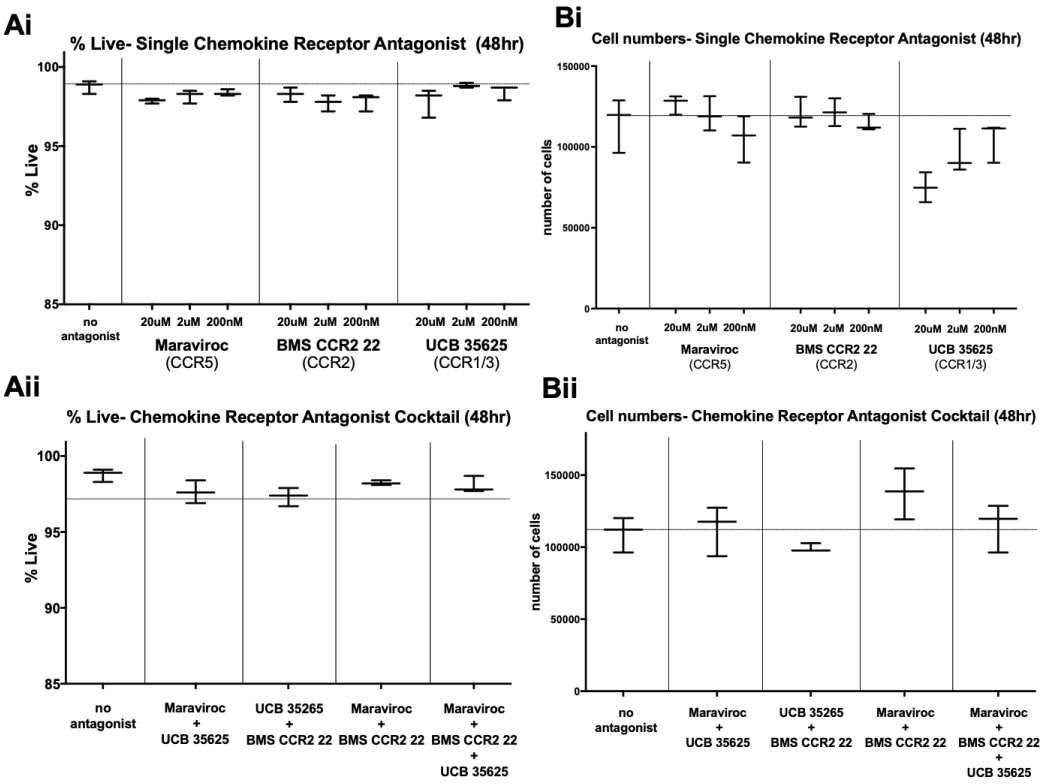

Figure 6-15 Determining non-toxic iCCR antagonist concentrations- A*- % Live cells in WT GM-CSF DC cultures after 48 hour exposure to chemokine antagonists, at varying concentrations, singly or in specific combinations, as measured via Luna Cell Counter.* ***B****- Cell numbers in WT GM-CSF DC cultures after 48 hour exposure to chemokine antagonists, at varying concentrations, singly or in specific combinations, as measured via Luna Cell Counter.*

Once a safe concentration was determined, WT GM-CSF cultures were exposed to an antagonist cocktail inhibiting CCR1, CCR2, CCR3 and CCR5 simultaneously. The media was changed every two days to ensure maintenance of the receptor occupancy. As seen from figure 6-16 B, both WT+ antagonist and iCCR$^{-/-}$ cultures showed no proliferation clusters by day 5 of culture, while the WT and single$^{-/-}$ culture proliferation clusters developed normally (Figure 6-14, 6-16A).

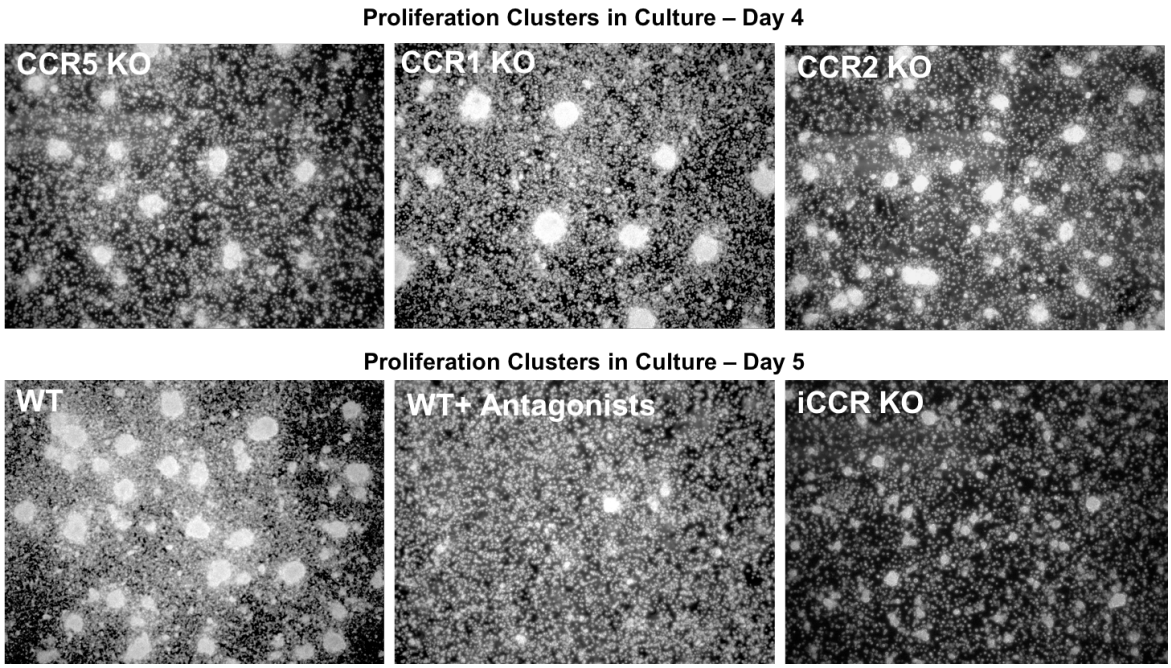

Figure 6-16 Proliferation Clusters after iCCR Antagonist Treatment- A- *Representative bright field images of proliferation clusters in GM-CSF cultures at x40 magnification from CCR5, CCR1 and CCR2$^{-/-}$ bone marrow at day 4.* ***B-*** *Representative bright field images of proliferation clusters in GM-CSF cultures at x40 magnification from WT, iCCR$^{-/-}$ and WT+ iCCR Antagonist treated bone marrow cells.*

Next, different chemokine antagonist cocktails were administered to WT cultures to tease out the effect of specific iCCRs on the development of proliferation clusters. As seen from Figure 6-17B (Representative fields of view 6-17A), cluster number in culture falls after simultaneous inhibition of 2 chemokine receptors, and the effects are most pronounced when CCR5 inhibition is involved.

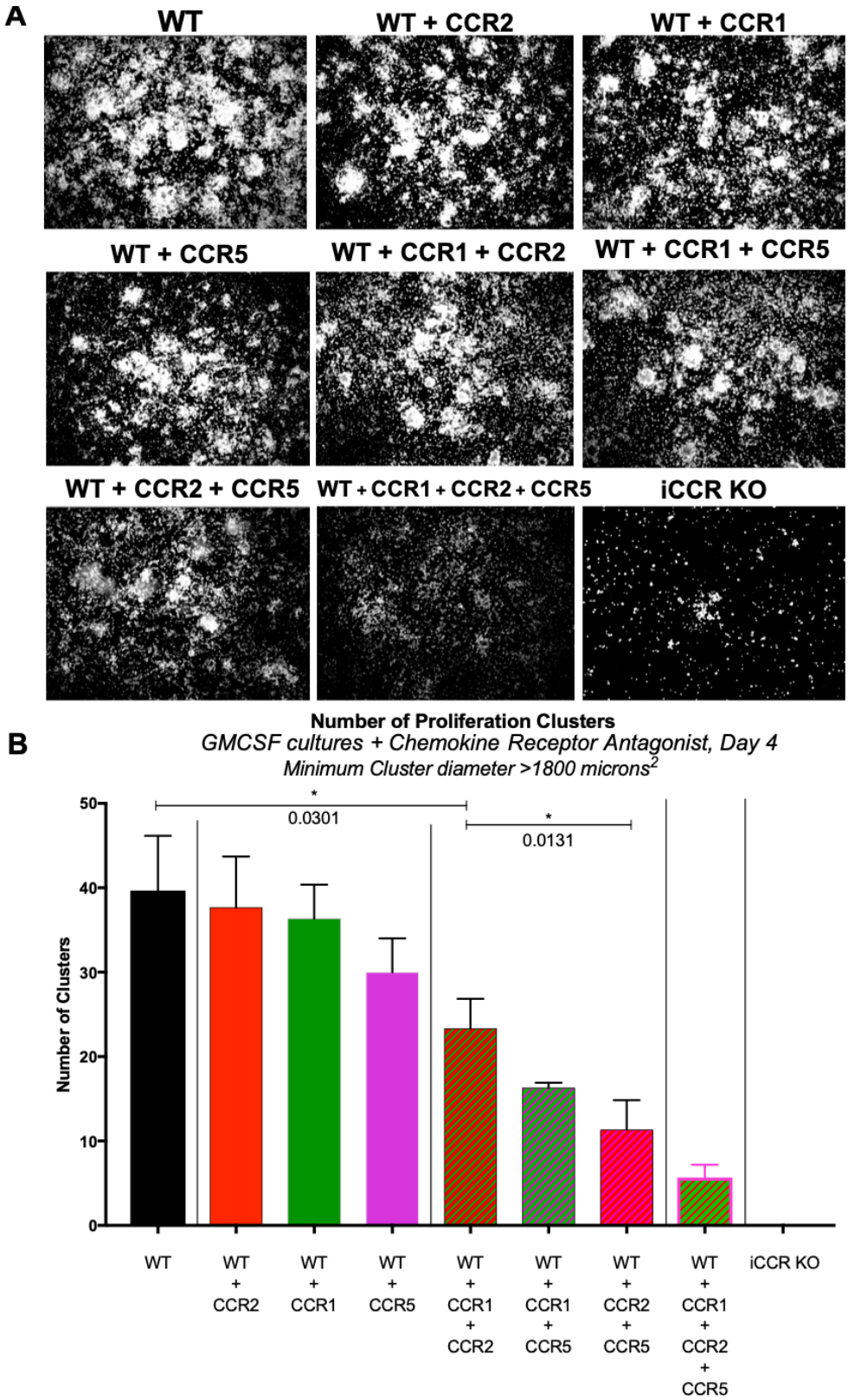

Figure 6-17 Proliferation Clusters with Various iCCR Antagonist Combinations- A- *Representative bright field images of proliferation clusters in GM-CSF cultures (12 well plates) at x40 magnification from WT cells treated with different combinations of chemokine receptor antagonists.* ***B-*** *Graph showing the quantification of clusters in WT GM-CSF cultures in 12 well plates treated with different receptor antagonist combination. The same WT bone marrow was used for each culture, each condition was repeated in triplicate, and 5 fields of view taken from each well. Each value in the graph represents an average of 18 points. Cluster quantification was performed with Fiji Software's particle analysis by selecting clusters larger than 1800microns^2. Ordinary one-way ANOVA with Dunnett's multiple comparisons test was performed to determine statistical significance, with a p value of 0.05 determined as significant.*

While no effect on cytotoxicity was observed at 48 hours, it was hypothesised that exposure to multiple chemokine receptor antagonists for several days could still have a negative effect on proliferation cluster survival. To minimise the amount of iCCR antagonist in culture, the experiment was repeated using CCR2$^{-/-}$ bone marrow. As CCR2 is knocked out, the only antagonists required in culture to reproduce the phenotype would be CCR1/3 (UCB35265) and CCR5 (Maraviroc).

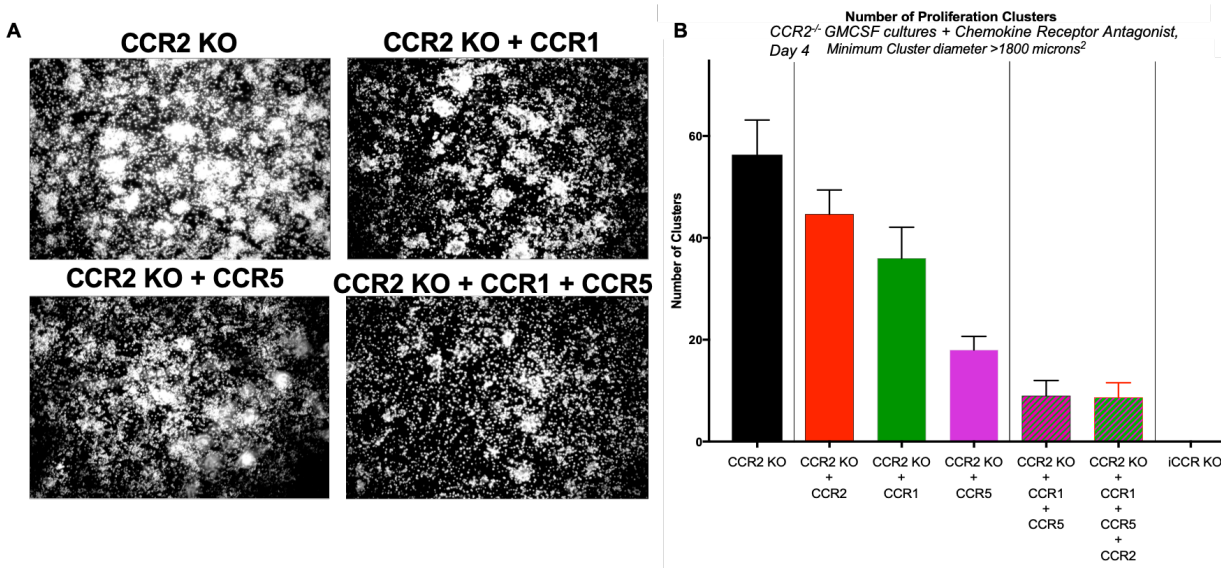

Figure 6-18 Proliferation Clusters in CCR2$^{-/-}$ Cultures Treated with iCCR Antagonists- ***A-*** *Representative bright field images of proliferation clusters in GM-CSF cultures (12 well plates) at x40 magnification from CCR2$^{-/-}$ cells treated with different combinations of chemokine receptor antagonists.* ***B-*** *Graph showing the quantification of clusters in WT GM-CSF cultures in 12 well plates treated with different receptor antagonist combination. The same WT bone marrow was used for each culture, each condition was repeated in triplicate, and 5 fields of view taken from each well. Each value in the graph represents an average of 18 points. Cluster quantification was performed with Fiji Software's particle analysis by selecting clusters larger than 1800microns^2. Ordinary one-way ANOVA with Dunnett's multiple comparisons test was performed to determine statistical significance, with a p value of 0.05 determined as significant*

As seen in Figure 6-18B (Representative fields of view 6-16A), the 'cluster-less' phenotype was recapitulated in CCR2$^{-/-}$ cultures treated with CCR1/3 and CCR5 antagonists, showing that the absence of cluster formation is the result of iCCR inhibition, and not chemokine receptor antagonist toxicity.

Figure 6-19A also compares the CCR2$^{-/-}$ findings with WT cultures treated with different antagonist combination. For example, the number of proliferation clusters in WT cultures treated with CCR1 and CCR2 antagonist is similar to the number of clusters in CCR2$^{-/-}$ cells treated with CCR1 antagonist alone. Again, this confirms that the cluster-less phenotype observed in WT cells treated with different antagonists was induced by iCCR inhibition and

not by a metabolic burden or toxicity caused by simultaneous exposure to different iCCR antagonists.

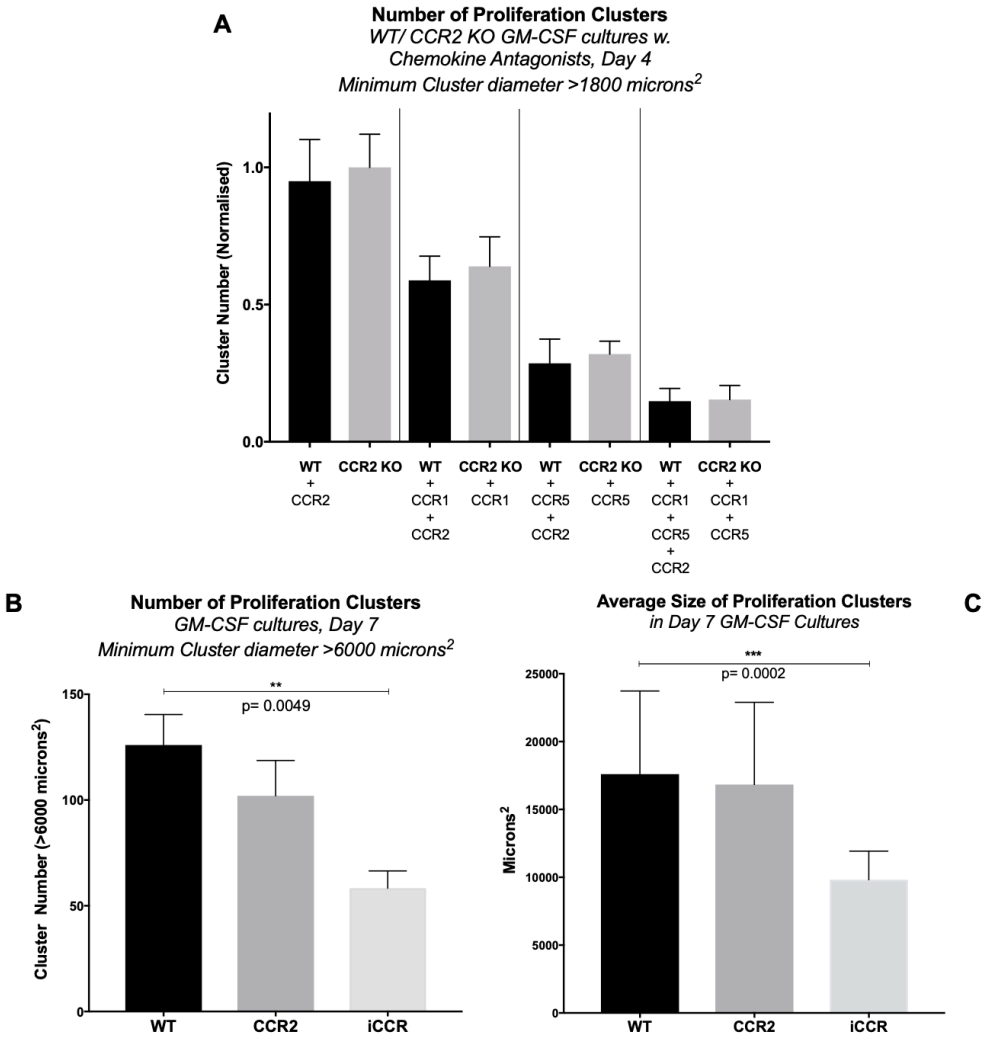

Figure 6-19 Size and Number of Proliferation Clusters in Culture- A- *Graph showing the quantification of clusters in WT compared to CCR2$^{-/-}$ GM-CSF cultures in 12 well plates treated with different antagonist combination.* ***B -*** *Graph showing the quantification of clusters in day 7 GM-CSF cultures grown in 12 well plates from WT, CCR2 and ICCR$^{-/-}$ cells. Each condition was repeated in triplicate, with 5 fields of view per condition. Each value in the graph represents an average of 18 points.* ***C-*** *Graph showing day 7 cluster size in WT, CCR2 and iCCR$^{-/-}$ cultures. Cluster quantification and size determination was performed with Fiji Software's particle analysis by selecting clusters larger than 6000microns^2. Ordinary one-way ANOVA with Dunnett's multiple comparisons test was performed to determine statistical significance, with a p value of 0.05 determined as significant.*

After 7-8 days of culture, iCCR$^{-/-}$ cells eventually form proliferation clusters, but they are fewer in number and half the size of an average WT cluster (Figure 6-19 B,C). This suggests that towards later stages of culture, some other mechanism might be overriding the absence of iCCRs to mediate cluster formation.

Thus, the data so far suggest that the absence of the iCCRs CCR1, CCR2 and CCR5 prevents cluster formation, and that inhibiting these receptors in WT or $CCR2^{-/-}$ cells with chemokine receptor antagonists replicates the phenotype.

6.7 Discussion- How can multiple iCCRs be involved?

Treating WT bone marrow cells with a combination of iCCR inhibitors induces a 'clusterless' phenotype in culture. No effect on proliferation is seen when inhibiting a single chemokine receptor, and the phenotype only becomes apparent when two or more iCCRs are inhibited. These data suggest that CCR1, CCR2, CCR3, and especially CCR5, are required for optimal proliferation and differentiation of monocytes in GM-CSF cultures.

However, flow cytometry on iREP bone marrow shows that inflammatory monocytes are exclusively CCR2+ (Figure 6-20A). If CCR1 and CCR5 are not expressed, how is their inhibition altering proliferation and differentiation? Are they upregulated in the early stages of culture *in vitro*? To test this, iREP bone marrow was cultured in GM-CSF for 7 days and clustered cells were analysed for expression of iCCRs via flow cytometry.

6.7.1 GGiREP GM-CSF Cultures

As seen from Figure 6-20A, inflammatory monocytes on Day 0 are CD11b+ CD11c- Ly6C++ and only express CCR2. After two days of culture in GM-CSF, those same Ly6C++ monocytes have upregulated CCR1 and CCR5 and 40% of Ly6C++ cells are now triple positives for iCCRs (Figure 6-20B). Also at day 2, some inflammatory monocytes have started downregulating Ly6C and upregulating CD11c, initiating the DC differentiation pathway. Up to 65% of these CD11c+ mo-DC precursors express CCR1 CCR2 and CCR5 simultaneously (Figure 6-20C).

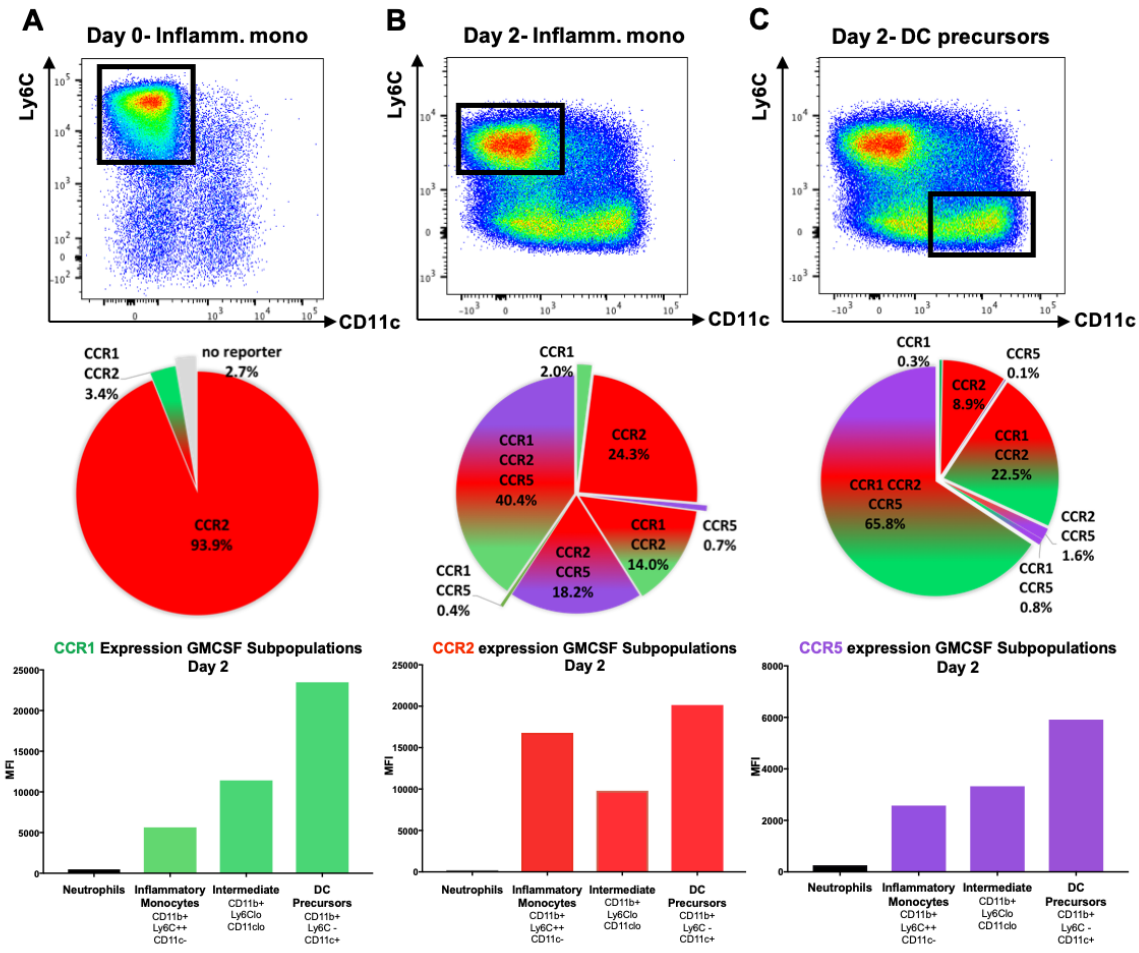

Figure 6-20 Change in iCCR Expression in differentiating Inflammatory Monocytes- Ai: *Representative FACS plot showing inflammatory monocyte Ly6C and CD11c expression in WT bone marrow after extraction and gating out of B-cells (B220+) and Neutrophils (Ly6G+).* ***Aii****: Pie chart showing expression of fluorescent reporter mRuby, corresponding to CCR2 expression, on Ly6C++ inflammatory monocytes extracted from bone marrow before culture in GM-CSF.* ***Bi****: Representative FACS plot showing Ly6C and CD11c expression on day 2 inflammatory monocytes.* ***Bii:*** *Pie chart showing expression of fluorescent reporter mRuby (red, CCR2), Clover (green, CCR1), IRFP682 (purple, CCR5) on inflammatory monocytes after 2 days of culture.* ***Ci****: Representative FACS plot showing Ly6C and CD11c expression on day 2 DC precursors.* ***Cii****: Pie chart showing expression of fluorescent reporter mRuby (red, CCR2), Clover (green, CCR1), IRFP682 (purple, CCR5) on DC precursors after 2 days of culture.* ***Di, ii, iii****-Charts showing fluctuating iCCR expression measured as reporter MFI via flow cytometry in each subpopulation of Ly6C/CD11c cell in GM-CSF cultures at day 2.*

After an additional 3 days of culture (Day 6), most inflammatory monocytes have downregulated Ly6C completely (Figure. 6-21A) and have fully upregulated CD11c and MHCII (Figure 6-21B, 6-21C), giving rise to the 4 subpopulations previously described: the mature mo-DCs, the immature mo-DCs, the CD11c+ macrophages and the monocytes. In immature mo-DCs, a third of cells co-express all 3 iCCRs, with CCR2 being the dominant chemokine receptor, possibly because of its early role in egress of monocytes from the bone marrow. At day 9, when most of the immature DCs have become mature CD11c+

MHCII++ mo-DCs, CCR5 is markedly upregulated, with 90% of mature DCs positive (a 40% rise from immature Day 6 DCs) (Figure 6-21C).

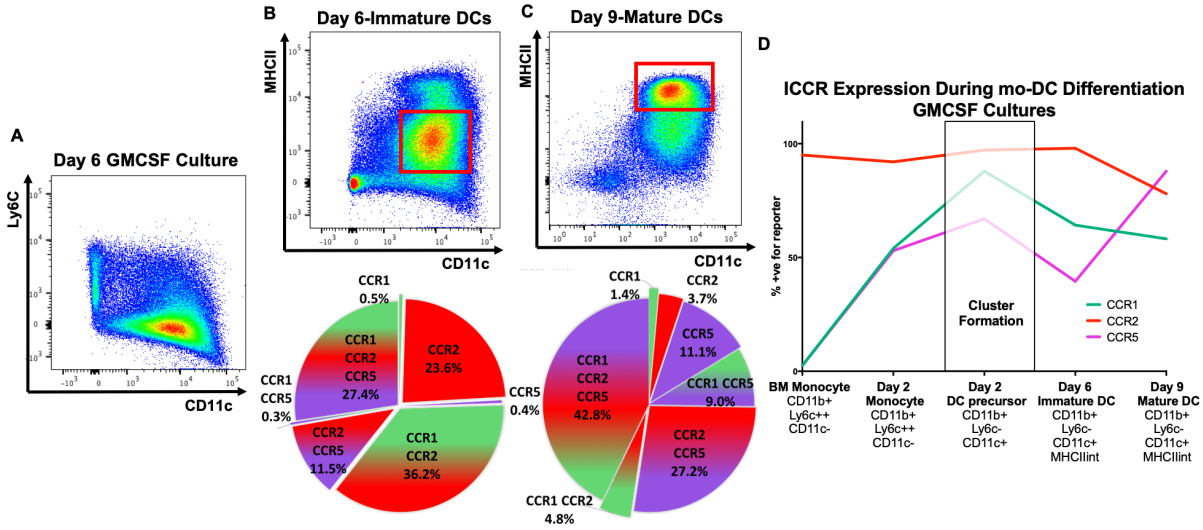

Figure 6-21 Change in iCCR Expression in Immature and Mature mo-DCs- A: *Representative FACS plot showing expression of Ly6C and CD11c in Day6 GM-CSF cultures.* ***B:*** *Representative FACS plot showing immature mo-DCs (CD11c+ MHCII+) in Day 6 GM-CSF cultures and their iCCR expression through detection of fluorescent reporter mRuby (red, CCR2), Clover (green, CCR1), IRFP682 (purple, CCR5).* ***C:*** *Representative FACS plot showing mature mo-DCs (CD11c+ MHCII++) in Day 9 GM-CSF cultures and their iCCR expression through detection of fluorescent reporter mRuby (red, CCR2), Clover (green, CCR1), IRFP682 (purple, CCR5).* ***D:*** *Line graphs summarising iCCR expression fluctuation as differentiation in GM-CSF cultures progresses from the early bone marrow inflammatory monocyte stage to the late day 9 mature mo-DC stage.*

Therefore, iCCR expression fluctuates as mo-DCs mature in culture. Starting from an exclusively CCR2+ population of inflammatory monocytes, the first few days of culture are characterised by an upregulation of CCR1 and CCR5 in developing cells, resulting in approximately 65% of mo-DC precursors co-expressing all 3 receptors simultaneously (Figure 6-21D).

Cluster development in culture also appears between days 2-4, thus suggesting that CCR1, CCR2 and CCR5 are all involved in cluster formation (while CCR3 was not detected at any point). Rapid upregulation of CCR1 and CCR5 at the beginning of differentiation explains why multiple chemokine receptor antagonists need to be administered simultaneously, as inhibiting a single iCCR would still allow signalling via the remaining iCCRs.

These findings might also explain why cluster formation is delayed in iCCR$^{-/-}$ cultures, as developing mo-DCs need time to upregulate other 'late-stage' receptors (such as CCR7 or MHC II) to overcome the lack of signalling caused by the deletion of CCR1, CCR2 and CCR5.

However, what is the cause? Is there a cellular mechanism involving chemokine receptor signalling that would cause changes in adhesion/clustering, phagocytosis, proliferation and that is affected by multiple chemokine receptors?

6.8 A Role for Integrins in Macrophage and mo-DC differentiation

Integrins are transmembrane receptors that facilitate cell-to-cell and cell-to-extracellular matrix interaction[405]. Integrins are involved in a variety of processes: mediating arrest and stable adhesion of leukocytes to blood vessels[406], stabilisation of the immune synapse allowing for prolonged contact between leukocytes, and they mediate cytoskeletal rearrangement for migration and phagocytosis[407]. Most integrins are not constitutively active, and their activity is controlled bidirectionally by either 'inside out signalling' or 'outside in signalling', which ultimately modifies the interactions between the alpha and beta subunits and changes their ligand binding affinity[408]. This ability to turn 'on' and 'off' is vital for their biological function, as integrins can be activated in milliseconds to their active state without having to be synthesised *de novo*[409]. This is especially important in circulating leukocytes, as immediate integrin activation allows for timely adhesion to vasculature and egress towards the site of inflammation. This bidirectional control of integrin function is mediated by two different kinds of signalling:

Inside-out signalling: when an extracellular signal (such as engagement of a chemokine receptor or a T-cell receptor) activates an intracellular signalling cascade in the cytoplasm which is then relayed to the integrin, inducing a change in its extracellular binding site, changing its affinity and avidity and mediating cell adhesion[408].

Outside-in signalling: ligand binding to integrins initiates a signal transduction cascade that promotes cell cycle regulation, organisation of the intracellular cytoskeleton and cellular differentiation[408].

6.8.1 Integrins on leukocytes

Integrins involved in cellular adhesion are primarily expressed on leukocytes, with $\beta 2$ integrins preferentially expressed by rolling leukocytes to bind to endothelial cellular adhesion molecules (ICAMs) just before transendothelial migration [410]. The importance of the $\beta 2$ subunit is highlighted in Leukocyte Adhesion Deficiency (LAD), a rare autosomal recessive disorder that causes a deficiency in the $\beta 2$ integrin subunit and thus a defect in the formation of $\beta 2$ integrins (LFA-1, Mac-1). Patients affected by LAD suffer from recurrent bacterial infections from birth and generally do not survive past infancy, highlighting the importance of cell to cell interactions for chemotaxis and phagocytosis[411].

LFA-1: Lymphocyte function-associated antigen-1 (integrin LFA-1) is expressed exclusively by leukocytes, and plays a role in leukocyte recruitment to inflammatory sites and lymphoid tissues [406]. LFA-1 is involved in various cell-cell interactions, such as between T cells and antigen presenting cells[412], or natural killer (NK) cells and target cells[413], and binds to intercellular adhesion molecules (ICAMs), including ICAM-1, ICAM-2, ICAM-3, ICAM-4, ICAM-5 and JAM-1[66]. LFA-1 also plays a major role in the formation of a stable immunological synapse between T-cells and antigen presenting cells, allowing for the prolonged contact required for the development of immunological memory[414].

Mac-1: Macrophage antigen-1 is expressed by cells of the myeloid lineage, including neutrophils, monocytes, macrophages and dendritic cells. Mac-1 is also expressed, to a limited extent, by certain subsets of T lymphocytes[415]. While LFA-1 has been shown to play a dominant role over Mac-1 in mediating the firm adhesion of leukocytes to the endothelium, Mac-1 interacts with ICAM-1 to mediate intraluminal crawling after leukocyte arrest[416]. In addition, upon binding to cognate ligand, Mac-1 promotes differentiation of monocytes to macrophages by downregulating the transcriptional repressor FoxP1, allowing for phagocyte differentiation from monocytes to macrophages as the cell moves towards the site of inflammation[417] **.**

A lack of engaged chemokine receptors would diminish 'inside-out' integrin signalling, resulting in fewer integrins undergoing a conformational change from low affinity to high affinity. Low affinity integrins would not be able to bind to their cognate ligand, which in turn would also affect 'outside-in' signalling. With both 'inside out' and 'outside in' feedback mechanisms removed, cell growth, differentiation, adhesion, migration, phagocytosis and degranulation could all be negatively affected in maturing monocytes.

Integrin mediated cell-cell interactions could allow for the formation of mo-DC clusters in GM-CSF cultures. Inside the cluster, concentrations of cytokines and growth factors are high, promoting correct proliferation and differentiation. A lack of integrins or integrins with low affinity would result in a decrease in frequency and strength of cell-cell contact, either resulting in incomplete cell activation or at least lack of proliferative signals.
Integrin engagement is also required for optimal actin remodelling for phagocytosis. An abundance of low affinity integrins and lack of 'outside in' signalling might fail to reorganise the actin cytoskeleton thus compromising efficient internalisation. This might explain the defect in phagocytosis observed in immature and mature iCCR$^{-/-}$ DCs.

6.8.2 Integrin Staining on ICCR$^{-/-}$ and WT proliferation clusters

Two different antibody clones for LFA-1 exist for flow cytometry, one for the low affinity version (2D7), while another that binds to both the high affinity and low affinity versions (M174) [418] . As seen in Figure 6-22, undifferentiated cells (CD11c-, Figure 6-22A) in iCCR$^{-/-}$ and WT+antagonist cultures express lower levels of integrin LFA-1 overall (Figure 6-22 Bi,Bii) when compared to untreated WT CD11c-cells. However, those same cells have a higher MFI for the low affinity version of the LFA-1 (Figure 6-22Ci,Cii), indicating that not only do undifferentiated monocytes have decreased integrin expression when iCCRs are absent or inhibited, but the ones that do express LFA-1 express higher levels of the low affinity version.

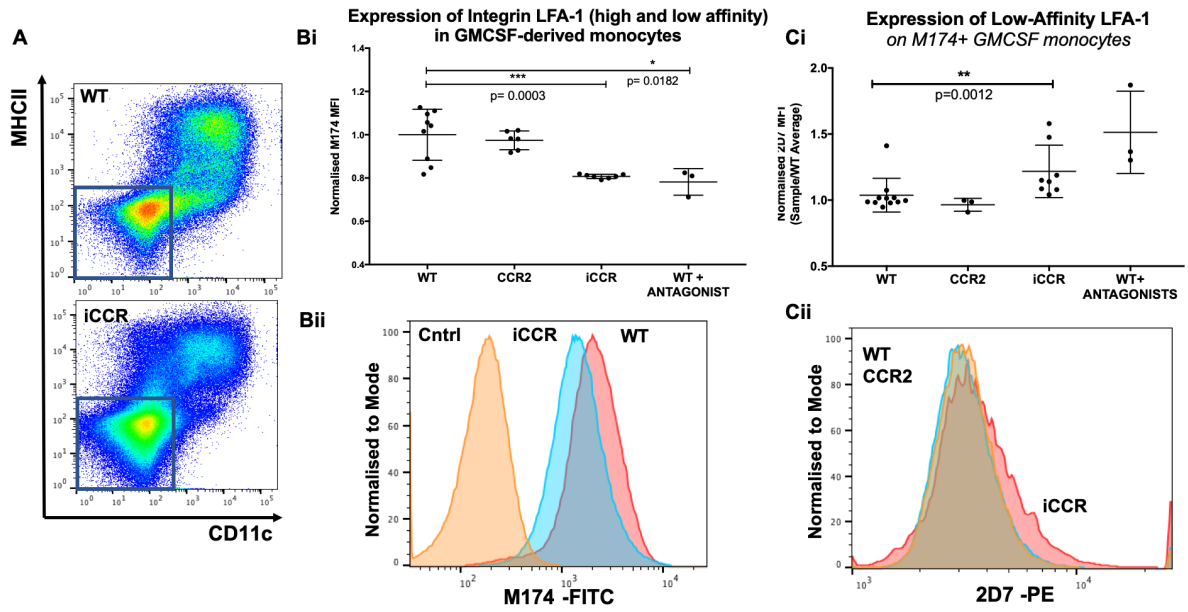

Figure 6-22 Integrin Expression in GM-CSF cultures- A- *Representative FACS plots showing the WT and iCCR$^{-/-}$ monocyte population analysed for integrin expression (blue box).* ***Bi:*** *Expression of integrin LFA-1 on CD11c- monocytes from GM-CSF cultures of WT, CCR2$^{-/-}$, ICCR$^{-/-}$ and WT+ Antagonist treated cells, measured as MFI via flow cytometry (M174 clone).* ***Bii****: : Representative overlaid histograms showing LFA-1 expression (M174 clone) in WT and iCCR$^{-/-}$ monocytes in GM-CSF cultures (red: WT, blue: iCCR$^{-/-}$, orange: control)* ***Ci, Cii:*** *Expression of low affinity LFA-1 on CD11c- monocytes from GM-CSF cultures of WT, CCR2$^{-/-}$, ICCR$^{-/-}$ and WT+ Antagonist treated cells, measured as MFI via flow cytometry.* ***E:*** *Overlaid histograms showing low affinity LFA-1 (2D7 clone) expression in WT and iCCR$^{-/-}$ monocytes in GM-CSF cultures (red: iCCR$^{-/-}$, blue: WT, orange: CCR2$^{-/-}$). Results elaborated from an average of at least 3 independent GM-CSF cultures for both WT and iCCR$^{-/-}$ samples. Student T-test was performed to determine statistical significance, with a p value of 0.05 determined as significant.*

6.8.3 Static Adhesion Assay and Intracellular Staining on Developing Monocytes

To further determine if the decrease of LFA-1 expression and activation in iCCR$^{-/-}$ cultures correlates with a decrease in cell adhesion, a static adhesion assay was performed by adding mo-DCs from WT and iCCR cultures to coverslips coated with the LFA-1 ligand ICAM-1. After a brief 30 minute incubation at 37°C, the coverslips were extensively washed in PBS and the number of remaining immobilised cells was counted using Fiji Image Software. As seen in Figure 6-23A and B, iCCR mo-DCs were found in lower numbers when compared to WT DCs, suggesting that their interaction with immobilised ICAM-1 is weaker and less stable. In addition, reduced staining against CD18, the $\beta 2$ subunit of integrins CD11b, CD11c and LFA-1 (Figure 6-23C), further confirms that iCCR$^{-/-}$ monocytes fail to upregulate integrins while proliferating in GM-CSF cultures.

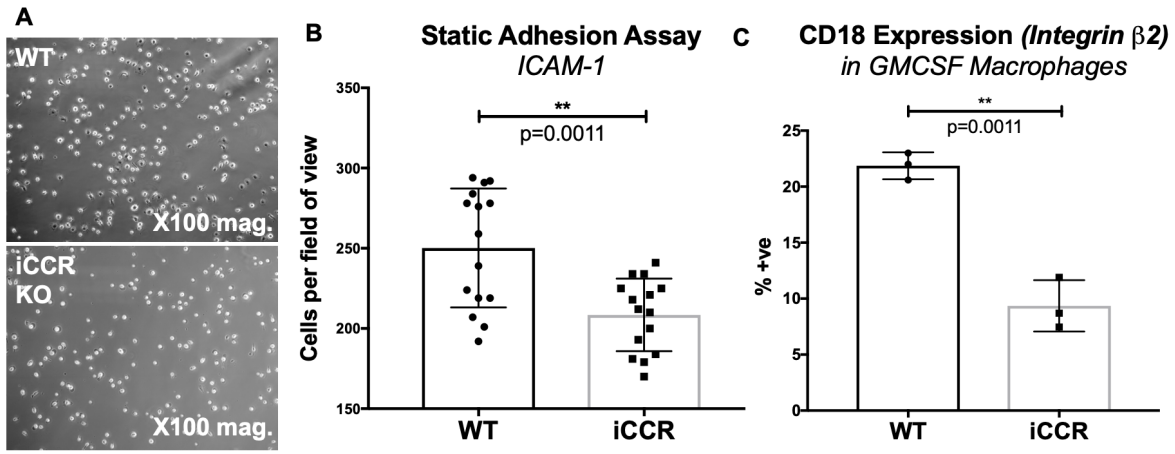

Figure 6-23 Static Adhesion Assay- ***A-*** *Representative bright field images at x100 magnification of WT and iCCR cells on an ICAM-1 coated coverslip.* ***B-*** *Bar graph summarising cell number counts in bright field images detected through 'Particle Count' program on Fiji Image Software (number of cells larger than 50μm^2). 5 fields of view were taken for each coverslip, resulting in an average of 15 total measurements for both WT and iCCR.* ***C-*** *Bar graph showing expression of $\beta 2$ integrin CD18, measured as % positive on CD11c+MHCII int macrophages in WT/iCCR$^{-/-}$ GM-CSF cultures. Results elaborated from an average of at least 3 independent GM-CSF cultures for both WT and iCCR$^{-/-}$ samples. Student T-test was performed to determine statistical significance, with a p value of 0.05 determined as significant.*

Lack of integrin engagement should also result in decreased cell growth (measurable via intracellular marker Ki67) and an increased expression of transcriptional repressor FoxP1. FoxP1 is highly expressed in monocytes and is downregulated as integrins become engaged, allowing for transcription of lineage specific genes that would result in monocyte to macrophage differentiation. As shown in Figure 6-24A, intracellular flow cytometry on iCCR$^{-/-}$ clustered cells reveals a decrease in Ki67 expression (indicating reduced proliferation) and an increase in FoxP1 expression (Figure 6-24B), indicating that clustered monocytes in iCCR$^{-/-}$ cultures are less proliferative and less mature that WT or CCR2$^{-/-}$ clustered monocytes.

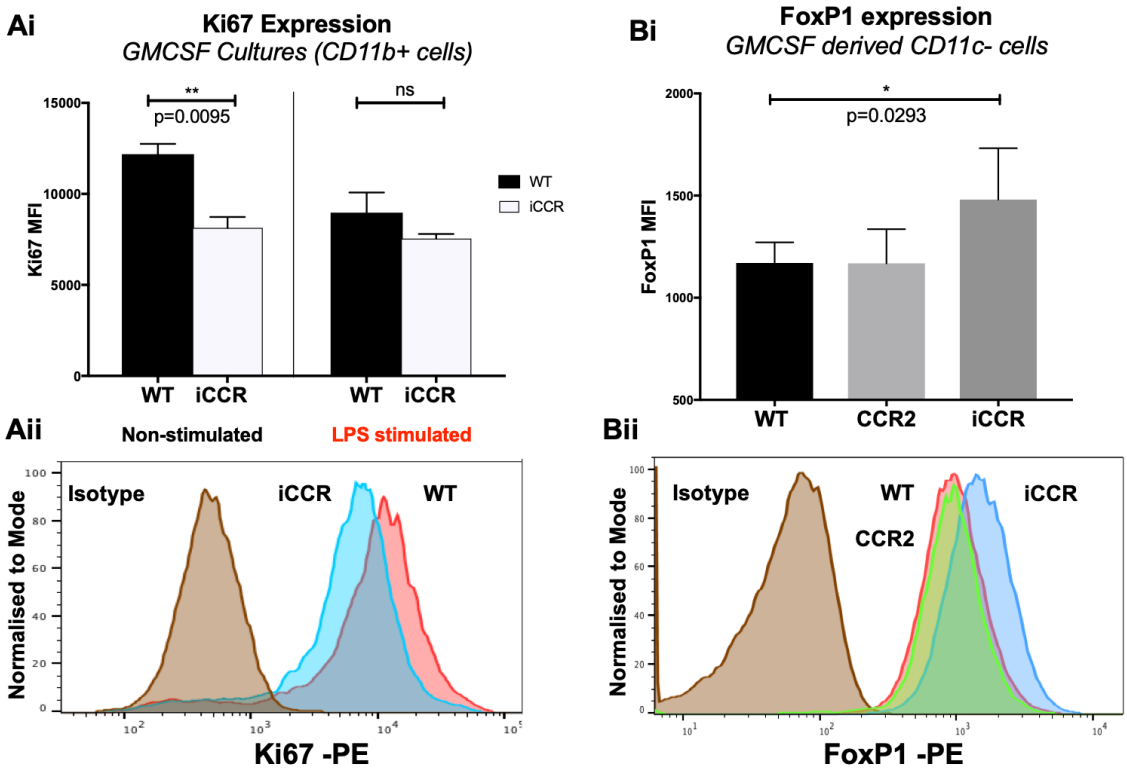

Figure 6-24 Intracellular Staining on developing monocytes- A: *Graph summarising Ki67 expression on clustered cells (CD11b+) in WT and iCCR$^{-/-}$ GM-CSF cultures, before and after LPS stimulation for 12 hours, measured as MFI via intracellular flow cytometry.* ***Aii****: Overlaid histograms showing MFI for Ki67 expression in WT and iCCR$^{-/-}$ clustered cells (iCCR:blue, WT: red, isotype control: brown), measured via intracellular flow cytometry.* ***Bi****: Graph summarising FoxP1 expression in clustered monocytes (CD11c-) from WT, iCCR$^{-/-}$ and CCR2$^{-/-}$ GM-CSF cultures, measured as MFI via intracellular flow cytometry.* ***Bii:*** *Overlaid histograms showing MFI for FoxP1 expression in WT and iCCR$^{-/-}$ clustered monocytes (CD11c-) (iCCR:blue, WT: red, CCR2: green, isotype control: brown), measured via intracellular flow cytometry. Results elaborated from an average of at least 3 independent GM-CSF cultures for both WT and iCCR$^{-/-}$ samples. Student T-test was performed to determine statistical significance, with a p value of 0.05 determined as significant.*

6.8.4 PCR Array on iCCR$^{-/-}$ and WT Proliferation Clusters

A PCR array was also performed on clustered WT and iCCR$^{-/-}$ cells to uncover any other potential difference. No major change in gene expression was observed, but several genes involved in inflammation, such as genes encoding for monocyte chemoattractant proteins and pro-inflammatory cytokines were slightly downregulated ($p>0.05$) (Figure 6-15A,B). This again suggests that monocytes in GM-CSF cultures lacking the main iCCRs are less activated and more immature.

A

Gene	Fold Change	Gene	Fold Change
Ccl12	-1.90	Cxcl10	-2.55
Ccl17	1.57	Cxcl2	-1.99
Ccl2	-1.87	Cxcr4	-1.70
Ccl3	0.04	Fcgrt	2.35
Ccl4	1.16	Flt3l	1.41
Ccl5	0.51	Icam1	0.59
Ccl7	-2.48	Icam2	-1.09
Ccl8	-1.43	Il12b	-1.68
Ccr9	-0.76	Il16	-1.33
Cd1d1	1.29	Il6	-1.43
Cd2	0.62	Irf7	-1.64
Cd209a	3.09	Itgam	-0.70
Cd28	-2.04	Nfkb1	1.36
Cd44	-0.62	Tgfb1	1.18
Cd74	1.76	Tlr1	0.87
Cd80	1.25	Tlr2	-1.27
Cd86	1.25	Tlr7	-1.33
Clec4b2	-1.99	Tlr9	1.43
Csf1r	0.61	Tnf	1.38

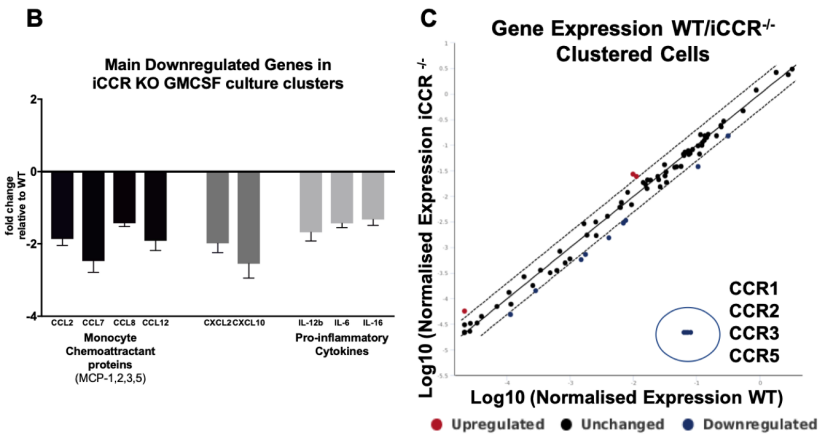

Figure 6-25: Main Upregulated and Downregulated Genes in Clustered Cells- A: *Table summarising most of the genes analysed via PCR-Array. Green: increased expression, Red: decreased expression)* ***B:*** *Graphs summarising the fold change of the most downregulated genes in iCCR$^{-/-}$ clusters compared to WT clustered cells.* ***C:*** *Scatterplot summarising the gene expression of iCCR$^{-/-}$ clusters compared to WT clustered cells. Genes above and below the dotted lines are significantly ($p<0.05$) either upregulated (red) or downregulated (blue). The Scatterplot showing the gene expression of WT and iCCR Clustered Cells and the statistical analyses were all performed according to the manufacturer's instructions using the Qiagen Data Analysis Centre Software. Results elaborated from an average of 2 independent GM-CSF cultures for both WT and iCCR$^{-/-}$ samples.*

Only a few genes were found to be upregulated in iCCR$^{-/-}$ mo-DCs, a DC specific non-integrin intercellular adhesion molecule CD209a (DC-SIGN), a low affinity IgG Fc receptor FcyRt and the invariant chain of MHCII (CD74) also found on DCs (Figure 6-25A). While initially it was unclear why an intercellular adhesion molecule and a low affinity Fc receptor would be upregulated in iCCR$^{-/-}$ cultures, the findings might corroborate the discovery by Menezes et al. of a novel naïve monocyte subpopulation discovered in murine bone marrow [312]. Not only are the monocytes described CD209a+ and GM-CSF dependent, but they also require iCCRs to egress from the bone marrow. This novel monocytic population was also seen and described previously, as CD209a+ monocytes were detected in CCR2$^{-/-}$ air pouch membranes but were missing completely from iCCR$^{-/-}$ air-pouch membranes.

Thus, it could be argued that a lack of CCR1 and CCR5 in the iCCR$^{-/-}$ strain might have caused an accumulation of this specific monocyte subset in the bone marrow, resulting in a higher proportion of CD209+ FcyR+ monocytes differentiating in GM-CSF cultures into CD209+ FcyR+ mo-DCs.

6.9 Discussion & Conclusion

Collectively, the data in this chapter provide a potential mechanism of action for the effects of CCR1, CCR2, CCR3 and CCR5 inhibition on the development and maturation of monocytes into mo-DCs *in vitro*.

Developing monocytes in culture upregulate expression of CCR1 and CCR5 in as little as 48 hours after exposure to GM-CSF. Chemokines secreted by developing cells, such as CCL2 and CCL5, bind to their cognate chemokine receptors, initiating a signaling cascade. The signal from the iCCRs gets relayed to low affinity integrins (inside-out signaling), which change conformation to a high affinity state and promotes inter-cellular adhesion. Integrin upregulation stabilises the formation of proliferation clusters, allowing developing monocytes to stay in close contact and to deliver signals and mediators to each other more effectively, thus promoting differentiation and proliferation of mo-DCs and macrophages.

In the absence or inhibition of iCCRs, integrins decrease in both expression and affinity, reducing cell-cell contact and inhibiting cluster formation, resulting in an accumulation of undifferentiated monocytes. The cells that do manage to differentiate have normal costimulatory molecule expression but decreased phagocytic ability. iCCR cluster formation is generally delayed from day 2-4 in WT, to day 5-7, as by this stage developing mo-DCs have upregulated other receptors, such as CCR7, which could override the lack of iCCR signaling experienced in the initial stages of culture.

6.9.1 Relevance *in vivo*

While it is unclear if the conditions seen *in vitro* would replicate *in vivo*, lack of efficient cell-cell contact between monocytes and the stroma might explain an unexpected phenotype observed in iCCR$^{-/-}$ bone marrow. CCR2 is required for inflammatory monocyte egress from the bone marrow[73][74], and accumulation of monocytes in CCR2-/- bone marrow has already been observed by many groups[280].

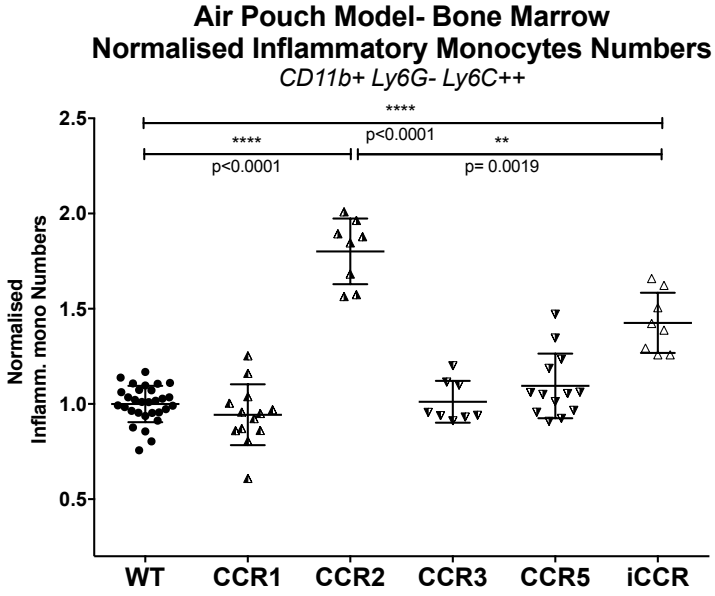

Figure 6-26 Number of inflammatory monocytes in the bone marrow during air-pouch inflammation. *Numbers for each single-/- are normalised with their own WT to mitigate strain-specific phenotypes. Measured via flow cytometry. Ordinary one-way ANOVA with Dunnett's multiple comparisons test was performed to determine statistical significance, with a p value of 0.05 determined as significant*

iCCR$^{-/-}$ monocytes were also expected to accumulate, possibly in even higher numbers, as they are also lacking 3 other iCCRs. Figure 6-26 shows that while monocytes were indeed found in higher numbers in iCCR$^{-/-}$ bone marrow in inflammation compared to WT, CCR2 single knock-out monocyte numbers were found at an even higher proportion. While other explanations to this phenotype were discussed previously, one of the possible mechanisms considered was weaker cell-cell contact in iCCR$^{-/-}$, as proliferating monocytes require constant cross-talking with the bone marrow stroma to develop properly. While monocytes do accumulate in iCCR$^{-/-}$ bone marrow due to lack of CCR2, they might also fail to proliferate as fast by having weaker interactions with supporting stromal cells, reducing their numbers.

6.9.1.1 Granuloma Formation

One potential mechanism involving extensive cell-cell contact between macrophages, monocytes and DCs *in vivo* is the formation of a granuloma following infection with *M.Tuberculosis*[419]. Granulomas are specialised structures that localise and contain *M.tb* while concentrating the immune response to a limited area [420] and consist mainly of blood-derived macrophages, differentiated macrophages and multinucleated giant cells, surrounded by T lymphocytes [421]. These Multinucleated Giant Cells (MGC) are formed by the fusion of macrophages and play important roles in a number of physiological and pathological processes, including the phagocytosis of large foreign particles that would be too big to phagocytose for a normal macrophage[422]. $\beta 1$ and $\beta 2$ integrins have been shown to mediate adhesion during macrophage fusion and multinucleated giant cell formation [423][424], so it would be interesting to see if iCCR$^{-/-}$ leukocytes are capable of forming a granulomas and multinucleated cells to contain *M.Tuberculosis* infection.

7 Final Discussion and Future Directions

7.1 Overview

The Inflammatory CC-chemokine receptors (CCR1, CCR2, CCR3 and CCR5) are regarded as major contributors to *in vivo* inflammatory cell recruitment. However, the totality of their combined contribution to the overall orchestration of the inflammatory responses, and residual inflammatory cell recruitment in their absence, has not yet been defined. In addition, despite over 25 years of pharmaceutical targeted research, no antagonist of inflammatory chemokine receptors has been licensed for use in treating inflammatory diseases such as rheumatoid arthritis or other conditions in which excessive inflammation is the main driver of the pathology. This failure as been attributed to an incomplete understanding of the inflammatory chemokine receptor system and confounding effects attributable to the promiscuity of each receptor[4].

To investigate the contribution each iCCR has on the development of the inflammatory response, this project has focused on characterising two novel mouse models recently developed by the Chemokine Research Group, an iCCR$^{-/-}$ strain and an iCCR reporter strain.

7.2 Role of iCCRs in Homeostasis

Analysis of resting iCCR$^{-/-}$ showed little evidence of a combinatorial effect of iCCRs on homeostatic recruitment and leukocyte distribution in various tissues. In both bone marrow and blood, iCCR$^{-/-}$ mice were generally a pheno-copy of CCR2$^{-/-}$ mice, with the exception of impaired eosinophil recruitment in the iCCR$^{-/-}$ which is controlled by CCR3. In other words, while iCCR-/- mice showed defects in recruitment of inflammatory monocytes and eosinophils, all phenotypes observed were attributable to the absence of either CCR2 or CCR3, with no evidence of contribution from CCR1 or CCR5.

Under resting conditions, the only evidence so far of a possible contribution of other iCCRs in directing leukocytes to different organs was found in the skin, where a decrease in skin tissue macrophages in iCCR$^{-/-}$ mice could not be completely explained by the absence of CCR2. It was still unclear however how much of this difference could be attributed to a combinatorial effect of the simultaneous deletion of CCR1, CCR2 and CCR5 on monocyte recruitment, or if the absence of CCR1 and CCR5 was affecting the proliferation of the newly recruited monocytes, further decreasing their overall numbers resulting in a phenotype that is more profound than in the CCR2$^{-/-}$ mice. The *in vitro* findings discussed in the previous chapter appear to support this hypothesis, as iCCR$^{-/-}$ monocytes or WT monocytes exposed to specific iCCR chemokine receptor antagonists grown into GM-CSF-derived DCs and macrophages show decreased proliferation rates and are unable to differentiate effectively.

Expectedly, deletion of iCCRs had no effect on tissue resident macrophage populations which are generally thought of as iCCR independent, such as alveolar macrophages in the lung or red pulp macrophages in the spleen.

7.3 Role of iCCRs in Inflammation

While both the Imiquimod and the air pouch models showed defects in recruitment of inflammatory monocytes, dendritic cells, eosinophils and macrophages, there was again little evidence of redundancy in receptor involvement, as most of the phenotypes were explained by an absence of either CCR2 or CCR3. Thus, the data highlight the overwhelming importance of CCR2 as a recruiter of monocytic cells (and CCR3 for eosinophils) to acutely inflamed sites. Macrophages were the only leukocyte subset hinting at a possible combinatorial role for iCCRs, as the numbers in iCCR$^{-/-}$ air pouch membrane were lower than in the CCR2$^{-/-}$ mice.

Differences in lymphoid cell recruitment were not observed in the air pouch model. After Imiquimod application to the skin however, activated T-cells were found elevated in blood, potentially indicating they were trapped in the circulation. This could suggest that certain ligands can induce the upregulation of specific sets of iCCRs on a leukocyte, and changing the inflammatory conditions by using, for example, a model for bacterial or fungal

infections, might reveal more profound phenotypes in more leukocyte subsets than was observed in iCCR$^{-/-}$ mice exposed to Imiquimod or carrageenan. Indeed, preliminary experiments performed with Dr. Pingen using Semliki Forest Virus to induce encephalitis in iCCR-/- mice revealed defects in recruitment into the brain of multiple leukocyte subsets, including monocytes, B-cells, CD4 and CD8 T-cells and even neutrophils. These preliminary findings suggest that the choice of inflammatory stimulus might be critical for the upregulation of certain CC-chemokine receptors, and further studies using a systematic approach to identify iCCR-inducing inflammation models will probably help identify the additional combinatorial roles of CCR1 and CCR5.

7.4 Role of CCR1 and CCR5

While CCR2 and CCR3 are expressed in resting 'classical' inflammatory monocytes and eosinophils, CCR1 and CCR5 are generally thought to be upregulated only during differentiation and after activation with inflammatory mediators. Thus, for example, while CCR1 has been shown to aid trans-endothelial migration of inflammatory monocytes[298][318], those same cells are probably able to migrate just as efficiently using other receptors, and the absence of CCR1 would not stop chemotaxis but delay it slightly.

Similarly, while CCR5's role in macrophage function has been well documented[321]–[323], the receptor itself might be dispensable or at least perform a function that overlaps with other receptors, allowing the leukocyte to function in its absence[324].

In other words, while the experiments on the iCCR$^{-/-}$ mouse model identified the pivotal roles of CCR2 and CCR3, it might not be the best model to uncover the role of CCR1 and CCR5 as these receptors are only upregulated during a specific temporal window in the life of a leukocyte and their effect might have gone undetected. To address this issue, a novel iCCR reporter murine strain developed by Dr. Medina-Ruiz was validated and used to determine leukocyte iCCR expression in various organs both at rest and during inflammation.

Fluorescent reporter proteins were detected in several leukocyte subsets, including inflammatory monocytes (CCR2), eosinophils (CCR3), B-cells (CCR1), and patrolling

macrophages (CCR2). Under resting conditions, detection of fluorescent reporter proteins via flow cytometry on the surface of leukocytes revealed high CCR2 expression (>95%) in inflammatory monocytes and high CCR3 in eosinophils (>90% in circulation), in line with expectations. More importantly, CCR2 and CCR1 co-expression was detected in both resting and inflamed iREP inflammatory monocytes, and the number of CCR1 expressing monocytes in various tissues offered hints on its function. During inflammation, up to 16% of inflammatory monocytes in the bone marrow express CCR1. The percentage drops to 10% in circulation but increases to 40% in the air-pouch membrane, finally reaching maximal CCR1 expression (70%) in macrophages of the air pouch fluid. The decrease in CCR1 expression in circulation raises two possibilities: either CCR1 is upregulated briefly during inflammatory monocyte bone marrow egress and downregulated rapidly while in circulation, and re-upregulated as the monocytes reaches the site of inflammation, or, more simply, CCR1+ monocytes are 'stuck' along the inflamed vessel walls, leaving mainly CCR2+ monocytes in extracted blood.

This mechanism of action is described in Figure 7-1. CCR2+ and CCR2+CCR1+ double positive inflammatory monocytes are both capable of bone marrow egress and enter the circulation. In the blood, CCR2+CCR1+ monocytes are better capable of interacting with the inflamed vessel lumen, binding more strongly and migrating more effectively than CCR2 single positive monocytes. As a result, the inflamed area becomes 'enriched' with CCR2+CCR1+ co-expressing monocytes. As these monocytes continue to migrate towards the site of inflammation, they differentiate into macrophages and begin expressing CCR5 (expressed on around 15% of air-pouch fluid macrophages). CCR2 single positive monocytes on the other hand do not migrate as effectively and probably stay in circulation, eventually seeding distal organs in the periphery, such as the gut, the spleen or the lungs.

Thus, while the experiments performed on the full iCCR$^{-/-}$ have underlined the importance of CCR2, the findings on iREP mice suggest an important role for CCR1 in monocyte trans-endothelial migration.

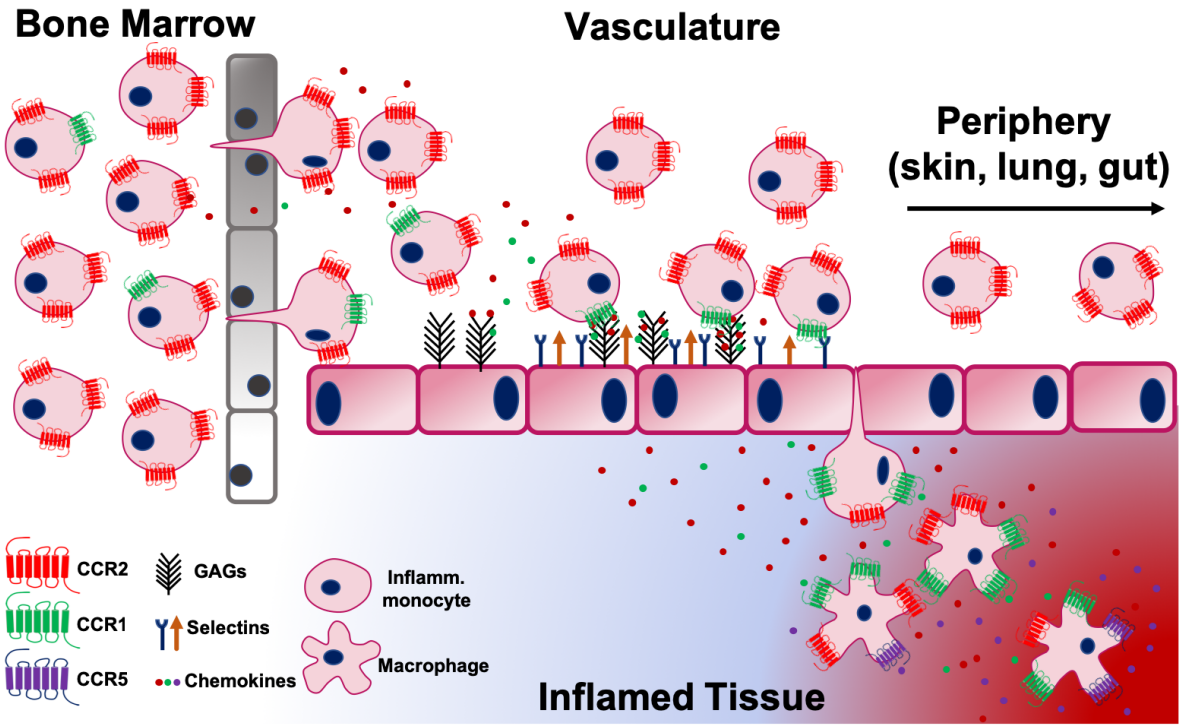

Figure 7-1- Schematic of inflammatory monocyte chemotaxis from the bone marrow to an acutely inflamed area. *CCR2+ and CCR1+CCR2+ inflammatory monocytes are both capable of bone marrow egress. Once in circulation, CCR1+CCR2+ monocytes preferentially bind to the activated endothelium and initiate chemotaxis. As the CCR2+CCR1+ monocytes move towards the site of inflammation, they differentiate into macrophages and increase expression of CCR5.*

7.5 Future Directions

The iCCR network is still incredibly complex. and while iCCR$^{-/-}$ and iREP mice have answered some questions, many still remain: what is the role of CCR5 in macrophages? What is the role of CCR1 in B-cells?

In vivo work on iREP mice has also shown that macrophages can express various combination of chemokine receptors, with CCR2+, CCR1+CCR2+ and CCR1, CCR2 and CCR5 triple positive populations all present simultaneously in the air pouch membrane. Are these different subsets phenotypically identical, or does the expression of particular chemokine receptors alter the macrophage's differentiation and effector function? Further experiments using another novel murine strain developed at the CRG by Dr. Schutte will address these issues.

7.5.1 A novel iCCR Conditional Knock-Out Strain

RiKO (Reversible iCCR Knock-Out, generated by Dr. Schutte), is novel murine strain which had the endogenous iCCR locus removed and replaced with a 'recombineered' locus in which transcriptional 'stop' cassettes, flanked by different recombinase recognition sites placed next to each receptor gene (Figure 7-2). These mice are therefore conditionally-null at each receptor allele. By crossing with appropriate recombinase-expressing mice, it will also be possible to switch on these individual receptors, and thus define their individual contributions to leukocyte migration. It will be also possible to cross multiple recombinase expressing mice, allowing for the generation of mice expressing specific combinations of iCCRs (for example, a CCR1+CCR2+CCR3-CCR5- strain).

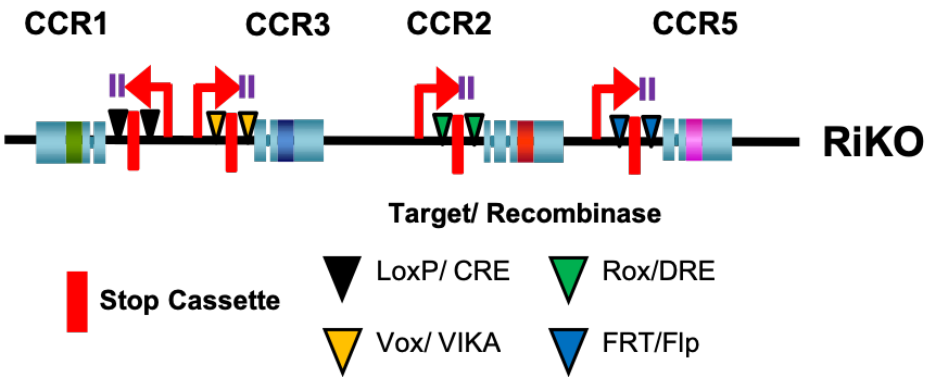

Figure 7-2- Generating A Reversible iCCR Knock-Out Strain (RIKO)- *Schematic provided by Dr. Schutte showing how the novel conditional iCCR murine strain was designed. The endogenous iCCR locus was removed and replaced with a recombineered locus which contains transcriptional stop cassettes (red) flanked by different recombinase recognition sites (inverted triangles in front of each gene). In the absence of the correct recombinase, the stop cassette does not allow for expression of a particular iCCR. Crossing with the appropriate recombinase-expressing mice switches on an individual chemokine receptor.*

The availability of these three novel mouse strains (iCCR$^{-/-}$, iREP, RiKO) provides a unique opportunity to define iCCR involvement in orchestrating the inflammatory response: experiments on the iCCR$^{-/-}$ mice have provided a 'clean slate' to assess the development of the immune response in the absence of CCR1, CCR2, CCR3 and CCR5 while studies on the iREP strain allowed for direct visualisation in changes of iCCR expression. Future studies on the RiKO mice will finally be able to address the issues of iCCR co-expression and redundancy.

Taken together, experiments on these three novel iCCR strains will provide much needed clarity to our current understanding of the orchestration of the chemokine driven inflammatory responses. The results will not only increase our understanding of basic chemokine biology, but will also inform future pharmacological intervention on the chemokine system.

References

[1] M. D. Turner, B. Nedjai, T. Hurst, and D. J. Pennington. Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. *Biochim. Biophys. Acta. Mol. Cell Res.* 2014;1843(11):2563–2582.

[2] R. Westhovens and L. Annemans. Costs of drugs for treatment of rheumatic diseases. *RMD Open*. 2016;2(2):1–4.

[3] E. M. Williamson and J. R. Berger. Infection Risk in Patients on Multiple Sclerosis Therapeutics. *CNS Drugs*. 2015;29(3):229–244.

[4] M. C. Lebre *et al.* Why CCR2 and CCR5 blockade failed and why ccr1 blockade might still be effective in the treatment of rheumatoid arthritis. *PLoS One*. 2011;6(7):8-13.

[5] M. C. Miller and K. H. Mayo. Chemokines from a structural perspective. *Int. J. Mol. Sci.* 2017;18(10):1–16.

[6] M. A. Thomas *et al.* Structure-function analysis of CCL28 in the development of post-viral asthma. *J. Biol. Chem.* 2015;290(7):2015.

[7] D. T. Groves and Y. Jiang. Chemokines, a Family of Chemotactic Cytokines. *Crit. Rev. Oral Biol. Med.* 1995;6(2):109-118

[8] C. R. Mackay. Chemokine receptors and T cell chemotaxis. *J. Exp. Med.* 1996;184(3):799-802.

[9] C. R. Mackay. Chemokines: What chemokine is that? *Curr. Biol.* 2004;7(6):384-386

[10] J. M. Morganti *et al.* The soluble isoform of CX3CL1 is necessary for neuroprotection in a mouse model of Parkinson's disease. *J. Neurosci.* 2012;32(42):14592-14601

[11] C. A. Haskell, M. D. Cleary, and I. F. Charo. Unique role of the chemokine domain of fractalkine in cell capture. Kinetics of receptor dissociation correlate with cell adhesion. *J. Biol. Chem.* 2000;275(44):34183-34189.

[12] M. F. Bachmann, M. Kopf, and B. J. Marsland. Opinion: Chemokines: More than just road signs. *Nat. Rev. Immunol.* 2006;6(2):159-164.

[13] J. L. Williams, D. W. Holman, and R. S. Klein. Chemokines in the balance: maintenance of homeostasis and protection at CNS barriers. *Front. Cell. Neurosci. 2014;8*(154):1-12.

[14] K. Chen *et al*. Chemokines in homeostasis and diseases. *Cell. Mol. Immunol.* 2017;15(4):324-334.

[15] E. S. Baekkevold *et al.* The CCR7 ligand elc (CCL19) is transcytosed in high endothelial venules and mediates T cell recruitment. *J. Exp. Med.* 2001;193(9):1105-1112.

[16] L. A. Johnson and D. G. Jackson. Inflammation-induced secretion of CCL21 in lymphatic endothelium is a key regulator of integrinmediated dendritic cell transmigration. *Int. Immunol.* 2010;22(10):839-849.

[17] A. Zlotnik, O. Yoshie, and H. Nomiyama. The chemokine and chemokine receptor superfamilies and their molecular evolution. *Genome Biol.* 2006;7(12):243.

[18] N. van Nuland *et al.* Function, diversity and therapeutic potential of the N-terminal domain of human chemokine receptors. *Biochem. Pharmacol.* 2012;84(10):1366-1380.

[19] C. Murdoch and A. Finn. Chemokine receptors and their role in inflammation and infectious diseases. *Blood*. 2000;95(10):3032-3043.

[20] H. Arai and I. F. Charo. Differential regulation of G-protein-mediated signaling by chemokine receptors. *J. Biol. Chem.* 1996;271(36):21814-21819.

[21] M. P. Crump *et al.* Solution structure and basis for functional activity of stromal cell-derived factor-1: dissociation of CXCR4 activation from binding and inhibition of HIV-1. *EMBO J.* 1997;16(23):6996-7007.

[22] F. S. Monteclaro and I. F. Charo. The Amino-terminal Extracellular Domain of the MCP-1 Receptor, but Not the RANTES/MIP-1α Receptor, Confers Chemokine Selectivity. *J. Biol. Chem.* 2002;271(32):19084-19092.

[23] M. Arimont *et al.* Structural Analysis of Chemokine Receptor-Ligand Interactions. *J. Med. Chem.* 2017;60(12):4735-4779.

[24] J. Sanchez *et al.* Evaluation and extension of the two-site, two-step model for binding and activation of the chemokine receptor CCR1. *J. Biol. Chem.* 2019;294(10):3464-3475.
[25] A. B. Kleist *et al.* New paradigms in chemokine receptor signal transduction: Moving beyond the two-site model. *Biochem. Pharmacol.* 2016;15(114)53–68.
[26] L. Rajagopalan and K. Rajarathnam. Structural basis of chemokine receptor function-a model for binding affinity and ligand selectivity. *Biosci. Rep.* 2006;26(5):325-339
[27] M. Arimont *et al.* Structural Analysis of Chemokine Receptor-Ligand Interactions. *J. Med. Chem.* 2017;60(12):4735-4779
[28] J. H. Kehrl. Heterotrimeric G protein signaling: Roles in immune function and fine- tuning by RGS proteins. *Immunity*. 1998;8(1):1-10.
[29] C. Moratz, K. Harrison, and J. H. Kehrl. Regulation of chemokine-induced lymphocyte migration by RGS proteins. *Methods Enzymol.* 2004;389:15-22.
[30] G. Servant *et al*. Polarization of Chemoattractant Receptor Signaling During Neutrophil Chemotaxis. *Science*. 2000;287(5455);1037-1040.
[31] J. K. Foskett *et al.* "Inositol trisphosphate receptor Ca2+ release channels. *Physiol. Rev.* 2007;87(2):593-658.
[32] A. P. Curnock, M. K. Logan, and S. G. Ward. Chemokine signalling: pivoting around multiple phosphoinositide 3-kinases. *Immunology*. 2002;105(2):125-136.
[33] Y. Wang, Y. Zhou, and D. T. Graves. FOXO transcription factors: their clinical significance and regulation. *Biomed Res. Int.* 2014;2014:925350.
[34] J. Ren *et al.* Protein kinase C-δ (PKCδ) regulates proinflammatory chemokine expression through cytosolic interaction with the NF-κB subunit p65 in vascular smooth muscle cells. *J. Biol. Chem.* 2014;289(13):9013-9026.
[35] B. A. Hemmings and D. F. Restuccia. PI3K-PKB / Akt Pathway. *Cold Spring Harb. Pespect. Biol*. 2012;4(9):1–4
[36] J. Patel, K. M. Channon, and E. McNeill. The Downstream Regulation of Chemokine Receptor Signalling: Implications for Atherosclerosis. *Mediators Inflamm.* 2013;2013:1-12.
[37] T. Jin and D. Hereld. Moving toward understanding eukaryotic chemotaxis. *Eur. J. Cell Biol.* 2006;85(9):905-913.
[38] J. Corbisier *et al.* Biased signaling at chemokine receptors. *J. Biol. Chem.* 2015;290(15):9542-9554.
[39] L. D. Bennett, J. M. Fox, and N. Signoret. Mechanisms regulating chemokine receptor activity. *Immunology*. 2011;134(3):246-256
[40] L. Ma and G. Pei. Beta-Arrestin Signaling and Regulation of Transcription. *J. Cell Sci.* 2007;120(2):213-218.
[41] S. S. G. Ferguson. Evolving Concepts in G Protein-Coupled Receptor Endocytosis: The Role in Receptor Desensitization and Signaling. *Pharmacol. Rev.* 2001;53(1):1-24.
[42] A. Sorkin and M. von Zastrow. Signal transduction and endocytosis: close encounters of many kinds. *Nat. Rev. Mol. Cell Biol.* 2002;3(8):600-614.
[43] N. Signoret *et al.* Endocytosis and Recycling of the HIV Coreceptor Ccr5. *J. Cell Biol.* 2000;151(6):1281-1294.
[44] N. Zimmermann, J. J. Conkright, and M. E. Rothenberg. CC chemokine receptor-3 undergoes prolonged ligand-induced internalization. *J. Biol. Chem.* 1999;274(18):12611-12618.
[45] D. Kimmig *et al.* Differential Activation of CC Chemokine Receptors by AOP-RANTES. *J. Biol. Chem.* 2002;275(11):7787-7794.
[46] V. V Gurevich and E. V Gurevich. The structural basis of arrestin-mediated regulation of G-protein-coupled receptors. *Pharmacol. Ther.* 2006;110(3):465-502.
[47] Bachelerie F. *et al.* International Union of Pharmacology. LXXXIX. Update on the Extended Family of Chemokine Receptors and Introducing a New Nomenclature for Atypical Chemokine Receptors. *Pharmacol. Rev.* 2013;66(1):1-79.
[48] R. J. B. Nibbs and G. J. Graham. Immune regulation by atypical chemokine receptors. *Nat. Rev. Immunol.* 2013;13(11):815-829.
[49] J.Wilson *et al.* Atypical chemokine receptor ACKR2 controls branching morphogenesis in the developing mammary gland. *Development*. 2016;144(1):74-82.

[50] R. J. B. Nibbs *et al.* The atypical chemokine receptor D6 suppresses the development of chemically induced skin tumors. *J. Clin. Invest.* 2007;117(7):1884-1892.
[51] A. Rot. Contribution of Duffy antigen to chemokine function. *Cytokine Growth Factor Rev.* 2005;16(6):687-694.
[52] R. Bonecchi, M. Massara, and M. Locati. Atypical Chemokine Receptors. *Encycl. Immunobiol.* 2016;2(5):579-585
[53] M. Thelen *et al.* The biochemistry and biology of the atypical chemokine receptors. *Immunol. Lett.* 2012;145(1):30-38.
[54] M. B. Lawrence and T. A. Springer. Leukocytes roll on a selectin at physiologic flow rates: Distinction from and prerequisite for adhesion through integrins. *Cell*. 1991;65(5):859-873.
[55] A. D. Blann, S. K. Nadar, and G. Y. H. Lip. The adhesion molecule P-selectin and cardiovascular disease. *Eur. Heart J.* 2003;24(24):2166-2179.
[56] M. P. Bevilacqua *et al.* Identification of an inducible endothelial-leukocyte adhesion molecule. *Proc. Natl. Acad. Sci.* 1987;84(24):9238-9242.
[57] B. Engelhardt. Adhesion and signalling molecules controlling the extravasation of leukocytes across the endothelium. *Transfus. Med. Hemotherapy*. 2008;35(2):73-75.
[58] Y. Tanaka, D. H. Adams, and S. Shaw. Proteoglycans on endothelial cells present adhesion-inducing cytokines to leukocytes. *Immunol. Today*. 1993;14(3):111-115.
[59] Y. Monneau, F. Arenzana-Seisdedos, and H. Lortat-Jacob. The sweet spot: how GAGs help chemokines guide migrating cells. *J. Leukoc. Biol.* 2016;99(6):935-953.
[60] M. Wolf and B. Moser. Antimicrobial activities of chemokines: Not just a side-effect?. *Front. Immunol.* 2012;23(3):1–12.
[61] R. Shamri *et al.* Lymphocyte arrest requires instantaneous induction of an extended LFA-1 conformation mediated by endothelium-bound chemokines. *Nat. Immunol.* 2005;6(5):497-506.
[62] R. M. Ransohoff. Chemokines and Chemokine Receptors: Standing at the Crossroads of Immunobiology and Neurobiology. *Immunity*. 2009;31(5):711-721.
[63] W. A. Muller. Getting Leukocytes to the Site of Inflammation. *Vet. Pathol.* 2013;50(1):7-22.
[64] O.Florey, J. Durgan, and W. Muller. Phosphorylation of leukocyte PECAM and its association with detergent-resistant membranes regulate transendothelial migration. *J Immunol*,. 2010;185(3):1878-1886.
[65] A. Woodfin *et al.* Endothelial cell activation leads to neutrophil transmigration as supported by the sequential roles of ICAM-2, JAM-A, and PECAM-1. *Blood*. 2009;113(24):6246-6257.
[66] G. Ostermann *et al.* JAM-1 is a ligand of the β2 integrin LFA-1 involved in transendothelial migration of leukocytes. *Nat. Immunol.* 2002;3(2):151-158.
[67] Z. Mamdouh, A. Mikhailov, and W. A. Muller. Transcellular migration of leukocytes is mediated by the endothelial lateral border recycling compartment. *J. Exp. Med.* 2009;206(12):2795-2808.
[68] J. Millán *et al.* Lymphocyte transcellular migration occurs through recruitment of endothelial ICAM-1 to caveola- and F-actin-rich domains. *Nat. Cell Biol.* 2006;8(2):113-123.
[69] M.D. Filippi. Mechanism of Diapedesis: Importance of the Transcellular Route. *Adv. Immunol.* 2016;129:25-53.
[70] T. M. Handel *et al.* Regulation of Protein Function by Glycosaminoglycans- as exemplified by chemokines. *Annu. Rev. Biochem.* 2005;74(1):385-410.
[71] E. F. Foxman, J. J. Campbell, and E. C. Butcher. Multistep navigation and the combinatorial control of leukocyte chemotaxis. *J. Cell Biol.* 1997;139(5):1349-1360.
[72] B. Y. T. Yoshimura *et al.* Purification and Amino Acid Analysis of two human glioma-derived monocyte chemoattractants. *J. Exp. Med.* 1998;169(4)1449-1459.
[73] D. R. Engel *et al.* CCR2 Mediates Homeostatic and Inflammatory Release of Gr1high Monocytes from the Bone Marrow, but Is Dispensable for Bladder Infiltration in Bacterial Urinary Tract Infection. *J. Immunol.* 2008;181(8):5579-5586.
[74] N. V. Serbina and E. G. Pamer. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat. Immunol.* 2006;7(3):311-317.

[75] H. H. Zhang *et al.* CCR2 Identifies a Stable Population of Human Effector Memory CD4+ T Cells Equipped for Rapid Recall Response. *J. Immunol.* 2010;185(11):6646-6663.
[76] S. J. Connor *et al.* CCR2 expressing CD4+ T lymphocytes are preferentially recruited to the ileum in Crohn's disease. *Gut*. 2004;53(9):1287-1294.
[77] C. H. Kim *et al.* Rules of chemokine receptor association with T cell polarization in vivo. *J. Clin. Invest.* 2001;108(9):1331-1339.
[78] R. Bonecchi *et al.* Differential Expression of Chemokine Receptors and Chemotactic Responsiveness of Type 1 T Helper Cells (Th1s) and Th2s. *J. Exp. Med*. 1998;187(1):129-134.
[79] M. Mack *et al.* Expression and Characterization of the Chemokine Receptors CCR2 and CCR5 in Mice. *J. Immunol.* 2001;166(7):4697-4704.
[80] S. J. Collington *et al.* The Role of the CCL2/CCR2 Axis in Mouse Mast Cell Migration In Vitro and In Vivo. *J. Immunol.* 2010;184(11):6114-6123.
[81] J. Talbot *et al.* CCR2 expression in neutrophils plays a critical role in their migration into the joints in rheumatoid arthritis. *Arthritis Rheumatol.* 2015;67(7):1751-1759.
[82] M. J. G. van Helden, D. M. W. Zaiss, and A. J. A. M. Sijts. CCR2 Defines a Distinct Population of NK Cells and Mediates Their Migration during Influenza Virus Infection in Mice. *PLoS One*. 2012;7(12):e52027
[83] L. Flaishon *et al.* Expression of the chemokine receptor CCR2 on immature B cells negatively regulates their cytoskeletal rearrangement and migration. *Blood*. 2014;104(3):933-941.
[84] S. C. Bischoff *et al.* Monocyte chemotactic protein 1 is a potent activator of human basophils. *J. Exp. Med.* 1992;175(5):1271-1275.
[85] K. S. C. Weber *et al.* Implications for MCP-1 Mediated Wound Injury Repair and In Vivo. *Arterioscler. Thromb. Vasc. Biol.* 1999;19(9):2085-2093.
[86] R. D. Loberg *et al.* CCL2 is a potent regulator of prostate cancer cell migration and proliferation. *Neoplasia*. 2006;8(7):578-586.
[87] Y. Lu *et al.* CCR2 expression correlates with prostate cancer progression. *J. Cell. Biochem.* 2007;101(3):676-685.
[88] L. Boring *et al.* Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *J. Clin. Invest.* 1997;100(10):1552-2561.
[89] W. Peters *et al.* Chemokine receptor 2 serves an early and essential role in resistance to Mycobacterium tuberculosis. *Proc.Natl.Acad.Sci.U.S.A*. 2001;98(14):7958-7963.
[90] J. Miotla Zarebska *et al.* CCL2 and CCR2 regulate pain-related behaviour and early gene expression in post-traumatic murine osteoarthritis but contribute little to chondropathy. *Osteoarthr. Cartil.* 2017;25(3):406-412.
[91] J. El Khoury *et al.* Ccr2 deficiency impairs microglial accumulation and accelerates progression of Alzheimer-like disease. *Nat. Med.* 2007;13(4):432-438
[92] B. T. Fife *et al.* CC chemokine receptor 2 is critical for induction of experimental autoimmune encephalomyelitis. *J. Exp. Med.* 2000;192(6):899-905.
[93] L. Boring *et al*. Decreased lesion formation in CCR2(-/-) mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature*. 1998;394(6696):894–897.
[94] P. G. Andres *et al.* Mice with a selective deletion of the CC chemokine receptors 5 or 2 are protected from dextran sodium sulfate-mediated colitis: lack of CC chemokine receptor 5 expression results in a NK1.1+ lymphocyte-associated Th2-type immune response in the intestine. *J. Immunol.* 2000;164(12):6303-6312.
[95] C. Abbadie *et al.* Impaired neuropathic pain responses in mice lacking the chemokine receptor CCR2. *Proc. Natl. Acad. Sci. U. S. A.* 2003;100(13):7947-7952.
[96] J. A. Belperio *et al.* Critical role for the chemokine MCP-1/CCR2 in the pathogenesis of bronchiolitis obliterans syndrome. *J. Clin. Invest.* 2001;108(4):547-556.
[97] M. Schlesinger *et al.* Inhibition of chemokine receptor CCR2 reduces sarcoma cell transendothelial migration and metastasis to the lungs. *Int. J. Clin. Pharmacol. Ther.* 2015;53(12):1046-1048.
[98] T. Kurihara *et al.* Defects in Macrophage Recruitment and Host Defense in Mice Lacking the CCR2 Chemokine Receptor. *J. Exp. Med.* 1997;186(10):1757-1762.

[99] J. K. Lim *et al.* Chemokine Receptor Ccr2 Is Critical for Monocyte Accumulation and Survival in West Nile Virus Encephalitis. *J. Immunol.* 2011;186(1):471-478.
[100] K. S. Warmington *et al.* Effect of C-C chemokine receptor 2 (CCR2) knockout on type-2 (schistosomal antigen-elicited) pulmonary granuloma formation: analysis of cellular recruitment and cytokine responses. *Am. J. Pathol.* 1999;154(5);1407-1416.
[101] W. Peters, M. Dupuis, and I. F. Charo. A Mechanism for the Impaired IFN-γ Production in C-C Chemokine Receptor 2 (CCR2) Knockout Mice: Role of CCR2 in Linking the Innate and Adaptive Immune Responses. *J. Immunol.* 2000;165(12):7071-7077.
[102] B. C. Chiu *et al.* Impaired lung dendritic cell activation in CCR2 knockout mice. *Am. J. Pathol.* 2004;165(4):1199-1209.
[103] M. D. Wareing *et al.* Chemokine regulation of the inflammatory response to a low-dose influenza infection in CCR2-/- mice. *J. Leukoc. Biol.* 2006;81(3):793-801.
[104] I. R. Dunay, A. Fuchs, and L. David Sibley. Inflammatory monocytes but not neutrophils are necessary to control infection with Toxoplasma gondii in mice. *Infect. Immun.* 2010;78(4):1564-1570.
[105] E. Belnoue *et al.* Chemokine receptor CCR2 is not essential for the development of experimental cerebral malaria. *Infect. Immun.* 2003;71(6):3648-3651
[106] U. A. Maus *et al.* Monocytes Are Potent Facilitators of Alveolar Neutrophil Emigration During Lung Inflammation: Role of the CCL2-CCR2 Axis. *J. Immunol.* 2003;170(6):3273-3278.
[107] C. T. Gilliland *et al.* The chemokine receptor CCR1 is constitutively active, which leads to G protein-independent, β-arrestin-mediated internalization. *J. Biol. Chem.* 2013;288(45):32194-32210.
[108] K. Furuichi *et al.* Chemokine Receptor CCR1 Regulates Inflammatory Cell Infiltration after Renal Ischemia-Reperfusion Injury. *J. Immunol.* 2008;181(12):8670-8676.
[109] V. Vielhauer *et al.* CCR1 blockade reduces interstitial inflammation and fibrosis in mice with glomerulosclerosis and nephrotic syndrome. *Kidney Int.* 2004;66(6):2264-2278.
[110] K. Yanaba *et al.* Role of C-C chemokine receptors 1 and 5 and CCL3/macrophage inflammatory protein-1α in the cutaneous Arthus reaction: Possible attenuation of their inhibitory effects by compensatory chemokine production. *Eur. J. Immunol.* 2004;34(12):3553-3561.
[111] D. J. Mahad *et al.* Expression of Chemokine Receptors CCR1 and CCR5 Reflects Differential Activation of Mononuclear Phagocytes in Pattern II and Pattern III Multiple Sclerosis Lesions. *J. Neuropathol. Exp. Neurol.* 2004;63(3):262-173.
[112] F. Wieser *et al.* Expression and regulation of CCR1 in peritoneal macrophages from women with and without endometriosis. *Fertil. Steril.* 2005;83(6):1878-1881.
[113] M. A. Schaller, L. E. Kallal, and N. W. Lukacs. A key role for CC chemokine receptor 1 in T-cell-mediated respiratory inflammation. *Am. J. Pathol.* 2008;172(2):386-394.
[114] M. Uguccioni *et al.* High expression of the chemokine receptor CCR3 in human blood basophils: Role in activation by eotaxin, MCP-4, and other chemokines. *J. Clin. Invest.* 1997;100(5):1137-1143.
[115] A. de Paulis *et al.* Expression of the Chemokine Receptor CCR3 on Human Mast Cells. *Int. Arch. Allergy Immunol.* 2001;124(1):146-150.
[116] T. Miyagaki *et al.* Eotaxins and CCR3 interaction regulates the Th2 environment of cutaneous T-Cell lymphoma. *J. Invest. Dermatol.* 2010;130(9):2304-2311.
[117] M. F. Gurish *et al.* CCR3 is required for tissue eosinophilia and larval cytotoxicity after infection with Trichinella spiralis. *J. Immunol.* 2002;168(11):5730-5736.
[118] W. Ma *et al.* CCR3 is essential for skin eosinopilia and airway hyperresponsiveness in a murine model of allergic skin inflammation. *J. Clin. Invest.*2002;109(5):621-628.
[119] A. A. Humbles *et al.* The murine CCR3 receptor regulates both the role of eosinophils and mast cells in allergen-induced airway inflammation and hyperresponsiveness. *Proc. Natl. Acad. Sci.* 2002;99(3):1479-1484.
[120] R. L. Contento *et al.* CXCR4-CCR5: A couple modulating T cell functions. *Proc. Natl. Acad. Sci.* 2008;105(29):10101-10106.
[121] F. Barmania and M. S. Pepper. C-C chemokine receptor type five (CCR5): An emerging

target for the control of HIV infection. *Appl. Transl. Genomics*. 2013;2(1):3-16.
[122] R. Abdi *et al.* Chemokine receptor polymorphism and risk of acute rejection in human renal transplantation. *J. Am. Soc. Nephrol.* 2002;13(3):754-758.
[123] P. Srivastava *et al.* Association of CCR5Δ32 with reduced risk of childhood but not adult asthma. *Thorax*. 2003;58:222–226.
[124] J.-E. Turner *et al.* CCR5 Deficiency Aggravates Crescentic Glomerulonephritis in Mice. *J. Immunol.* 2008;181(9):6546-6556.
[125] M. Petrek *et al.* CC chemokine receptor 5 and interleukin-1 receptor antagonist gene polymorphisms in patients with primary Sjogren's syndrome. *Clin. Exp. Rheumatol.* 2002;20(5):701-703.
[126] I. Zapico *et al.* CCR5 (chemokine receptor-5) DNA-polymorphism influences the severity of rheumatoid arthritis. *Genes Immun.* 2000;1(4):288-289.
[127] Z. E. Karaali *et al.* Effect of genetic variants of chemokine receptors on the development of myocardial infarction in Turkish population. *Mol. Biol. Rep.* 2010;37(7):3615-3619.
[128] A. D. Sullivan, J. Wigginton, and D. Kirschner. The coreceptor mutation CCR5Delta32 influences the dynamics of HIV epidemics and is selected for by HIV. *Proc. Natl. Acad. Sci. U. S. A.* 2001;98(18):10214-10219.
[129] M. Petrek *et al.* CC chemokine receptor gene polymorphisms in Czech patients with pulmonary sarcoidosis" *Am. J. Respir. Crit. Care Med*. 2000;162(3):1000-1003.
[130] R. Gade-Andavolu *et al.* Association of CCR5 Δ32 deletion with early death in multiple sclerosis. *Genet. Med.* 2004;6(3):126-131.
[131] M. K. Arababadi *et al.* No association between CCR5-Δ32 mutation and multiple sclerosis in patients of Southeastern Iran. *Lab. Med.* 2010;41(1):31-33.
[132] F. Atzeni *et al.* CC chemokine receptor 5 polymorphism in Italian patients with Beḥet's disease. *Rheumatol.* 2012;51(12):2141-2145.
[133] W. G. Glass *et al.* CCR5 deficiency increases risk of symptomatic West Nile virus infection. *J. Exp. Med.* 2006;203(1):35-40.
[134] D. A. Dorward *et al*. The role of formylated peptides and formyl peptide receptor 1 in governing neutrophil function during acute inflammation. *Am. J. Pathol.* 2015;185(5):1172-1184.
[135] M. Liu *et al.* Formylpeptide receptors are critical for rapid neutrophil mobilization in host defense against Listeria monocytogenes. *Sci. Rep.* 2012;2:786.
[136] C. Gemperle *et al.* Regulation of the Formyl Peptide Receptor 1 (FPR1) Gene in Primary Human Macrophages. *PLoS One*. 2012;7(11):e50195.
[137] F. Colazzo *et al.* Role of the Cysteinyl Leukotrienes in the Pathogenesis and Progression of Cardiovascular Diseases. *Mediators Inflamm.* 2017;2017:1-13.
[138] A. Stsiapanava, B. Samuelsson, and J. Z. Haeggström. Capturing hydrolase in action: Insights to the chemistry and dynamics of chemotactic synthesis. *Proc. Natl. Acad. Sci.* 2017;114(36):9689-9694.
[139] P. V. Afonso *et al.* LTB4 Is a Signal-Relay Molecule during Neutrophil Chemotaxis. *Dev. Cell*. 2012;22(5):1079-1091.
[140] E. Ricciotti and G. A. FitzGerald. Prostaglandins and inflammation. *Arterioscler. Thromb. Vasc. Biol.* 2011;31(5):986-1000.
[141] Y. S. Bakhle and R. M. Botting. Cyclooxygenase-2 and its regulation in inflammation. *Mediators Inflamm.* 1996;5(5):305-323.
[142] M. F. Yam *et al.* General Pathways of Pain Sensation and the Major Neurotransmitters Involved in Pain Regulation. *Int. J. Mol. Sci.* 2018;18(8):2164
[143] O. Dormond *et al.* Prostaglandin E2 promotes integrin αvβ3-dependent endothelial cell adhesion, Rac-activation, and spreading through cAMP/PKA-dependent signaling. *J. Biol. Chem.* 2002;277(48):45838-45846.
[144] K. D. Stone, C. Prussin, and D. D. Metcalfe. IgE, mast cells, basophils, and eosinophils. *J. Allergy Clin. Immunol.* 2010;125(2):73-80.
[145] C. M. Mikelis *et al.* RhoA and ROCK mediate histamine-induced vascular leakage and anaphylactic shock. *Nat. Commun.* 2015;6:6725.
[146] A. C. Branco *et al.* Role of Histamine in Modulating the Immune Response and

Inflammation. *Mediators Inflamm.* 2018;201:1-10
[147] P. Ling *et al.* Histamine H4 receptor mediates eosinophil chemotaxis with cell shape change and adhesion molecule upregulation. *Br. J. Pharmacol.* 2004;142(1):161-171.
[148] G. Caron *et al.* Histamine Induces CD86 Expression and Chemokine Production by Human Immature Dendritic Cells. *J. Immunol.* 2001;166(10):6000-6006.
[149] M. M. Markiewski and J. D. Lambris. The role of complement in inflammatory diseases from behind the scenes into the spotlight. *Am. J. Pathol.* 2007;171(3):715-727.
[150] M. Noris and G. Remuzzi. Overview of complement activation and regulation. *Semin. Nephrol.* 2013;33(6):479-492.
[151] C. Bayly-Jones, D. Bubeck, and M. A. Dunstone. The mystery behind membrane insertion: a review of the complement membrane attack complex. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*, 2017;372(1726):e20160221
[152] R. G. DiScipio *et al.* A Comparison of C3a and C5a-Mediated Stable Adhesion of Rolling Eosinophils in Postcapillary Venules and Transendothelial Migration In Vitro and In Vivo. *J. Immunol.* 1999;162(2):1127-1136.
[153] K. Hartmann *et al.* C3a and C5a stimulate chemotaxis of human mast cells. *Blood*. 1997;89(8):2863-2870.
[154] S. Huang *et al.* Genomic analysis of the immune gene repertoire of amphioxus reveals extraordinary innate complexity and diversity. *Genome Res.* 2008;18(7):1112-1126.
[155] K. J. Nordström, R. Fredriksson, and H. B. Schiöth. The amphioxus (Branchiostoma floridae) genome contains a highly diversified set of G protein-coupled receptors. *BMC Evol. Biol.* 2008;8(1):9
[156] M. E. DeVries *et al.* Defining the Origins and Evolution of the Chemokine/Chemokine Receptor System. *J. Immunol.* 2006;176(1):401-415.
[157] M. O. Huising *et al.* Three novel carp CXC chemokines are expressed early in ontogeny and at nonimmune sites. *Eur. J. Biochem.* 2004;271(20):4094-4106.
[158] B. Bajoghli. Evolution and function of chemokine receptors in the immune system of lower vertebrates. *Eur. J. Immunol.* 2013;43(7):1686-1692.
[159] B. Taddese *et al.* Evolution of chemokine receptors is driven by mutations in the sodium binding site. *PLOS Comput. Biol.* 2018;14(6): e1006209.
[160] H. Nomiyama, N. Osada, and O. Yoshie. Systematic classification of vertebrate chemokines based on conserved synteny and evolutionary history. *Genes to Cells*. 2013;18(1):1-16.
[161] T. Lapidot and I. Petit. Current understanding of stem cell mobilization: The roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. *Exp. Hematol.* 2002;30(9):973-981.
[162] M. Doitsidou *et al.* Guidance of primordial germ cell migration by the chemokine SDF-1. *Cell*. 2002;111(5):647-659.
[163] T. Ara *et al.* Impaired colonization of the gonads by primordial germ cells in mice lacking a chemokine, stromal cell-derived factor-1 (SDF-1). *Proc. Natl. Acad. Sci.* 2003;100(9):5319-5323.
[164] V. A. Robert and A. Casadevall. Vertebrate Endothermy Restricts Most Fungi as Potential Pathogens. *J. Infect. Dis.* 2009;200(10):1623-1626.
[165] A. Casadevall. Fungal virulence, vertebrate endothermy, and dinosaur extinction: Is there a connection? *Fungal Genet. Biol.* 2006;42(2):98-106.
[166] A. Alcami. Viral mimicry of cytokines, chemokines and their receptors. *Nat. Rev. Immunol.* 2003;3(1):36-50.
[167] S. K. Ahuja and P. M. Murphy. Molecular piracy of mammalian interleukin-8 receptor type B by herpesvirus saimiri. *J. Biol. Chem.* 1993;268(28):20691-20694.
[168] P. M. Murphy. Molecular piracy of chemokine receptors by herpesviruses. *Infect. Agents Dis.* 1994;3(2):137-154.
[169] V. González-Motos, K. A. Kropp, and A. Viejo-Borbolla. Chemokine binding proteins: An immunomodulatory strategy going viral. *Cytokine Growth Factor Rev.* 2016;30:71-80
[170] I. Damon, P. M. Murphy, and B. Moss. Broad spectrum chemokine antagonistic activity of a human poxvirus chemokine homolog. *Proc. Natl. Acad. Sci. U. S. A.* 1998;95(11):6403-6407.
[171] Q. Jin *et al.* Role for the conserved N-terminal cysteines in the anti-chemokine activities by

the chemokine-like protein MC148R1 encoded by Molluscum contagiosum virus. *Virology*, 2011;417(2):449-456.
[172] A. Alcamí *et al.* Blockade of Chemokine Activity by a Soluble Chemokine Binding Protein from Vaccinia Virus. *J. Immunol.* 1998;160(2):624-633.
[173] D. R. Dewin, J. Catusse, and U. A. Gompels. Identification and Characterization of U83A Viral Chemokine, a Broad and Potent -Chemokine Agonist for Human CCRs with Unique Selectivity and Inhibition by Spliced Isoform. *J. Immunol.* 2014;176(1):544-556.
[174] H. R. Lu *et al.* Kaposi Sarcoma-associated Herpes Virus Targets the Lymphotactin Receptor with Both a Broad Spectrum Antagonist vCCL2 and a Highly Selective and Potent Agonist vCCL3. *J. Biol. Chem*. 2007;282(24):17794-17805.
[175] K. Nakano *et al.* Kaposi's sarcoma-associated herpesvirus (KSHV)-encoded vMIP-I and vMIP-II induce signal transduction and chemotaxis in monocytic cells. *Arch. Virol.* 2003;148(5):871-890.
[176] C. J. C. de Haas *et al.* Chemotaxis inhibitory protein of Staphylococcus aureus, a bacterial antiinflammatory agent. *J. Exp. Med.* 2004;199(5):687-695.
[177] P. Kurupati *et al.* Chemokine-cleaving Streptococcus pyogenes protease SpyCEP is necessary and sufficient for bacterial dissemination within soft tissues and the respiratory tract. *Mol. Microbiol.* 2010;76(6):1387-1397.
[178] J. Bestebroer *et al.* Staphylococcal SSL5 inhibits leukocyte activation by chemokines and anaphylatoxins. *Blood*. 2009;113(2):328-337.
[179] S. Mangmool and H. Kurose. Gi/o protein-dependent and -independent actions of pertussis toxin (ptx). *Toxins.* 2011;3(7):884-899.
[180] C. Andreasen and N. H. Carbonetti. Pertussis toxin inhibits early chemokine production to delay neutrophil recruitment in response to Bordetella pertussis respiratory tract infection in mice. *Infect. Immun.* 2008;76(11):5139-5148.
[181] P. Smith *et al.* Schistosoma mansoni secretes a chemokine binding protein with antiinflammatory activity. *J. Exp. Med.* 2005;202(10):1319-1325.
[182] F. J. Culley *et al.* Eotaxin Is Specifically Cleaved by Hookworm Metalloproteases Preventing Its Action In Vitro and In Vivo. *J. Immunol.* 2000;165(11):6447-6453.
[183] J. R. O. Eaton *et al.* The N-terminal domain of a tick evasin is critical for chemokine binding and neutralization and confers specific binding activity to other evasins. *J. Biol. Chem.* 2018;293(16):6134-6146.
[184] R. Horuk. Chemokine receptor antagonists: Overcoming developmental hurdles. *Nat. Rev. Drug Discov.* 2009;8(1):23-33.
[185] Raport CJ *et al.* Molecular cloning and functional characterization of a novel human CC chemokine receptor (CCR5) for RANTES, MIP-1beta, and MIP-1alpha. *J. Biol. Chem.* 1996;271(29):17161-17166.
[186] F. J. Pixley. Macrophage migration and its regulation by CSF-1. *Int. J. Cell Biol.* 2012;2012: e501962.
[187] J. Yang *et al.* Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. *Biomark. Res.* 2014;2(1):1
[188] A. Kaufmann *et al.* Increase of CCR1 and CCR5 expression and enhanced functional response to MIP-1alpha during differentiation of human monocytes to macrophages. *J. Leukoc. Biol.* 2001;69(2):248-252.
[189] P. Italiani and D. Boraschi. From monocytes to M1/M2 macrophages: Phenotypical vs. functional differentiation. *Front. Immunol.* 2014;17(5):1-22.
[190] N. D. Kim and A. D. Luster. The role of tissue resident cells in neutrophil recruitment. *Trends Immunol.* 2015;36(9):547-555.
[191] E. Hoffmann *et al*. Multiple control of interleukin-8 gene expression. *J. Leukoc. Biol.* 2002;72(5):847-855.
[192] M. W. Nasser *et al.* Differential Activation and Regulation of CXCR1 and CXCR2 by CXCL8 Monomer and Dimer. *J. Immunol.* 2009;183(5):3425-3432.
[193] G. E. White, A. J. Iqbal, and D. R. Greaves. CC Chemokine Receptors and Chronic Inflammation—Therapeutic Opportunities and Pharmacological Challenges. *Pharmacol. Rev.* 2013;65(1):47-89.

[195] R. Ross. Atherosclerosis- An Inflammatory Disease. *N. Engl. J. Med.* 1999;340(2):115-126.
[196] M. A. Gimbrone Jr and G. García-Cardeña. Endothelial Cell Dysfunction and the Pathobiology of Atherosclerosis. *Circ. Res.* 2016;118(4):620-636.
[197] M. Mudau *et al.* Endothelial dysfunction: the early predictor of atherosclerosis. *Cardiovasc. J. Afr.* 2012;23(4):222-231.
[198] J. Gaboury *et al.* Nitric oxide prevents leukocyte adherence: role of superoxide. *Am. J. Physiol. Circ. Physiol.*,n 1993;265(3):862-867.
[199] I. Tabas and A. H. Lichtman. Monocyte-Macrophages and T Cells in Atherosclerosis. *Immunity*. 2017;47(4):621-634.
[200] K. J. Moore and I. Tabas. Macrophages in the pathogenesis of atherosclerosis. *Cell*. 2011;145(3):341-355.
[201] A. Tedgui and Z. Mallat. Cytokines in Atherosclerosis: Pathogenic and Regulatory Pathways. *Physiol. Rev.*, 2006;86(2):515-581.
[202] Zhou X. and Hansson GK. Detection of B Cells and Proinflammatory Cytokines in Atherosclerotic Plaques of Hypercholesterolaemic Apolipoprotein E Knockout Mice. *Scand. J. Immunol.* 1999;50(1):25-30.
[203] W. Insull. The Pathology of Atherosclerosis: Plaque Development and Plaque Responses to Medical Treatment. *Am. J. Med.* 2009;122(1):3-14.
[204] B. J. Fog *et al.* Mechanisms of Plaque Formation and Rupture. *Circ. Res.* 2014;114(12):1852-1866.
[205] F. Tacke *et al.* Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. *J. Clin. Invest.* 2007;117(1):185-194.
[206] T. C. Dawson *et al.* Absence of CC chemokine receptor-2 reduces atherosclerosis in apolipoprotein E-deficient mice. *Atherosclerosis*. 1999;143(1):205-211.
[207] P. Stéphane *et al.* Role of Bone Marrow–Derived CC-Chemokine Receptor 5 in the Development of Atherosclerosis of Low-Density Lipoprotein Receptor Knockout Mice. *Arterioscler. Thromb. Vasc. Biol.* 2006;26(8):1858-1863.
[208] R. J. Aiello *et al.* Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 1999;19(6):1518-1525
[209] K. J. Katschke *et al.* Differential expression of chemokine receptors on peripheral blood, synovial fluid, and synovial tissue monocytes/macrophages in rheumatoid arthritis. *Arthritis Rheum.* 2001;44(5):1022-1032.
[210] E. Choy. Understanding the dynamics: pathways involved in the pathogenesis of rheumatoid arthritis. *Rheumatology*. 2012;51(5):3-11.
[211] S. Kerlan-Candon *et al.* HLA-DRB1 gene transcripts in rheumatoid arthritis. *Clin. Exp. Immunol.* 2011;124(1):142-149.
[212] J. S. Smolen *et al.* New therapies for treatment of rheumatoid arthritis. *Lancet*. 2007;370(9602):1861-1874.
[213] G. A. Bishop and B. S. Hostager. B lymphocyte activation by contact-mediated interactions with T lymphocytes. *Curr. Opin. Immunol.* 2001;13(3):178-285.
[214] X. Li and R. P. Kimberly. Targeting the Fc receptor in autoimmune disease. *Expert Opin. Ther. Targets*,. 2014;18(3):335-350.
[215] G. Murphy *et al.* Matrix metalloproteinases in arthritic disease. *Arthritis Res.* 2002;4(3):39-49.
[216] R. M. Pope *et al.* IL-17-Mediated Monocyte Migration Occurs Partially through CC Chemokine Ligand 2/Monocyte Chemoattractant Protein-1 Induction. *J. Immunol.* 2010;184(8): 4479-4487.
[217] D. D. Patel, J. P. Zachariah, and L. P. Whichard. CXCR3 and CCR5 Ligands in Rheumatoid Arthritis Synovium. *Clin. Immunol.* 2001;98(1):39-45.
[218] M. C. Kraan *et al.* The development of clinical signs of rheumatoid synovial inflammation is associated with increased synthesis of the chemokine CXCL8 (interleukin-8). *Arthritis Res. Ther.* 2000;3(1):65.
[219] S. Prahalad. Negative association between the chemokine receptor CCR5-Delta32 polymorphism and rheumatoid arthritis: a meta-analysis. *Genes Immun.* 2006;7(3):264-268.

[220] D. Galimberti *et al.* Serum MCP-1 levels are increased in mild cognitive impairment and mild Alzheimer's disease. *Neurobiol. Aging* 2006;27(12):1763-1768.
[221] A. Arakelyan *et al.* Serum levels of the MCP-1 chemokine in patients with ischemic stroke and myocardial infarction. *Mediators Inflamm.* 2005;2005(3):175-179.
[222] M. Mellado *et al.* Chemokine receptor 2 blockade prevents asthma in a cynomolgus monkey model. *J. Pharmacol. Exp. Ther.* 2008;324(2):769–775.
[223] K. Kaikita *et al.* Targeted Deletion of CC Chemokine Receptor 2 Attenuates Left Ventricular Remodeling after Experimental Myocardial Infarction. *Am. J. Pathol.* 2004;165(2):439-447.
[224] D. J. Mahad and R. M. Ransohoff. The role of MCP-1 (CCL2) and CCR2 in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE). *Semin. Immunol.* 2003;15(1):23-32.
[225] C. T. Chan *et al.* Reversal of vascular macrophage accumulation and hypertension by a CCR2 antagonist in deoxycorticosterone/salt-treated mice. *Hypertension*. 2012;60(5):1207-1212.
[226] J. H. Gong, R. Yan, and J. D. Waterfield .Post-onset inhibition of murine arthritis using combined chemokine antagonist therapy. *Rheumatology*. 2004;43(1):39-42.
[227] C. Plater-Zyberk *et al.* Effect of a CC chemokine receptor antagonist on collagen induced arthritis in DBA/1 mice. *Immunol. Lett.* 1997;57(1):117-120.
[228] C. M. Brodmerkel *et al.* Discovery and Pharmacological Characterization of a Novel Rodent-Active CCR2 Antagonist, INCB3344. *J. Immunol.* 2005;175(8):5370-5378.
[229] H. Brühl *et al.* Dual role of CCR2 during initiation and progression of collagen-induced arthritis: evidence for regulatory activity of CCR2+ T cells. *J. Immunol.* 2004;172(2):890-898.
[230] Y. Cho and E. Lolis. When anti-CCR2 treatment for arthritis strikes out. *Arthritis Rheum.* 2011;63(1):23-25.
[231] C. E. Vergunst *et al.* Modulation of CCR2 in rheumatoid arthritis: A double-blind, randomized, placebo-controlled clinical trial. *Arthritis Rheum.* 2008;58(7):1931-1939.
[232] M. Xia and Z. Sui. Recent developments in CCR2 antagonists. *Expert Opin. Ther. Pat.* 2009;19(3):295-303.
[233] H. Lassmann, W. Brück, and C. Lucchinetti. Heterogeneity of multiple sclerosis pathogenesis: Implications for diagnosis and therapy. *Trends Mol. Med.* 2001;7(3):115-121.
[234] J. A. Hamilton and P. P. Tak. The dynamics of macrophage lineage populations in inflammatory and autoimmune diseases. *Arthritis Rheum.* 2009;60(5):1210-1221.
[235] D. M. Gerlag *et al.* Preclinical and clinical investigation of a CCR5 antagonist, AZD5672, in patients with rheumatoid arthritis receiving methotrexate. *Arthritis Rheum.* 2010;62(11):3154-3160.
[236] P. P. Tak *et al.* Chemokine receptor CCR1 antagonist CCX354-C treatment for rheumatoid arthritis: CARAT-2, a randomised, placebo controlled clinical trial. *Ann. Rheum. Dis.*. 2013;72(3):337-344.
[237] J. J. Haringman. Chemokine blockade and chronic inflammatory disease: proof of concept in patients with rheumatoid arthritis. *Ann. Rheum. Dis.* 2003;62(8):715-721.
[238] C. E. Vergunst, "MLN3897 plus methotrexate in patients with rheumatoid arthritis: Safety, efficacy, pharmacokinetics, and pharmacodynamics of an oral CCR1 antagonist in a phase IIa, double-blind, placebo-controlled, randomized, proof-of-concept study. *Arthritis Rheum.* 2009;60(12):3572-3581.
[239] R. M. Thurlings *et al.* Monocyte scintigraphy in rheumatoid arthritis: The dynamics of monocyte migration in immune-mediated inflammatory disease. *PLoS One*. 2009;4(11):1-6.
[240] R. P. Gladue, M. F. Brown, and S. H. Zwillich. CCR1 Antagonists: What Have We Learned From Clinical Trials. *Curr. Top. Med. Chem.* 2010;10(13):1268-1277.
[241] D. J. Dairaghi *et al.* Pharmacokinetic and pharmacodynamic evaluation of the novel CCR1 antagonist CCX354 in healthy human subjects: Implications for selection of clinical dose. *Clin. Pharmacol. Ther.* 2011;89(5):726-734.
[242] J. Hesselgesser *et al.* Identification and characterization of small molecule functional antagonists of the CCR1 chemokine receptor. *J. Biol. Chem.* 1998;273(25):15687-15692.
[243] M. Giorelli, P. Livrea, and M. Trojano. Dopamine fails to regulate activation of peripheral

blood lymphocytes from multiple sclerosis patients: effects of IFN-beta. *J. Interferon Cytokine Res.* 2005;25(7):395-406.
[244] F. Nimura *et al.* Cross-Linking Cell Surface Chemokine Receptors Leads to Isolation, Activation, and Differentiation of Monocytes into Potent Dendritic Cells. *Exp. Biol. Med.* 2006;231(4):431-443.
[245] B. Berman and C. Caperton. Safety, efficacy, and patient acceptability of imiquimod for topical treatment of actinic keratoses. *Clin. Cosmet. Investig. Dermatol.* 2011;4:35-40.
[246] M. Romano *et al.* Carrageenan-induced acute inflammation in the mouse air pouch synovial model. Role of tumour necrosis factor. *Mediators Inflamm.* 1997;6(1):32-38.
[247] A. Alloatt *et al.* Evaluation of Cross-presentation in Bone Marrow-derived Dendritic Cells in vitro and Splenic Dendritic Cells ex vivo Using Antigen-coated Beads. *Bio. Protoc.* 2016;6(22):1-13.
[248] S. Rm. K. Clarke *et al.* Characterization of the ovalbumin-specific TCR transgenic line OT-I: MHC elements for positive and negative selection. *Immunol. Cell Biol.* 2000;78(2):110-117.
[249] M. J. Barnden *et al.* Defective TCR expression in transgenic mice constructed using cDNA-based α- and β-chain genes under the control of heterologous regulatory elements. *Immunol. Cell Biol.* 1998;76(1):34-40.
[250] K. Fukada *et al.* Functional Expression of the Chemokine Receptor CCR5 on Virus Epitope-Specific Memory and Effector CD8+ T Cells. *J. Immunol.* 2002;168(5):2225-2232.
[251] K. V. Sawant *et al.* Chemokine CXCL1 mediated neutrophil recruitment: Role of glycosaminoglycan interactions. *Sci. Rep.* 2016;6:4-11.
[252] D. Hartl *et al.* Infiltrated Neutrophils Acquire Novel Chemokine Receptor Expression and Chemokine Responsiveness in Chronic Inflammatory Lung Diseases. *J. Immunol.* 2008;181(11):8053-8067.
[253] S. S. Cheng *et al.* Granulocyte-Macrophage Colony Stimulating Factor Up-Regulates CCR1 in Human Neutrophils *J. Immunol.* 2001;166(2):1178-1184.
[254] B. Johnston *et al.* Chronic inflammation upregulates chemokine receptors and induces neutrophil migration to monocyte chemoattractant protein-1. *J. Clin. Invest.* 1999;103(9):1269-1276.
[255] B. Lamkhioued *et al.* The CCR3 Receptor Is Involved in Eosinophil Differentiation and Is Up-Regulated by Th2 Cytokines in CD34+ Progenitor Cells. *J. Immunol.* 2003;170(1):537-547.
[256] P. C. Fulkerson *et al.* A central regulatory role for eosinophils and the eotaxin/CCR3 axis in chronic experimental allergic airway inflammation. *Proc. Natl. Acad. Sci. U. S. A.* 2006;103(44):16418-16423.
[257] C. Shi and E. G. Pamer. Monocyte Recruitment Suring Infection and Inflammation. *Nat Rev Immunol*. 2014;11(11):762-774.
[258] M. Mellado *et al.*, "Chemokine receptor homo- or heterodimerization activates distinct signaling pathways," *EMBO J.* 2011;20(10):2497-2507.
[259] N. V Serbina *et al*. Monocyte-Mediated Defense Against Microbial Pathogens. *Annu. Rev. Immunol.* 2008;26(1):421-452.
[260] N. V. Serbina *et al.* TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. *Immunity*. 2003;19(1):59-70.
[261] J. A. Stefater *et al.* Regulation of angiogenesis by non-canonical Wnt-Flt1 pathway in myeloid cells. *Nature*. 2011;474(7352):511-515.
[262] D. Castellana, R. Paus, and M. Perez-Moreno. Macrophages Contribute to the Cyclic Activation of Adult Hair Follicle Stem Cells. *PLoS Biol.* 2014;12(12):e1002002.
[263] A. S. Care *et al.* Macrophages regulate corpus luteum development during embryo implantation in mice. *J. Clin. Invest.* 2013;123(8):3472-3487.
[264] L. Boulter, O. Govaere, and T. Bird. Macrophage derived Wnt signalling opposes Notch signalling in a Numb mediated manner to specify HPC fate in chronic liver disease in human and mouse. *Nat. Med.* 2012;18(4):572-579.
[265] T. R. L. Klei *et al.* From the cradle to the grave: The role of macrophages in erythropoiesis and erythrophagocytosis. *Front. Immunol.* 2017;8(2):73.
[266] C. Gordy *et al.* Regulation of steady-state neutrophil homeostasis by macrophages. *Blood*. 2011;117(2):618-629.

[267] J. A. Stefater *et al.* Regulation of angiogenesis by a non-canonical Wnt - Flt1 pathway in myeloid cells. *Nature*. 2011;474(7352):511-516.
[268] Y. Li *et al.* Reciprocal Regulation between Resting Microglial Dynamics and Neuronal Activity In Vivo. *Dev. Cell*. 2012;23(6):1189-1202.
[269] R. C. Paolicelli *et al.* Synaptic pruning by microglia is necessary for normal brain development. *Science*. 2011;333(6049):1456-1458.
[270] J. W. Pollard. Trophic macrophages in development and disease. *Nat. Rev. Immunol.* 2009;9(4):159-270.
[271] D. P. Dyer *et al.* Chemokine Receptor Redundancy and Specificity Are Context Dependent. *Immunity*. 2019;50(2):378-389.
[272] R. Möhle *et al.* The chemokine receptor CXCR-4 is expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. *Blood*. 1998;91(12):4523-4530.
[273] T. Sugiyama *et al.* Maintenance of the Hematopoietic Stem Cell Pool by CXCL12-CXCR4 Chemokine Signaling in Bone Marrow Stromal Cell Niches. *Immunity*. 2006;25(6):977-988.
[274] D. E. Wright *et al.* Hematopoietic Stem Cells Are Uniquely Selective in Their Migratory Response to Chemokines. *J. Exp. Med. 2002;195(9):1145-1154*.
[275] T. Kitamura *et al.* Inactivation of chemokine (C-C motif) receptor 1 (CCR1) suppresses colon cancer liver metastasis by blocking accumulation of immature myeloid cells in a mouse model. *Proc. Natl. Acad. Sci.* 2010;107(29):13063-13068.
[276] Y. Si *et al.* CCR2 mediates hematopoietic stem and progenitor cell trafficking to sites of inflammation in mice. *J. Clin. Invest.* 2010;120(4):1192-1203.
[277] L. B. Ford *et al.* Characterization of conventional and atypical receptors for the chemokine CCL2 on mouse leukocytes. *J. Immunol.* 2014;193(1):400-411.
[278] T. Jia *et al.* Additive Roles for MCP-1 and MCP-3 in CCR2-Mediated Recruitment of Inflammatory Monocytes during Listeria monocytogenes Infection. *J. Immunol.* 2008;180(10):6846-6853.
[279] F. Licata *et al.* CX3CR1 reduces Ly6C high -monocyte motility within and release from the bone marrow after chemotherapy in mice. *Blood*. 2013;122(5):674-684.
[280] C. L. Tsou *et al.* Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *J. Clin. Invest*. 2007;117(4):902-909.
[281] B. J. Gao *et al.* Impaired Host Defence, hematopoiesis, granulomatous inflammation and type-1 and type-2 cytokine balance in mice lacking CC Chemokine Receptor 1. *J. Exp. Med.* 1997;185(11):1959-1968.
[282] D. Kurotaki, T. Uede, and T. Tamura. Functions and development of red pulp macrophages. *Microbiol. Immunol.* 2015;59(2):55-62.
[283] C. Stremmel *et al.* Yolk sac macrophage progenitors traffic to the embryo during defined stages of development. *Nat. Commun.* 2018;9(1):75.
[284] F. Ginhoux *et al.* Fate mapping analysis reveala that adult microglia derive from primitive macrophages. *Science*. 2010;330(6005):841-845.
[285] S. Yona *et al.* Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity*. 2013;38(1):79-91.
[286] D. Hashimoto *et al.* Adult Life With Minimal Contribution From Circulating Monocytes. *Immunity*. 2013;38(4):792-804.
[287] U. A. Maus *et al.* Resident alveolar macrophages are replaced by recruited monocytes in response to endotoxin-induced lung inflammation. *Am. J. Respir. Cell Mol. Biol.* 2006;35(2):227-235.
[288] T. Röszer. Understanding the Biology of Self-Renewing Macrophages. *Cells*. 2018;7(8):e103
[289] M. Guilliams *et al.* Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF. *J. Exp. Med.* 2013;210(10):1977-1992.
[290] Y. Wang *et al.* The Toll-Like Receptor 7 (TLR7) Agonist, Imiquimod, and the TLR9 Agonist, CpG ODN, Induce Antiviral Cytokines and Chemokines but Do Not Prevent Vaginal Transmission of Simian Immunodeficiency Virus When Applied Intravaginally to Rhesus Macaques. *J. Virol.* 2005;79(22):14355-14370.

[291] V. Oldfield, G. M. Keating, and C. M. Perry. Imiquimod: In superficial basal cell carcinoma. *Am. J. Clin. Dermatol.* 2005;6(3):195-200.
[292] M. Solomon, B. Balasa, and N. Sarvetnick. CCR2 and CCR5 chemokine receptors differentially influence the development of autoimmune diabetes in the NOD mouse. *Autoimmunity*. 2010;43(2):156-163.
[293] J. Schumann *et al*. Differences in CD44 Surface Expression Levels and Function Discriminates IL-17 and IFN-γ Producing Helper T Cells. *PLoS One*. 2015;10(7):e0132479
[294] F. Sallusto *et al.* Switch in chemokine receptor expression upon TCR stimulation reveals novel homing potential for recently activated T cells. *Eur. J. Immunol.* 1999;29(6):2037-2045.
[295] R. D. Berahovich *et al.* Evidence for NK Cell Subsets Based on Chemokine Receptor Expression. *J. Immunol.* 2006;177(11):7833-7840.
[296] F. Castellino *et al.* Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. *Nature*. 2006;440(7086):890-895.
[297] F. Geissmann *et al.* Development of monocytes, macrophages and dendritic cells. *Science*. 2010;327(5966):656-661.
[298] C. Weber *et al.* Specialized roles of the chemokine receptors CCR1 and CCR5 in the recruitment of monocytes and T H 1-like / CD45RO + T cells Specialized roles of the chemokine receptors CCR1 and CCR5 in the recruitment of monocytes and T H 1-like / CD45RO ƨ T cells. *Blood*. 2011;97(4):1144-1146.
[299] A. Walter *et al.* Aldara activates TLR7-independent immune defence. *Nat. Commun.* 2013;4:1-13.
[300] L. Grine *et al.* Topical imiquimod yields systemic effects due to unintended oral uptake. *Sci. Rep.* 2016;6:20134
[301] X. Hu *et al*. IFN -Primed Macrophages Exhibit Increased CCR2-Dependent Migration and Altered IFN- Responses Mediated by Stat1. *J. Immunol.* 2005;175(6):3637-3647.
[302] X. Hu, S. D. Chakravarty, and L. B. Ivashkiv. Regulation of interferon and Toll-like receptor signaling during macrophage activation by opposing feedforward and feedback inhibition mechanisms. *Immunol. Rev.* 2008;226:41-56.
[303] A. Dallagi *et al.* The activating effect of IFN-γ on monocytes/macrophages is regulated by the LIF-trophoblast-IL-10 axis via Stat1 inhibition and Stat3 activation. *Cell. Mol. Immunol.* 2015;12(3):326-341.
[304] Y. M. Sin and S. L. Chio. Effect of carrageenan-induced acute inflammation on corticosterone levels in mice. *Agents Actions*. 1990;29(3):196-200.
[305] N. M. A. Hassimotto *et al.* Inhibition of carrageenan-induced acute inflammation in mice by oral administration of anthocyanin mixture from wild mulberry and cyanidin-3-glucoside. *Biomed Res. Int.* 2013;2013:e146716.
[306] K. Matsumoto *et al.* Anti-inflammatory effects of linezolid on carrageenan-induced paw edema in rats. *J. Infect. Chemother.* 2015;21(12):889-891.
[307] I. A. Care and C. River. Models of Inflammation : Adjuvant-Induced. *Pharmacology*. 2001;317(5):1-5.
[308] L. C. Loram *et al.* Cytokine Profiles During Carrageenan-Induced Inflammatory Hyperalgesia in Rat Muscle and Hind Paw. *J. Pain*. 2007;8(2):127-136.
[309] E. Ghadrdan et al. Inhibitory effects of oxytocin on the inflammatory parameters and vascular endothelial growth factor (VEGF) in the rat air pouch model of inflammation. *Physiol. Pharmacol*. 2016;20(1):48-56.
[310] I. Kim *et al.* Vascular Endothelial Growth Factor Expression of Intercellular Adhesion Molecule 1 (ICAM-1), Vascular Cell Adhesion Molecule 1 (VCAM-1), and E-selectin through Nuclear Factor-κB Activation in Endothelial Cells. 2001;276(10):7614-7620.
[311] M. C. Jamur *et al.* Identification and characterization of undifferentiated mast cells in mouse bone marrow. *Blood*. 2005;105(11):4282-4289.
[312] S. Menezes *et al.* The Heterogeneity of Ly6C(hi) Monocytes Controls Their Differentiation into iNOS(+) Macrophages or Monocyte-Derived Dendritic Cells. *Immunity*. 2016;45(6):1205-1218.
[313] C. W. Winkler *et al.* Cutting Edge: CCR2 Is Not Required for Ly6C ^{hi} Monocyte Egress from

the Bone Marrow but Is Necessary for Migration within the Brain in La Crosse Virus Encephalitis. *J. Immunol 2018;200(2):471-476.*
[314] L. K. Weaver, N. Chu, and E. M. Behrens. TLR9-mediated inflammation drives a Ccr2-independent peripheral monocytosis through enhanced extramedullary monocytopoiesis. *Proc. Natl. Acad. Sci.* 2016;113(39):10944-10949.
[315] M. Aswad *et al.* CCL5 Promotes Resolution-Phase Macrophage Reprogramming in Concert with the Atypical Chemokine Receptor D6 and Apoptotic Polymorphonuclear Cells. *J. Immunol.* 2017;199(4):1393-1404.
[316] M. Rådinger *et al.* Local proliferation and mobilization of CCR3+ CD34+ eosinophil-lineage-committed cells in the lung. *Immunology*. 2011;132(1):144-154.
[317] D. Voehringer, N. van Rooijen, and R. M. Locksley. Eosinophils develop in distinct stages and are recruited to peripheral sites by alternatively activated macrophages. *J. Leukoc. Biol.* 2007;81(6):1434-1444.
[318] H. J. Anders, V. Ninichuk, and D. Schlondorff. Progression of kidney disease: Blocking leukocyte recruitment with chemokine receptor CCR1 antagonists. *Kidney Int.* 2006;69(1):29-32.
[319] J. Yuan *et al.* In Vitro Immunological Effects of Blocking CCR5 on T Cells. *Inflammation*. 2015;38(2):902-910.
[320] T. Ota. CCR5: A novel player in the adipose tissue inflammation and insulin resistance? *Adipocyte*. 2013;2(2):99-103.
[321] Y. Zhou *et al.* Impaired macrophage function and enhanced T cell-dependent immune response in mice lacking CCR5, the mouse homologue of the major HIV-1 coreceptor. *J. Immunol.* 1998;160(8):4018-4025.
[322] D. Y. Choi *et al.* CCR5 knockout mice with C57BL6 background are resistant to acetaminophen-mediated hepatotoxicity due to decreased macrophages migration into the liver. *Arch. Toxicol.* 2015;89(2):211-250.
[323] B. Tomkowicz *et al.* The Src kinase Lyn is required for CCR5 signaling in response to MIP-1beta and R5 HIV-1 gp120 in human macrophages. *Blood*. 2006;108(4):1145-1150.
[324] H. M. S. Algood and J. L. Flynn. CCR5-deficient mice control Mycobacterium tuberculosis infection despite increased pulmonary lymphocytic infiltration. *J. Immunol.* 2004;173(5):3287-3296.
[325] A. T. Y. Yeung *et al.* Conditional-ready mouse embryonic stem cell derived macrophages enable the study of essential genes in macrophage function. *Sci. Rep.* 2015;5:1-11
[326] L. Zhuang *et al.* Pure populations of murine macrophages from cultured embryonic stem cells. Application to studies of chemotaxis and apoptotic cell clearance. *J. Immunol. Methods*. 2012;385(1):1-14.
[327] X. Wang and P. Yang. In vitro Differentiation of Mouse Embryonic Stem (mES) Cells Using the Hanging Drop Method. *J. Vis. Exp.* 2008;17:2-3.
[328] R. J. Phillips, M. Lutz, and B. Premack. Differential signaling mechanisms regulate expression of CC chemokine receptor-2 during monocyte maturation. *J. Inflamm.* 2005;2:14
[329] U. A. Maus *et al.* CCR2-positive monocytes recruited to inflamed lungs downregulate local CCL2 chemokine levels. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 2005;288(2):350-358.
[330] K. H. Han *et al.* Oxidized LDL reduces monocyte CCR2 expression through pathways involving peroxisome proliferator–activated receptor γ. *J. Clin. Invest.* 2000;106(6):793-802.
[331] A. Saccani *et al.* Redox regulation of chemokine receptor expression. *Proc. Natl. Acad. Sci. U. S. A.* 2000;97(6):2761-2766.
[332] G. Lehoux *et al.* Upregulation of expression of the chemokine receptor CCR5 by hydrogen peroxide in human monocytes. *Mediators Inflamm.* 2003;12(1):29-35.
[333] A. Sica *et al.* Bacterial lipopolysaccharide rapidly inhibits expression of C-C chemokine receptors in human monocytes. *J. Exp. Med.* 1997;185(5):969-974
[334] M. Heesen *et al.* Human endotoxemia induces down-regulation of monocyte CC chemokine receptor 2. *Clin. Vaccine Immunol.* 2006;13(1):156-159.
[335] B. R. Lane *et al.* TNF-α Inhibits HIV-1 Replication in Peripheral Blood Monocytes and

Alveolar Macrophages by Inducing the Production of RANTES and Decreasing C-C Chemokine Receptor 5 (CCR5) Expression. *J. Immunol.* 1999;163(7):3653-3661.
[336] S. Pasquereau, A. Kumar, and G. Herbein. Targeting TNF and TNF Receptor Pathway in HIV-1 Infection: from Immune Activation to Viral Reservoirs. *Viruses*. 2017;9(4):e64.
[337] B. L. Fantuzzi *et al.* Loss of CCR2 Expression and Functional Response to Monocyte Chemotactic Protein (MCP-1) During the Differentiation of Human Monocytes: Role of Secreted MCP-1 in the Regulation of the Chemotactic Response. *Blood. 1999;94(3):875-884*.
[338] J. W. Tyner *et al.* CCL5-CCR5 interaction provides antiapoptotic signals for macrophage survival during viral infection. *Nat. Med.* 2005;11(11):1180-1187.
[339] G. Jenster *et al.* Steroid receptor induction of gene transcription: A two-step model. *Proc. Natl. Acad. Sci.* 1997;94(15):7879-7884.
[340] H. Nagase *et al.* Regulation of Chemokine Receptor Expression in Eosinophils. *Int. Arch. Allergy Immunol.* 2001;125:29-32
[341] S. Su *et al.* Preparation of specific polyclonal antibodies to a C-C chemokine receptor, CCR1, and determination of CCR1 expression on various types of leukocytes. *J. Leukoc. Biol.* 1996;60(5):658-666.
[342] J. P. Pereira, L. M. Kelly, and J. G. Cyster. Finding the right niche: B-cell migration in the early phases of T-dependent antibody responses. *Int. Immunol.* 2010;22(6):413-419.
[343] I. Kholodnyuk *et al.* Expression of the chemokine receptors CCR1 and CCR2B is up-regulated in peripheral blood B cells upon EBV infection and in established lymphoblastoid cell lines. *Virology*. 2017;512:1-7.
[344] A. J. Lessard *et al.* Triggering of NOD2 Receptor Converts Inflammatory Ly6Chigh into Ly6Clow Monocytes with Patrolling Properties. *Cell Rep.* 2017;20(8):1830-1843.
[345] M. Guilliams *et al.* Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF. *J. Exp. Med.* 2013;210(10):1977-1992.
[346] M. Liebick, C. Schläger, and M. Oppermann. Analysis of chemokine receptor trafficking by site-specific biotinylation. *PLoS One*. 2016;11(6):1-16.
[347] J. Elsner *et al.* Differential activation of CC chemokine receptors by AOP-RANTES. *J. Biol. Chem.* 2000;175(11):7787-7794.
[348] S. Eltayeb *et al.* Temporal expression and cellular origin of CC chemokine receptors CCR1, CCR2 and CCR5 in the central nervous system: insight into mechanisms of MOG-induced EAE. *J. Neuroinflammation*. 2007;4(1):14.
[349] M. Thapa, W. a Kuziel, and D. J. J. Carr. Susceptibility of CCR5-deficient mice to genital herpes simplex virus type 2 is linked to NK cell mobilization. *J. Virol.* 2007;81(8):3704-37013.
[350] W. G. Glass and T. E. Lane. Functional Expression of Chemokine Receptor CCR5 on CD4+ T Cells during Virus-Induced Central Nervous System Disease. *J. Virol.* 2003;77(1):191-198.
[351] J. Surre *et al.* Strong increase in the autofluorescence of cells signals struggle for survival. *Sci. Rep.* 2018;8(1):1-14.
[352] T. Baltus *et al.* Oligomerization of RANTES is required for CCR1-mediated arrest but not CCR5-mediated transmigration of leukocytes on inflamed endothelium. 2003;102(6):1985-1988.
[353] J. Kitayama *et al.* The C-C chemokine receptor CCR3 participates in stimulation of eosinophil arrest on inflammatory endothelium in shear flow. *J. Clin. Invest.* 1998;101(9):2017-2024.
[354] T. Okada *et al.* Chemokine Requirements for B Cell Entry to Lymph Nodes and Peyer's Patches. *J. Exp. Med.* 2002;196(1):65-75.
[355] S. Epelman *et al.* Embryonic and adult-derived resident cardiac macrophages are maintained through distinct mechanisms at steady state and during inflammation. *Immunity*. 2014;40(1):91-104.
[356] S. Sprangers, T. J. D. Vries, and V. Everts. Monocyte Heterogeneity: Consequences for Monocyte-Derived Immune Cells. *J. Immunol. Res.* 2016;2016: e1475435
[357] A. Rivollier *et al.* Inflammation switches the differentiation program of Ly6C ^{hi} monocytes

from antiinflammatory macrophages to inflammatory dendritic cells in the colon. *J. Exp. Med.* 2012;209(1):139-155.
[358] G. J. Randolph, K. Inaba, D. F. Robbiani. Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo. *Immunity*. 1999:11(6):753-76.
[359] S. Manzanero, B. Ashman. Generation of Mouse Bone Marrow-Derived Macrophages. *Leukocytes* 2012;2012:177–181.
[360] D. C. Lacey *et al.* Defining GM-CSF- and Macrophage-CSF-Dependent Macrophage Responses by In Vitro Models. *J. Immunol.* 2012;188(11):5752-5765.
[361] I. Ushach and A. Zlotnik. Biological role of granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) on cells of the myeloid lineage. *J. Leukoc. Biol.* 2016;100(3):481-489.
[362] E. R. Stanley *et al.* Biology and action of colony-stimulating factor-1. *Mol. Reprod. Dev.* 1997;46(1):4-10.
[363] W. Wiktor-Jedrzejczak *et al.* Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. *Proc. Natl. Acad. Sci.* 1990;87(12):4828-4832.
[364] C. V. Jones and S. D. Ricardo. Macrophages and CSF-1: Implications for development and beyond. *Organogenesis*. 2013;9(4):249-260.
[365] E. Stanley *et al.* Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. *Proc. Natl. Acad. Sci.* 1994;91(12):5592-5596.
[366] A. M. LeVine *et al.* GM-CSF-deficient mice are susceptible to pulmonary group B streptococcal infection. *J. Clin. Invest.* 1999;103(4):563-569.
[367] J. Lee *et al.* The MHC class II antigen presentation pathway in human monocytes differs by subset and is regulated by cytokines. *PLoS One*. 2017;12(8):e0183594.
[368] M. Jaguin *et al.* Polarization profiles of human M-CSF-generated macrophages and comparison of M1-markers in classically activated macrophages from GM-CSF and M-CSF origin. *Cell. Immunol.* 2013;281(1):51-61.
[369] M. A. Schmid *et al.* Instructive cytokine signals in dendritic cell lineage commitment. *Immunol. Rev.* 2010;234(1):32-44.
[370] Y. Lavin *et al.* Regulation of macrophage development and function in peripheral tissues. *Nat. Rev. Immunol.* 2015;15(12):731-744.
[371] M. B. Lutz *et al.* An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods*. 1999;223(1):77-92.
[372] J. Helft *et al.* GM-CSF Mouse Bone Marrow Cultures Comprise a Heterogeneous Population of $CD11c^{+}MHCII^{+}$ Macrophages and Dendritic Cells. *Immunity*. 2015;42(6):1197-1211.
[373] Y. Xu *et al.* Differential Development of Murine Dendritic Cells by GM-CSF versus Flt3 Ligand Has Implications for Inflammation and Trafficking. *J. Immunol.* 2007;179(11):7577-7584.
[374] G. B. Li and G. X. Lu. Adherent cells in granulocyte-macrophage colony-stimulating factor-induced bone marrow-derived dendritic cell culture system are qualified dendritic cells. *Cell. Immunol.* 2010;264(1):4-6.
[375] V. Purvanov *et al.* Fluorescently Tagged CCL19 and CCL21 to Monitor CCR7 and ACKR4 Functions. *Int. J. Mol. Sci.* 2018;19(12):3876.
[376] E. Scandella *et al.* CCL19 / CCL21-triggered signal transduction and migration of dendritic cells requires prostaglandin E2. *Signal Transduct.* 2010;103(5):1595-1601.
[377] E. Langhoff and R. M. Steinman. Clonal expansion of human T lymphocytes initiated by dendritic cells. *J. Exp. Med.* 1989;169(1):315-320.
[378] F. Sallusto and A. Lanzavecchia. The instructive role of dendritic cells on T-cell responses. *Arthritis Res.* 2002;4(3): 127-32.
[379] A. Cerutti, X. Qiao, and B. He. Plasmacytoid dendritic cells and the regulation of immunoglobulin heavy chain class switching. *Immunol. Cell Biol.* 2005;83(5):554-562.
[380] M. Wykes and G. Macpherson. Dendritic cell-B-cell interaction: Dendritic cells provide B cells with CD40-independent proliferation signals and CD40-dependent survival signals. *Immunology*. 2000;100(1):1-3.

[381] L. Meyaard, H. Schuitemaker, and F. Miedema. T-cell dysfunction in HIV infection: anergy due to defective antigen-presenting cell function? *Immunol. Today*. 1993;14(4):161-164.
[382] A. Agrawal, S. Agrawal, and S. Gupta. Role of dendritic cells in inflammation and loss of tolerance in the elderly. *Front. Immunol.* 2017;8:1-8
[383] N. D. Pennock *et al.* T cell responses: naïve to memory and everything in between. *Adv. Physiol. Educ.* 2013;37(4):273-283.
[384] S. Goral. The three-signal hypothesis of lymphocyte activation/targets for immunosuppression. *Dial. Transplant.* 2011;40(1):14-16.
[385] A. Jain and C. Pasare. Innate Control of Adaptive Immunity: Beyond the Three-Signal Paradigm. *J. Immunol.* 2017;198(10):3791-3800.
[386] J. M. Curtsinger *et al.* Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. *J. Immunol.* 1999;162(6):3256-3262.
[387] J. S. Boomer and J. M. Green. An enigmatic tail of CD28 signaling. *Cold Spring Harb. Perspect. Biol.* 2010;2(8):1-20.
[388] S. Bromley, W. Burack, and K. Johnson. The immunological synapse. *Annu. Rev. Immunol.* 2011;19(11):375-396.
[389] S. K. Bromley *et al.* The immunological synapse and CD28-CD80 interactions. *Nat. Immunol.* 2001;2(12):1159-1166.
[390] J. Valenzuela, C. Schmidt, and M. Mescher. The Roles of IL-12 in Providing a Third Signal for Clonal Expansion of Naive CD8 T Cells. *J. Immunol.* 2002;169(12):6842-6849.
[391] C. S. Wagner, J. Grotzke, and P. Cresswell. Intracellular Regulation of Cross-Presentation during Dendritic Cell Maturation. *PLoS One*. 2013;8(10):1-13.
[392] K. Inaba *et al.* Generation of Large Numbers of Dendritic Cells from Mouse Bone Marrow Cultures Supplemented with Granulocyte/Macrophage Colony-stimulating Factor. *J. Exp. Med.* 1992;176(6):1693-1702.
[393] Y. R. Na *et al.* GM-CSF Grown Bone Marrow Derived Cells Are Composed of Phenotypically Different Dendritic Cells and Macrophages. *Mol. Cells*. 2016;39(10):734-741.
[394] M. Guilliams and B. Malissen. A Death Notice for In-Vitro-Generated GM-CSF Dendritic Cells? *Immunity*. 2015;42(6):988-990.
[395] M. Guilliams *et al.* Dendritic cells, monocytes and macrophages: A unified nomenclature based on ontogeny. *Nat. Rev. Immunol.* 2014;14(8):571-578.
[396] K. Shortman and S. H. Naik.Steady-state and inflammatory dendritic-cell development. *Nat. Rev. Immunol.* 2007;7(1):19-30.
[397] M. Schmid, A. K. Wege, and U. Ritter. Characteristics of 'Tip-DCs and MDSCs' and their potential role in leishmaniasis. *Front. Microbiol.* 2012;3:1-8.
[398] P. Parajuli *et al.* Flt3 ligand and granulocyte-macrophage colony-stimulating factor preferentially expand and stimulate different dendritic and T-cell subsets. *Exp. Hematol.* 2001;29(10):1185-1193.
[399] K. Brasel *et al.* Generation of murine dendritic cells from flt3-ligand – supplemented bone marrow cultures. *Blood*. 2014;96(9):3029-3039.
[400] Y. L. Chen *et al.* Efficient generation of plasmacytoid dendritic cell from common lymphoid progenitors by Flt3 ligand. *PLoS One*. 2015;10(8):1-18.
[401] B. J. Masten *et al.* Flt3 ligand preferentially increases the number of functionally active myeloid dendritic cells in the lungs of mice. *J. Immunol.* 2004;172(30):4077-4083.
[402] A. D'Amico and L. Wu. The Early Progenitors of Mouse Dendritic Cells and Plasmacytoid Predendritic Cells Are within the Bone Marrow Hemopoietic Precursors Expressing Flt3. *J. Exp. Med.* 2003;198(2):293-303.
[403] S. Volpe *et al.* CCR2 acts as scavenger for CCL2 during monocyte chemotaxis. *PLoS One*. 2012;7(5):1-10.
[404] M. Pons *et al.* Phosphorylation of filamin A regulates chemokine receptor CCR2 recycling. *J. Cell Sci.* 2017;130(2):490-501.
[405] M. Barczyk, S. Carracedo, and D. Gullberg. Integrins. *Cell Tissue Res.* 2010;339(1):269-280.
[406] T. A. Springer. Traffic signals for lymphocyte recirculation and leukocyte emigration: The multistep paradigm. *Cell*. 1994;76(2):301-314.
[407] L. Schittenhelm, C. M. Hilkens, and V. L. Morrison. B2Integrins As Regulators of Dendritic

Cell, Monocyte, and Macrophage Function. *Front. Immunol.* 2017;8:1866.
[408] B. Shen, M. K. Delaney, and X. Du. Inside-out, outside-in, and inside-outside-in: G protein signaling in integrin-mediated cell adhesion, spreading, and retraction. *Curr. Opin. Cell Biol.* 2012;24(5):600-606.
[409] R. Shamri *et al.* Lymphocyte arrest requires instantaneous induction of an extended LFA-1 conformation mediated by endothelium-bound chemokines. *Nat. Immunol.* 2005;6(5):497-506.
[410] A. Ala, A. P. Dhillon, and H. J. Hodgson. Role of cell adhesion molecules in leukocyte recruitment in the liver and gut. *Int. J. Exp. Pathol.* 2003;84(1):1-16.
[411] T. K. Kishimoto *et al.* "Heterogeneous mutations in the β subunit common to the LFA-1, Mac-1, and p150,95 glycoproteins cause leukocyte adhesion deficiency. *Cell*. 1987;50(2):193-202.
[412] B. L. Walling and M. Kim. LFA-1 in T cell migration and differentiation. *Front. Immunol.* 2018;9:e952.
[413] D. F. Barber, M. Faure, and E. O. Long. LFA-1 Contributes an Early Signal for NK Cell Cytotoxicity. *J. Immunol.* 2004;173(6):3653-3659.
[414] A. Scholer *et al.* Intercellular Adhesion Molecule-1-Dependent Stable Interactions between T Cells and Dendritic Cells Determine CD8+ T Cell Memory. *Immunity*. 2008;28(2):258-270.
[415] H. I. McFarland *et al.* CD11b (Mac-1): a marker for CD8+ cytotoxic T cell activation and memory in virus infection. *J. Immunol.* 1992;149(4):1326-1333.
[416] M. Phillipson *et al.* Intraluminal crawling of neutrophils to emigration sites: a molecularly distinct process from adhesion in the recruitment cascade. *J. Exp. Med.* 2006;203(12):2569-2575.
[417] C. Shi *et al.* Downregulation of the forkhead transcription factor Foxp1 is required for monocyte differentiation and macrophage function Downregulation of the Forkhead Transcription Factor Foxp1 is Required for Monocyte Differentiation and Macrophage Function Director. *Blood*. 2008;112(12):4699-4711.
[418] Y. Wang *et al.* LFA-1 affinity regulation is necessary for the activation and proliferation of naive T cells. *J. Biol. Chem.* 2009;284(19):12645-12653.
[419] M. Skold and S. M. Behar. Tuberculosis Triggers a Tissue-Dependent Program of Differentiation and Acquisition of Effector Functions by Circulating Monocytes. *J. Immunol.* 2008;181(9):5349-6360.
[420] E. Guirado and L. S. Schlesinger. Modeling the Mycobacterium tuberculosis granuloma - the critical battlefield in host immunity and disease. *Front. Immunol.* 2013;4:e98.
[421] M. Gonzalez-juarrero *et al.* Temporal and Spatial Arrangement of Lymphocytes within Lung Granulomas Induced by Aerosol Infection with *Mycobacterium tuberculosis* Temporal and Spatial Arrangement of Lymphocytes within Lung Granulomas Induced by Aerosol Infection with Mycobacteri. *Infect. Immun.* 2001;69(3):1722-1728.
[422] R. Milde *et al.* Multinucleated Giant Cells Are Specialized for Complement-Mediated Phagocytosis and Large Target Destruction. *Cell Rep.* 2015;13(9):1937-1948.
[423] A. Aghbali *et al.* IL-4 induces the formation of multinucleated giant cells and expression of β5 integrin in central giant cell lesion. *Med. Oral Patol. Oral Cir. Bucal*. 2017;22(1):1-6.
[424] A. K. McNally and J. M. Anderson. B1 and B2 Integrins Mediate Adhesion During Macrophage Fusion and Multinucleated Foreign Body Giant Cell Formation. *Am. J. Pathol.* 2002;160(2):621-630.