CC-chemokine receptors CCR1, CCR2, CCR3, and CCR5 in inflammation

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Abstract

The CC-chemokine receptors CCR1, CCR2, CCR3, and CCR5 (iCCRs) play a critical role in orchestrating the recruitment of leukocytes to inflamed areas. However, excessive leukocyte accumulation during inflammation can cause tissue damage and various inflammatory disorders, making iCCRs a promising target for therapeutic interventions. Unfortunately, despite extensive research, no iCCR antagonists have been approved for inflammatory and autoimmune diseases due to the apparent complexity of the chemokine system, where individual leukocytes can express multiple chemokine receptors simultaneously and the promiscuity of receptor-ligand interactions. In addition, the need for proper mouse models has hindered our understanding of how iCCRs coordinate the inflammatory response and the development of effective treatments.

The expression of iCCRs in response to specific inflammatory conditions was investigated using BMDMs *in vitro*. The results indicate that CCR1 and CCR5 are primarily regulated in response to various cytokines and TLR ligands, with CCR1 potentially being further enhanced in response to bacterial infections and CCR5 upregulated in response to viral infections. However, CCR2 and CCR3 did not fluctuate in response to the stimulating agents and remained stable.

Then, using iCCR-reporter mice (iREPs), the temporal changes of iCCR expression in monocytes and differentiated macrophages during sustained inflammation *in vivo* were assessed. The results show that inflammatory monocytes mainly express reporter CCR2, but a small fraction also co-expresses reporter CCR1 and CCR2, regardless of inflammatory state. We also identified a small subset of inflammatory monocytes that only express reporter CCR1 under sustained inflammation. The transcriptomic analysis of these monocyte subsets

shows that they have distinct transcriptional profiles, indicating that multiple iCCR expression does not represent redundant expression.

The findings presented in this thesis have added to our understanding of the role of iCCRs in inflammation by providing information on the regulation of iCCR expression in macrophages and the temporal regulation of iCCRs in monocytes and macrophages during inflammation, and the differential transcriptomes of cells expressing iCCRs. Additional insights into how CC-chemokines orchestrate inflammatory responses can be gained from future studies using the iREP mouse model. These insights will not only improve our understanding of basic chemokine biology but also have the potential to guide the development of pharmacological interventions targeting the chemokine system.

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Author's Declaration

I declare that the work presented in this thesis is entirely original and was produced as a result of my own efforts, except where explicit reference is made to the contributions of others. Also, I confirm that none of the data presented in this thesis has been submitted for any other degree, either at the University of Glasgow or any other institution.

Signature
Printed name: Heba Adnan Halawa

Abbreviations

A

ACK	Ammonium Chloride Potassium
ACKR	Atypical Chemokine Receptor
AHR	Airway Hyperresponsiveness
AIDS	Acquired Immune Deficiency Syndrome
AIM2	Absent in Melanoma-2
ALR	Absent in Melanoma-2-like Receptor
APC	Antigen Presenting Cell

B

BAC	Bacterial Artificial Chromosome
BCR	B Cell Antigen Receptor
BM	Bone Marrow

C

C5a	Complement Component 5a
CCL	CC Chemokine Ligand
CCR	CC Chemokine Receptor
CD	Cluster Differentiation
CLA	Cutaneous Lymphocyte-Associated Antigen
CLP	Common Lymphoid Progenitor
CLR	C-type Lectin Receptor
CM	Conditioned Media
CMP	Common Myeloid Progenitor
COPD	Chronic Obstructive Pulmonary Disease
CTCL	Cutaneous T-cell Lymphoma
CXCL	CXC Chemokine Ligand
CXCR	CXC Chemokine Receptor

D

DAMP	Damage-Associated Molecular Pattern
DARC	Duffy Antigen Receptor for Chemokines
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid

E

EAE	Experimental Allergic Encephalomyelitis
ECP	Eosinophil Cationic Protein
EDN	Eosinophil-Derived Neurotoxin
EDTA	Ethylenediaminetetraacetic Acid
EPO	Eosinophil Peroxidase

F

FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FMO	Fluorescence Minus One
FSC	Forward Scatter

G

GDP	Guanosine Diphosphate
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GMEM	Glasgow's Eagle's Minimal Essential Medium
GO	Gene Ontology
GPCR	G-Protein Coupled Receptor
GTP	Guanosine Triphosphate

H

HBSS	Hank's Balanced Salt Solution
HCC	Hepatocellular Carcinoma
HEK-293	Human Embryonic Kidney-293 Cell
HIV	Human Immunodeficiency Virus
HMW	High Molecular Weight
HSC	Hematopoietic Stem Cell

I

iCCR	Inflammatory CC Chemokine Receptor
IFN	Interferons
IL	Interleukin
iREP	Inflammatory CC Chemokine Fluorescent Reporter Mouse
IRF3	Interferon Regulatory Factor 3

L

LMW	Low Molecular Weight
LN	Lymph Nodes
LPS	Lipopolysaccharide
LPS	Lipopolysaccharide

M

M-CSF	Macrophage Colony-Stimulating Factor
MAC	Membrane Attack Complex
MAC	Membrane Attack Complex
MAPK	Mitogen-Activated Protein Kinase
MBP	Major Basic Protein
MDSC	Myeloid-Derived Suppressor Cell
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex

MS	Multiple Sclerosis

N

NEAA	Non-Essential Amino Acids
NET	Neutrophil Extracellular Trap
NET	Neutrophil Extracellular Trap
NF-κB	Nuclear Factor-kappa B
NK	Natural Killer Cell
NLR	Nucleotide Oligomerisation Domain-like receptor
NOD	Nucleotide Oligomerisation Domain
NOS	Reactive Nitrogen Species

O

ON	Overnight
OVA	Ovalbumin

P

PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate-Buffered Saline
PCA	principal component analysis
PCR	Polymerase Chain Reaction
PGE2	Prostaglandin E2
PMN	Polymorphonuclear
PMN	Polymorphonuclear
PRR	Pattern Recognition Receptor

R

RA	Rheumatoid Arthritis
RBC	Red Blood Cell
RGS	Regulators of G-protein Signalling

RIG-I	Retinoic Acid-inducible Gene-I
RLR	Retinoic Acid-inducible Gene-I-like receptor
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute Medium-
RSV	Respiratory Syncytial Virus
RT	Room Temperature

S

SLO	Secondary Lymphoid Organ
SSC	Side Scatter
STD	Standard

T

TGF	Transforming Growth Factor
TH	T Helper Cell
TIR	Toll/Interleukin-1 Receptor
TLR	Toll-like Receptor
TME	Tumour Microenvironment
TNF	Tumour Necrosis Factor

U

UV	Ultraviolet

V

VEGF	Vascular Endothelial Growth Factor

W

WNV	West Nile Virus
WT	Wild-Type

Chapter 1 Introduction

1.1 The immune system: innate and adaptive

The immune system involves a network of organs, cells, and secreted molecules that all work together to promote effective host defence against pathogens and damage. It is divided into two major arms: innate and adaptive immune responses[1-3]. The innate immune system provides immediate and general (non-specific) defence, while the adaptive immune system takes longer to develop and delivers a more specific and directed response[1-3]. All blood cells involved in both innate and adaptive immune functions are derived from hematopoietic stem cells (HSCs) in the bone marrow, where most develop and mature. HSCs can divide to generate two main types of progenitors: a common lymphoid progenitor (CLP) and common myeloid progenitor (CMP), in which CLP gives rise to the lymphoid lineage of leukocytes whilst CMP differentiates into myeloid cells[3, 4].

Inflammation is a normal immune response and occurs when leukocytes migrate from the circulation to the site of infection or injury. It eventually leads to four cardinal signs: redness (rubor), heat (calor), pain (dolor), and swelling (tumor), together with loss of tissue function (laesa functio)[5]. However, an inappropriate inflammatory response can be detrimental, resulting in excessive infiltration of leukocytes and the subsequent development of chronic inflammation. Therefore, it is very important for the immune system to maintain a balanced response[6, 7]. It must also be capable of distinguishing our body's own tissues "self " from "non-self " antigens to avoid misguided and overreacted inflammatory responses, which will lead to autoimmune diseases[6, 7].

1.1.1 Innate immune system

The innate immune system consists of basic components that act before the onset of infection. The skin and mucosal membranes, for example, are the first physical barriers to impede the entry of invading pathogens[8-10]. It also includes physiological barriers such as temperature, acidic pH, and chemical mediators, which control the spread and replication of microorganisms. If these barriers are overcome to establish an infection, the innate immune system responds with two defensive components: the humoral and cellular components[8-10].

Innate immune cells express pattern recognition receptors (PRRs), which allow them to initiate an immediate inflammatory response[11, 12]. These receptors can recognise a wide range of conserved microbial structures, known as pathogen-associated molecular patterns (PAMPs) or indirectly sense "danger" signals released from injured tissue or cell lysis in the form of damage-associated molecular patterns (DAMPs)[11, 12]. PRRs can be divided into different families: toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide oligomerisation domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and absent in melanoma-2 (AIM2)-like receptors (ALRs)[11, 12].

TLRs are a major family of PRRs. They were initially found to have an important role in the fruit flies' embryonic development and their fungal response[13, 14]. TLRs are transmembrane proteins with a cytoplasmic homology domain similar to interleukin 1 (IL-1) receptor, known as the Toll/IL-1 receptor (TIR) domain. TIR domain is required for signal transductions and can form a homodimer or heterodimer with other TIR domains of the TLR. Currently, there are 10 TLRs defined in humans and 12 in mice[15, 16]. They are either expressed

on the surface of cells as TLR1, TLR2, TLR4, TLR5 and TLR6 or intracellularly in endosomes like TLR3, TLR7, TLR8, and TLR9. TLRs can recognise several forms of PAMPs[15, 16]. For example, bacterial lipoteichoic acid and di- and triacylated lipopeptides are detected via TLR2/TLR1 or TLR2/TLR6 heterodimeric complexes[17-19]. TLR3 is activated by double-stranded RNA[20], whereas TLR4 is by lipopolysaccharide (LPS) of gram-negative bacteria[21]. TLR5 recognises bacterial flagellin[22]. TLR7 and TLR8 are both crucial for detection of single-stranded RNA[23], while TLR9 is for unmethylated CpG DNA[24].

Once activated, TLRs mediate innate immune response via the activation of several downstream signalling pathways, including nuclear factor kappa-B (NF-κB) and mitogen-activated protein kinase (MAPK)[15, 16]. These lead to the release of inflammatory cytokines and chemokines to control inflammation[15, 16]. Cytokines are small secreted proteins with multifunction in which they serve as activators of immune cells or chemoattractants to regulate their migration into infected sites[9]. They include interferons (IFNs), interleukins (ILs), tumour necrosis factors (TNFs), and transforming growth factors (TGFs) and can be classified as being either pro-inflammatory or anti-inflammatory cytokines[9].

IFNs are mainly associated with anti-viral defence and divide into three classes: type I IFNs such as IFNα and IFNβ, type II IFNs (IFNγ) and type III (IFNλ)[1]. IFNλ has also been implicated in anti-fungal immune responses[25]. ILs can regulate many aspects of cellular responses, such as growth, proliferation, and differentiation. IL-6, for example, activates neutrophils and prolongs their survival[26]. IL1β and IL1α also induce neutrophil recruitment and lymphocyte activation[27]. Further, IL-6 with TGFβ induces the differentiation of T helper 17 (Th17) cells[28]. IL-12 amplifies the immune responses by enhancing the production of IFNγ and $CD4^+$T cells differentiation into Th1 cells[29]. IL-10 has an

anti-inflammatory function that can inhibit macrophage proliferation and their secretion of IL-1β, TNF, and IL-6 after LPS stimulation[30]. However, IL-10 shows a pro-inflammatory effect during human endotoxemia as it stimulates the production of INFγ[31].

Furthermore, the CLRs family of PRRs is involved in recognising carbohydrate structures of bacteria, viruses, and fungi[32, 33]. NLRs are intracellular cytosolic proteins that are important for sensing the presence of DAMPs[15, 33]. Some NLRs are associated with the formation of a multiprotein complex called the inflammasome. It activates the caspase-1 pathway, which induces the secretion of IL-1β and IL-18 pro-inflammatory cytokines and cell death[34, 35]. RLRs mainly recognise RNA viruses and limit their replication through the induction of type I IFNs[36]. ALRs also form the inflammasome and control the infection with bacteria and DNA viruses[37].

1.1.1.1 Humoral component of innate immunity

Humoral immune defence can be mainly mediated by complement and involves other factors such as natural antibodies and lysosomes[9, 38]. Complement is a system that involves over 30 proteins found in plasma and on cell surfaces[39]. It can be activated by three pathways, all of which result in enhancing microbial killing and inflammation. The classical pathway starts when the complement component C1 binds to the antigen-antibody complex or pathogen surface, whereas the lectin pathway is initiated by recognising mannose on microbial surfaces[39, 40]. The activity of these two pathways spontaneously activates the alternative pathway[39, 40].

Activation of the complement cascade from all three pathways leads to the generation of a C3 convertase, which can cleave C3 into two fragment subunits:

C3a and C3b. C3a subunit is an anaphylatoxin with chemoattractant effects that induce leukocyte recruitment to the infected site and promote inflammation[39, 40]. C3b subunit coats or opsonises the pathogen surface to facilitate its phagocytosis and destruction. C3b can also join the C3 convertase to form another complex, termed a C5 convertase[39, 40]. It, in turn, cleaves C5 into C5a and C5b. C5a is another strong anaphylatoxin, attracting neutrophils, eosinophils and macrophages[41, 42]. Interaction of either C3a or C5a with mast cells triggers their degranulation[42, 43]. However, C5b interacts with other complement proteins to form a membrane attack complex (MAC), leading to membrane disruption and direct cell lysis[39, 40].

1.1.1.2 Cellular component of innate immunity

1.1.1.2.1 Neutrophils

Neutrophils are polymorphonuclear leukocytes (PMNs) with multilobed nuclei[44, 45]. These cells are the most abundant and first innate immune cells to leave the circulation and migrate towards the infected tissues[44, 45]. Neutrophils mediate the clearance of invading microorganisms by several mechanisms, including phagocytosis, degranulation, generation of reactive oxygen and nitrogen species (ROS/NOS), and formation of neutrophil extracellular traps (NETs)[44-46]. Three types of cytoplasmic granules are formed during different stages of neutrophil maturation, including primary (azurophilic), secondary (specific), and tertiary (gelatinase) granules[47]. These granules contain antimicrobial peptides and proteolytic enzymes such as myeloperoxidase, elastase, defensins, cathelicidin, and matrix metalloproteinase, which are secreted into the phagosome and aid in destroying the engulfed pathogens[47]. Also, several of these granules can induce the activation and migration of monocytes to the site

of infection[48]. NETs are a collection of chromosomal DNA, histones, and granules that provide an efficient antimicrobial defence[44, 45]. Neutrophils have been shown to induce the activation of long-lived macrophages that mediate a rapid parasitic clearance in the lung[49]. A subset of neutrophils is further found to shape the adaptive immune response, where they act as myeloid-derived suppressor cells (MDSCs) and dampen T cell proliferation and cytokine production[50]. However, splenic neutrophils can promote the development of B cells and antibody production[51]. Neutrophils are tightly controlled and cleared away once they complete their job by macrophages and dendritic cells via phagocytosis. IL-23 and IL-17, which are released by phagocytes and T cells, respectively, can both control neutrophil production through granulocyte-macrophage colony-stimulating factor (GM-CSF)[52].

1.1.1.2.2 Monocytes

Monocytes are recruited to the peripheral tissues shortly after neutrophils and persist longer at the site of inflammation[7]. The development of circulating monocytes is mainly controlled by a growth factor known as the macrophage colony-stimulating factor (M-CSF). Mice deficient either in M-CSF or its receptor display a reduction in the number of blood monocytes[53, 54]. Monocytes express immunoglobulin Fc and complement receptors, allowing them to phagocytose the opsonised pathogens[7]. Once inside tissues, monocytes can differentiate into macrophages or dendritic cells according to the environmental signals[55, 56]. Monocytes are heterogenous cells divided into two main subsets. In humans, they are defined based on the expression of CD14 and CD16 surface markers, in which classical or inflammatory monocytes are $CD14^{high}CD16^{-}$, whereas non-classical or patrolling monocytes are $CD14^{low}CD16^{high}$[57-59]. Similar subsets in mice are characterised as $ly6c^{high}CCR2^{+}$ and $ly6c^{low}CX3CR1^{high}$[57-59]. A third subset of

monocytes, termed intermediate monocytes (Ly6C^{int} in mice and CD14^{int}CD16^{int} in humans), is thought to be a transitional state during development between the other two[60, 61]. The classical monocytes secrete pro-inflammatory cytokines, including TNFα and IL1ß, and produce ROS[62]. However, non-classical monocytes are considered anti-inflammatory, where they resolve inflammation and promote wound healing[63].

1.1.1.2.3 Macrophages

Macrophages are highly phagocytic cells expressing FC and scavenger receptors, which facilitate the clearance of cellular debris, tumour cells, and other toxic substances from the circulation[64, 65]. They also possess a wide range of PRRs as TLRs and respond to several pathogenic stimuli[64, 65]. Once stimulated, tissue-resident macrophages produce pro-inflammatory cytokines such as TNFα, IL-1, and IL-6 to induce neutrophil recruitment and amplify the immune response[66]. These cells are initially thought to derive only from blood monocytes that have infiltrated the tissues. However, it became clear that tissue-resident macrophages can develop early during embryogenesis before HSCs, firstly from yolk sac-derived progenitors and later from fetal liver monocytes[67-69]. Tissue-resident macrophages differ in their names and phenotypes and gain special functions based on their anatomical location. For example, bone resident osteoclasts are involved in bone resorption, brain microglia regulate the development and maintenance of neural networks, and liver Kupffer cells break down red blood cells[70].

Macrophages can be broadly classified based on their functional phenotype as being either classically activated or pro-inflammatory (M1) macrophages and alternatively activated or anti-inflammatory (M2) macrophages[64, 71]. M1 macrophages are induced by IFNγ and TLR agonists like LPS, while IL-4 and IL-13

mainly activate M2 macrophages[72]. M1 macrophages mediate microbial killing and anti-tumour immunity and produce ROS, NOS, and pro-inflammatory cytokines such as IL-1β, IL-12, IL-6, and TNFα[64, 71]. In contrast, M2 macrophages promote immunosuppression, wound healing, tissue remodelling, and tumour growth and are characterised by the production of IL-10 and transforming growth factor-β (TGFβ)[64, 71].

1.1.1.2.4 Dendritic cells

Dendritic cells (DCs) are specialised antigen-presenting cells (APCs) characterised by their ability to capture and process antigens (Ags) into peptide fragments that are required to present them via the major histocompatibility complex (MHC) and regulate T-cell responses[73, 74]. In this way, they act as the first step to connect innate to adaptive immunity[75]. Once DCs are loaded with antigens, they undergo maturation and upregulate the surface expression of CD80/CD86 co-stimulatory molecules and their ability to produce cytokines. Then, they migrate to the secondary lymphoid organs (SLOs), such as lymph nodes and spleen, where they can present the extracellular antigenic peptides to $CD4^+$T-cells using MHC class II, and intracellular ones to $CD8^+$T cells via MHC class I[73, 74]. In the absence of pathogens, however, immature DCs control T-cell immune homeostasis and tolerance by inducing anergy or deletion to self-reactive T-cells[76].

1.1.1.2.5 Natural killer cells

Natural killer (NK) cells are lymphocyte-like subsets of the innate immune system that do not possess antigen-specific receptors. These cells were initially believed to arise only from CLP in the bone marrow. However, they were further found to develop and mature in the lymph nodes, spleen, tonsils, and liver[77, 78]. NK cells have a cytotoxic activity that directly kills virus and tumour cells. It

is either mediated by the production of perforin and several granzymes, which induce membrane destruction and cell lysis or by inducing a caspase-dependent apoptotic pathway[77, 78]. In addition, NK cells are an early high source of IFNγ, which subsequently shapes T-cell responses[79]. They also produce other cytokines such as TNFα, IL-6, IL-1β, and IL-10[80, 81]. Efficient activation of NK cells is stimulated by IL-12[82], IL-18[83], and IL-15 produced by DCs[84] or macrophages[85].

1.1.1.2.6 Mast cells

Mast cells (MCs) are resident cells found in most tissues, particularly close to surfaces that encounter the external environment, including skin, airways, and intestine[64, 86, 87]. They, therefore, serve as the frontline guard against invading pathogens or other environmental agents. These cells are derived from pluripotent HSCs in the bone marrow. Then mast cell precursors circulate in the blood and migrate into tissues, where they completely differentiate and mature[64, 86, 87]. MCs express several TLRs and complement receptors that can directly induce their activation and release of several pro-inflammatory mediators[88]. For example, TLR4 signalling induces MCs to secrete IL-1β, IL-6, IL-13, and TNFα, while their activation via TLR2 leads to degranulation and cytokine production[89, 90]. MCs secretory granules contain histamine, proteases, and TNF, as well as prostaglandins and leukotrienes, which are generated upon activation[87, 91]. Histamine and prostaglandin E2 (PGE2) have been found to reduce IFNγ secretion by DCs and facilitate the development of the Th2 cells[92]. MCs mainly participate in allergic and parasitic immune responses by expressing the high-affinity receptor for immunoglobulin E (IgE), known as FcεRI, which results in MCs degranulation via interaction with allergen-specific IgE[93].

1.1.1.2.7 Eosinophils

Eosinophils are circulating granulocytes that fully develop and mature in the bone marrow. These cells have a well-known role in the exacerbation of inflammatory responses during allergic asthma, leading to tissue damage. Their cytoplasm is rich in cytotoxic granules, including major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN), which directly mediate pathogen killing[94, 95]. In addition, they also contain several preformed cytokines available for immediate release upon activation, such as IL-4, IL-6, IL-10, IL-13, IL-12, TNFα, and IFNγ[96]. Eosinophils express FC and complement receptors and can respond to both PAMPs and DAMPs. For example, stimulation of TLR2, TLR5 and TLR7 promotes the production of IL-6, and IL1ß from eosinophils. While TLR2 signalling induces their ECP secretion[97]. Further, they can participate in antigen presentation via their expression of MHCII and co-stimulatory molecules (CD80, CD86 and CD40), triggering adaptive immunity[98, 99].

1.1.1.2.8 Basophils

Basophils are granulocytes that comprise the smallest percentage of all leukocytes in the blood, where they are less than 1%. But they can rapidly undergo proliferation in the bone marrow upon infection and migrate to the periphery as mature cells[100, 101]. Like MCs, they are characterised by their expression of FcεRI and a similar pattern of secreted mediators such as histamine and leukotrienes[100, 101]. Basophils mainly produce IL-4 and several other chemokines and cytokines, such as CCL3, CCL4, IL-13, IL-5, IL-6, and IL-9, promoting the recruitment of further effector cells[100, 101]. They have also been shown to function as APCs by presenting small peptides via MHCII to T-cells, leading to the induction of Th2 immunity[102].

1.1.2 Adaptive immune system

The acquired, or adaptive, immune response develops specific defences against infections. Additionally, it provides the body with an immunological memory that can last a lifetime and react quickly in the event of reinfection. Adaptive immunity relies on two main cell types: T cells and B cells, also known as lymphocytes, derived from CLPs in the bone marrow (BM)[7, 103, 104]. Immature T cells then migrate to the thymus, where they become fully mature and educated to distinguish between self- and non-self antigens to eliminate autoreactive T cells. B cells, in contrast, remain in the bone marrow to develop and complete their maturation in the spleen[7, 103, 104]. Both mature T cells and B cells can move through the bloodstream and traffic to the SLOs (lymph node and spleen), where they engage with antigens on the surface of APCs, leading to their activation and subsequent differentiation[7, 103, 104].

1.1.2.1 T cells

T cells are characterised by T cell receptor (TCR) expression. This receptor is generated through a coordinated series of processes called gene rearrangement that is required to create a functional TCR capable of recognising several antigenic peptides[103, 105]. There are two forms of TCR known as αß and γδ TCRs. While αß TCR makes up 95-99% of circulating T cells, γδ TCR is less common[104]. T cells expressing αß TCR can be further subdivided into two major subsets as being either $CD4^+$ or $CD8^+$ T cells. Full activation of T cells requires three consecutive signals delivered from APCs. Signal 1 (stimulation) is initiated when TCR on $CD4^+$ and $CD8^+$ T cells binds to target peptides presented by MHCII and MHCI molecules, respectively[104]. Signal 2 (survival) is mediated by the interaction of co-stimulatory molecules (CD80/CD86) on APCs with their cognate receptor CD28

expressed by T cells[106]. Finally, signal 3 (differentiation) involves APCs secreting cytokines to promote the polarisation of naïve T cells toward effector cells[74, 107].

CD8 T cells are cytotoxic (TC) and can directly kill infected cells. $CD4^+$ T cells, also known as T helper (Th) cells, perform this function and can further differentiate into different subsets in response to the cytokines driven by APCs. Th1 cells are characterised by their production of IL-2, IFNγ, TNFα and lymphotoxin, which promote microbial killing[108]. Whereas Th2 cells produce IL-4, IL5, IL-13, and IL9, these cytokines induce B cell antibody production, eosinophil recruitment, and M2 macrophage polarisation[108, 109]. Th17 cells secrete IL-17 and are important in autoimmune disease since IL-17 is found in the inflamed tissues of people with multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus[103, 110]. Regulatory T (Treg) cells, also $CD4^+$, can produce IL-10 and TGFß and suppress pro-inflammatory responses of other immune cells[111, 112].

1.1.2.2 B cells

B cells' primary function is to secrete antigen-specific antibodies against invading pathogens. Therefore, they have been linked to humoral immunity. But B cells also play an important role in cellular immunity, contributing to T cell activation by antigen presentation and cytokine release[7, 113, 114]. These cells have a B cell antigen receptor (BCR), a membrane-bound immunoglobulin (Ig) with a signalling subunit. Its structure is almost similar to the secreted antibody as both contain four identical protein chains: two heavy (H) and two light (L) joined together by disulfide bonds to form a Y-shaped molecule[103, 114]. Each of the H and L chains has constant and variable regions in which variable regions from both

combine to create a unique fragment antigen-binding site (Fab), giving the Ig molecule its antigen specificity[103, 114]. The constant regions of H chains make up the FC site that binds to the FC receptor of other immune cells and mediates the antibody functions. Once activated, B cells can either enter the germinal centre (GC) of SLOs or differentiate into transient plasma cells, which secrete low-affinity antibodies[113, 115, 116]. In GC, they go through somatic hypermutation and class switch recombination to generate plasma cells and memory B cells producing high-affinity antibodies[113, 115, 116]. There are five different classes or isotypes of antibodies, including IgM, IgA, IgD, IgE, and IgG. Naïve B cells also express IgD alongside IgM, but this expression directly switches after activation[114, 117]. IgM is mostly associated with early immune responses and is used as an inflammatory sign for acute infection. IgG is the most common isotype in the body and has four more subclasses: IgG1, IgG2, IgG3, and IgG4. IgE is linked to parasitic infections and hypersensitivity, whereas IgA is highly present on mucosal surfaces and in secretions such as breast milk and saliva[114, 117].

1.2 Chemokines and their receptors

Leukocytes are not randomly recruited to the sites of damage or infection. Instead, their movement process is well orchestrated, primarily by chemokines (or chemotactic cytokines)[118, 119]. They are a large family of small peptides with selective chemoattractant properties. Chemokines are typically released by Infected or damaged tissues[118, 119]. When blood leukocytes bind the chemokines via the expression of their specific chemokine receptors, they firmly adhere to the endothelium and subsequently extravasate into tissues[118, 120].

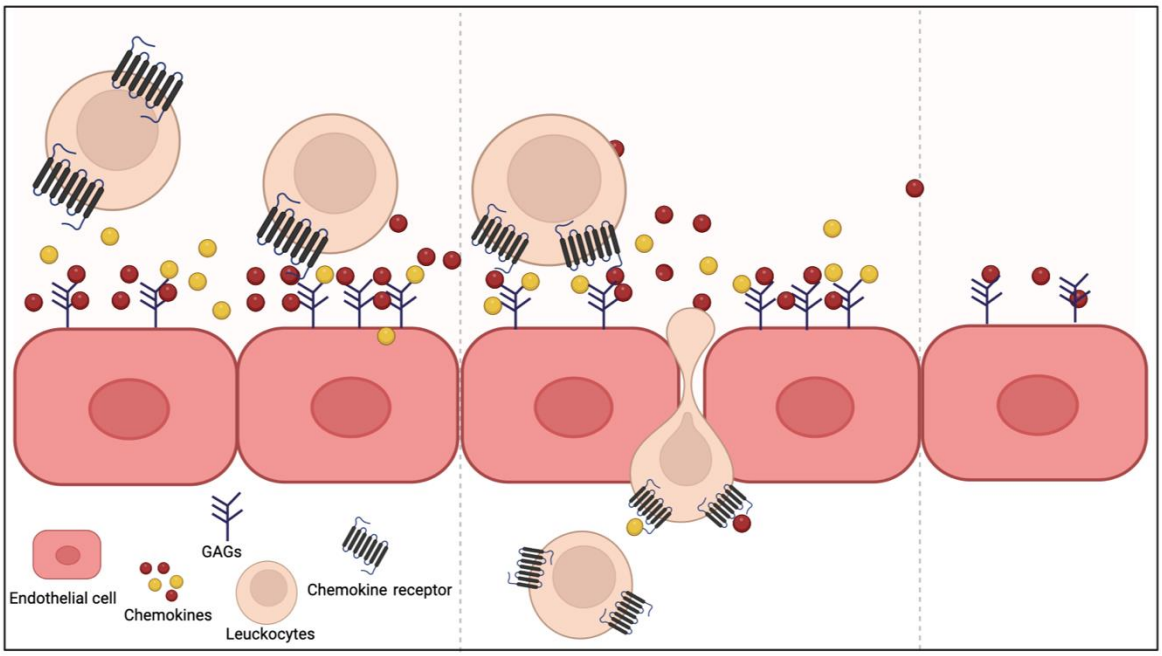

Figure 1-1 Migration of leukocytes in response to the inflammatory stimuli. Infected or damaged tissue release chemokines and become immobilised and present on the endothelial surface through glycosaminoglycans (GAGs) binding. Leukocytes roll along the endothelium upon chemokine binding and move into underlying tissues. Image adapted from "Leukocyte recruitment from the vasculature" by Handel et al.[120].

1.2.1 Structural divisions of chemokine

Chemokines are a group of small proteins (8-12 kDa) and are present in vertebrates from teleost fish to humans[121]. In 1999, a chemokine nomenclature system was defined based on the composition of the first two conserved cysteine residues in the N -terminal domain[121, 122]. This classified the chemokine fairly into four different groups; CXC, CC, CX3C and XC- with the letter L for ligand followed by a number according to when the gene was discovered[121, 122]. The CXC family has a variable amino acid sequence between the first two N-terminal cysteine residues, whereas the CC family has two adjacent cysteine residues. So far, nearly 50 chemokine ligands have been discovered and most of them belong to CC and CXC families[123, 124]. However, two more chemokine families have also been identified [125]. The CX3C family consists of three amino acids between the two cysteine residues. CXC3L1 is the only member of this family[122, 124, 126]. The fourth family is termed XC, in which only one conserved N-terminal

cysteine residue is present and has two highly related members: lymphotactin-α (XCL1) and lymphotactin-β (XCL2)[122, 124, 126]. The CC chemokines in general are chemo-attractants for myeloid cells with CCL2 attracting monocytes, whereas CCL11, CCL26 and CCL24 are chemo-attractants for eosinophils and involved in allergic inflammation[127]. Also, the CC- chemokine family regulates lymphocytes, natural killer cells (NK), and dendritic cells (DC) migrations[118]. The CXC family is further subdivided based on the presence or absence of the Glutamate-Leucine-Arginine (ELR) motif in the NH2 terminal domain[125, 128]. ELR-positive chemokines such as CXCL8 are angiogenic and chemo-attractant for neutrophils, whereas CXCL10 is ELR-negative, lacking such activity and attracting mainly lymphocytes[118, 128].

The tertiary structures of chemokines are broadly similar because of the disulfide bonds that hold the cysteine residues together[129]. These bonds are crucial for maintaining the structural integrity of the chemokine and ensuring receptor-ligand interaction and activity[118, 129, 130]. The sequence identity among chemokines is less than 30% between different subfamilies, while within the same family, the range of identity can vary from 30% to 99%[121]. Their common structure consists of a flexible N terminus and N terminal loop “signalling domain” followed by three antiparallel β sheets, which are overlaid by an α-helix in the C- terminal “core domain”[131, 132].

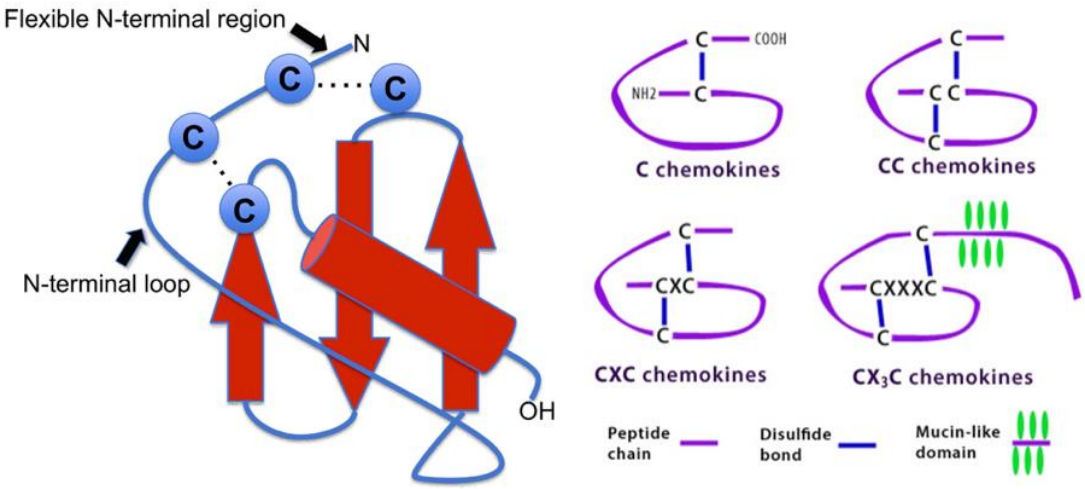

Figure 1-2 General structure of chemokines. In the model of the tertiary structure of chemokines, all ligands have a flexible N-terminal region and extended N-loop that contribute to the binding and activation of receptors, followed by the three-stranded β sheet and C-terminal α helix. The structural stability is maintained by the disulfide bonds (dotted lines), connecting the first N-terminal cysteine and the third and second cysteine to the fourth. The position of the first two cysteines closest to the N-terminal region split chemokine into groups CC, CXC, CX3C and XC. Image amended from "Tertiary structure of chemokines" in the Chemokines Handbook by Yung and Farber[121] and “Structure of chemokines” by Panda et al.[133].

1.2.2 Functional divisions of chemokine

Chemokines can be further classified as being either inflammatory or homeostatic based on their expression pattern and associated function[124, 134]. Homeostatic chemokines are constitutively produced to maintain leukocyte trafficking under normal conditions[124, 134]. For example, in the skin, CCL27 is predominately expressed to mediate homing of CLA^+ (cutaneous lymphocyte-associated antigen) T cells via CCR10[135]. Also, CCL19 and CCL21 and their receptor CCR7 are involved in DC and lymphocyte migration to the lymph node[136]. In the small intestine, CCL25-CCR9 mediates homing of lymphocytes[135]. Besides their role in regulating leukocyte recruitment, they are also important in immune surveillance, haematopoiesis, and the development of secondary lymphoid organs[119]. The homeostatic chemokine CXCL12 was first known as a pre-B cells growth factor and later was shown to have a critical role in

other homeostatic processes such as angiogenesis and embryogenesis[137]. Further, CXCL12 Knockout (KO) mice have severe deficiencies in hematopoiesis and neurogenesis, as well as a defect in cardiac development and they die close to birth [138, 139].

Unlike homeostatic chemokines, inflammatory chemokines are not constitutively produced[127, 134]. Such chemokines are produced by leukocytes or activated cells during inflammation and tissue damage[127, 134]. For example, CCL2 and CCL7 are induced after cytokine stimulation and recruit $CCR2^{+}$ inflammatory monocytes[119]. In general, inflammatory chemokines include CCL2, CCL3, CCL4, CCL5, CXCL2, CXCL3 and CXCL7 [128]. However, some chemokines can fall into both homeostatic and inflammatory categories making the structural-based classification preferable. Such chemokines have dual functions CXCL9, CXCL10, CCL17, and CCL20[128]. CCL21, despite being mentioned above as homeostatic chemokines, also shows induction in response to inflammation to regulate DC migration to the draining lymph node and induce the adaptive immune response[137].

1.2.3 The structure of the Chemokine Receptors

Chemokines exert their specific effects by binding to chemokine receptors that are members of the G-protein-coupled receptor (GPCRs) family, expressed on the surface of target cells[140]. GPCRs are considered the largest gene family in the human[141]and mouse[142]genomes and include receptors for hormones-inflammatory mediators, chemokines, neurotransmitters and even taste, odorant molecules and calcium ions. The structure of chemokine receptors is complex and characterised by seven-transmembrane(7TM)-spanning-α-helices and three extracellular and intracellular connecting loops[143, 144]. The extracellular

domain contains a short acidic N-terminus that is involved in controlling specificity and affinity for ligand binding[142, 143]. The intracellular region includes the C-terminus, which is composed of serine/threonine rich residues and acts on receptor signalling and internalisation[143]. Disulfide bonds link the highly conserved cysteine residues in the first and second extracellular loops, maintaining the structural stability of the receptors[142, 144]. The G-proteins that are involved in signal transduction are coupled through the C-terminus segment and probably the third intracellular loop[144]. All the chemokine receptors contain approximately 350 amino acids with around 40kDa molecular weight[140, 144]. According to their amino acid sequences, these receptors are part of the class A rhodopsin-like family[140, 145]. All the classical signalling chemokine receptors contain a highly conserved amino acid sequence (DRYLAIV motif) in the second intracellular loop, which is absent in atypical chemokine receptors (ACKRs). Such a motif is essential for signalling[145, 146]. Mutations within this motif can lead to impaired G-protein binding and can result in consecutive receptor signalling[142]. Thus far, 19 classical chemokine receptors have been defined and grouped into four subfamilies based on the chemokine ligand in which they bind; 10 reporters for CC-chemokines, 7 for CXC-chemokines and single receptors for CX3C-and XC-chemokines[147].

1.2.4 Activation of chemokine receptors

The binding of chemokine ligands to the extracellular domains of cognate receptors induces conformational changes that trigger intracellular signalling[140, 142]. A model proposed in the mid-1990s defined the interaction and activation of chemokine receptors with their ligands as a two-step/two-site activation mechanism[148, 149]. This model separates the binding process of

chemokine/receptor spatially (two-site) and functionally (two-step) [148, 149]. It is initiated through the interaction of N-loop residues of the ligand with the receptor N-terminus (site-1)[132, 150]. These regions are the recognition sites which control the specificity and affinity of the ligand-receptor binding (step-1)[132, 150]. Once the ligand specificity has been confirmed, activation of the receptor and the cellular response is mediated through the insertion of the ligand N-terminal region into the orthostatic pocket of the chemokine receptor (site-2) (step-2)[132, 150].

This classical model's concept helps us understand how GPCRs are generally activated, but it also oversimplifies how complicated chemokine/receptor interactions are. For instance, structural studies have revealed two independent sites for ligand binding and further interaction between chemokines and their receptors is probably required to ensure complete receptor activation[149, 151, 152]. Moreover, this two-site binding model is largely based on the idea that chemokine receptors only function as monomers. However, they can combine to form homodimers and heterodimers, affecting their downstream signalling and function[153].

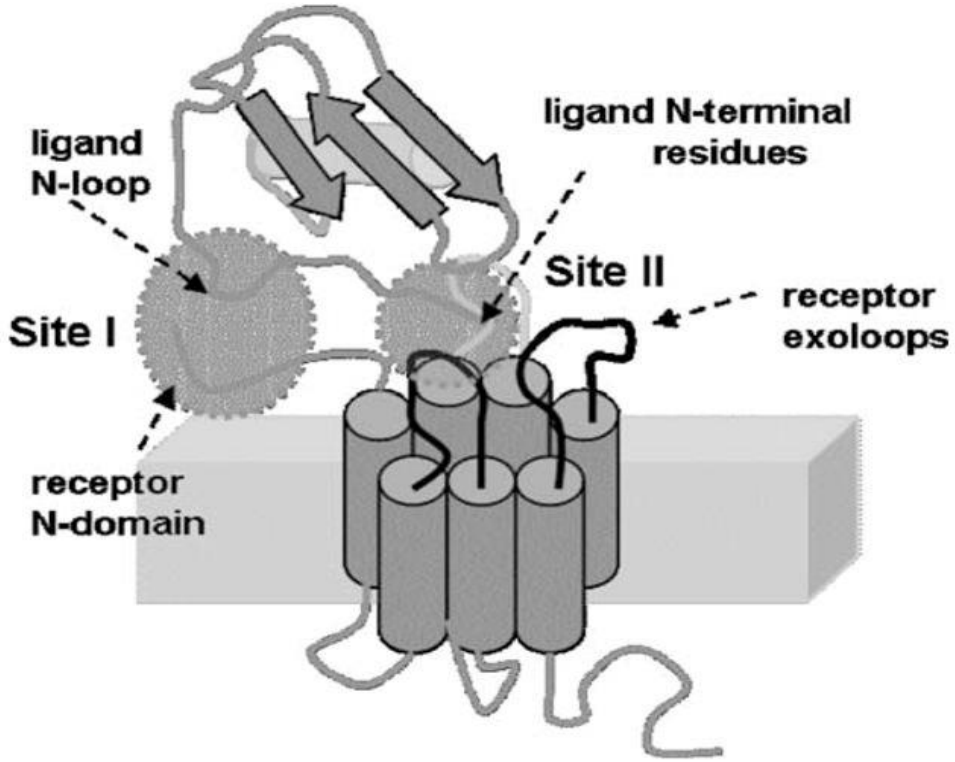

Figure 1-3 Chemokine ligand/receptor interaction model. Initially, the N-terminal residues of the ligand bind to the receptor's N-terminus domain (site-1). The chemokine ligand's N-terminus residues are subsequently inserted into the receptor's transmembrane domain (site-2). Adapted from "A model of chemokine ligand-receptor interaction" by Rajagopalan and Rajarathnam[150].

1.2.5 Signalling of chemokine receptors

Upon binding, chemokines induce intracellular signalling through coupling with pertussis toxin-sensitive heterotrimeric G-proteins Gα, Gβ, Gγ at the seven-transmembrane 7TM- domain[144, 154]. G-proteins then exchange the guanosine diphosphate (GDP) for guanosine triphosphate (GTP), which in turn results in the activation and dissociation of G-proteins into Gα and Gβγ subunits[148, 155]. Both subunits interact with many downstream effectors, eventually leading to cellular responses[148, 155].

1.2.6 Regulation of chemokine receptors

Chemokine receptor signalling induces a cellular response and drives intracellular events that negatively regulate the receptor activation itself to

prevent overstimulation[154, 155]. Typically, upon ligand binding, stimulation of G-protein mediated signalling triggers kinases such as protein kinase C (PKC) and G protein receptor kinases (GRKs) to phosphorylate the cytoplasmic C terminal domains of GPCRs. When the receptor is phosphorylated, it becomes more susceptible to β-arrestin binding, which in turn prevents the receptor from any further binding to G-proteins and results in signalling desensitisation "inactivation"[155-157]. β-arrestins are important in chemokine receptor regulation as they also act as an adaptor protein for receptor endocytosis by directing cell surface receptors to clathrin-coated pits for internalisation and endosomal degradation, which then downregulates the expression level of certain receptors [155-157]. The internalised receptor can then be dephosphorylated and recycled back to the cell surface. However, the internalisation and recycling rates can vary between receptors, indicating that the chemokine ligand itself can have varying effects on its targeting pathways. For example, compared to CCL21, CCR7 is more efficiently internalised through binding to its ligand CCL19[158]. Similarly, CCR1, CCR3, and CCR5 are all effectively internalised when bound to CCL5. However, they do so via different mechanisms since CCR5 is entirely recycled back into the surface of eosinophils, whilst CCR3 is only partially recycled back, and CCR1 is not[159, 160].

However, further regulation of GPCRs can stop the signal transduction through binding to the α subunit of activated G-protein without interacting with the receptor directly. Such a mechanism is controlled by proteins known as regulators of G protein signalling (RGS). RGS proteins enhance the intrinsic GTPase activity of the Gα subunit, which then promotes hydrolysis of GTP to GDP and rebinding to the Gβγ subunit. This subsequently leads to the inactivation of G-proteins and the termination of chemokine signalling[161, 162].

1.2.7 Atypical chemokine receptors

Atypical chemokine receptors (ACKR) are a subfamily of chemokine receptors that play an important role in regulating the chemokine system. However, in contrast to other chemokine receptors, these receptors were initially referred to as “silent” chemokine receptors because they cannot induce the classical signalling events following ligand binding, and thus are uncoupled from downstream cellular responses and migration. This apparent lack of signalling is due to structural modification, especially alteration of the DRYLAIV motif within the second intracellular loop[163-165]. This highly conserved domain is essential for G-protein binding and activation. Four atypical chemokine receptors have been identified, including ACKR1, ACKR2, ACKR3 and ACKR4, with two others called ACKR5 and ACKR6, under further functional investigation[147, 166]. ACKRs are mainly expressed by non-leukocyte cells such as endothelial cells and erythrocytes, even though leukocytes have been found to express ACKRs, particularly ACKR2 and ACKR3[167-169]. Like classical chemokine receptors, the ACKRs tend to be promiscuous in terms of their ligand binding. For example, ACKR1 can bind at least 20 CC- and CXC- chemokines, whereases ACKR2 binds many inflammatory CC-chemokines, allowing them to control a broad range of chemokines[147, 164].

ACKR1, previously called DARC, is thought to function as a sink for its ligands as there is clear evidence that inflammatory chemokines bind to circulating RBCs in an ACKR1-dependent manner, which limits inflammatory cell infiltration into the lung and liver after LPS challenge[170]. However, it also serves as a chemokine buffer, maintaining blood chemokine levels to regulate leukocyte mobilisation and extravasation across the endothelium[171]. Further, unlike other atypical chemokine receptors, it can mediate chemokine transcytosis by

transporting functionally intact ligands across endothelial cells, leading to engagement with their receptors on the apical surface without any apparent degradation of chemokines[172].

ACKR2, previously known as D6, is a chemokine scavenger and best exemplifies this function. It rapidly depletes extracellular chemokines during inflammation by internalising and transporting them to lysosomes for intracellular degradation[173, 174]. Consequently, ACKR2-deficient mice exhibit excessive inflammatory responses characterised by uncontrolled leukocyte infiltration in several tissues due to impaired chemokine clearance[175, 176].

ACKR3, or CXCR7, binds just two chemokine ligands: CXCL12 and CXCL11. Mice lacking ACKR3 display cardiac malformation and die shortly after birth[177], and display other developmental abnormalities in the brain and kidney[147]. Additionally, it has been found that ACKR3 can modulate the functional activity of CXCL12 directly by scavenging and indirectly by forming heterodimers with its receptor CXCR4 to modify CXCL12-driven signalling[178]. ACKR4 binds to CCL21 and CCL19, the ligands of CCR7 and can scavenge them to shape chemokine gradients required to facilitate APCs trafficking toward the draining LN[179, 180].

1.2.8 Evolution of chemokine receptors

Chemokine receptors first evolved between 650 and 564 million years ago, during the origin of vertebrates[181]. This period is marked by the development of more complex structures such as neural crest tissue, a multi-chambered heart, a closed circulatory system, and a hematopoietic system, which all call for the regulated migration of primitive cells. Homeostatic chemokines and their receptors emerged much earlier than inflammatory chemokines and have been conserved across species due to their important role in organogenesis and

survival[182, 183]. For instance, the primordial chemokine receptor CXCR4 and its ligand CXCL12, which have orthologs in vertebrates ranging from jawless fish to humans, are necessary for the embryonic development of germ cells, brain and heart as well as hematopoiesis[184, 185]. Each orthologous gene descended from a common ancestor, and as a result of local gene duplications, the chemokine receptor family expanded to include all other receptors[183, 186]. This rapid evolution explains why different vertebrate species have different numbers of chemokine receptor genes. For instance, humans have 24 receptor genes, whereas elephant sharks only have 14[182]. The inflammatory CC-chemokine receptors (CCR1, CCR2, CCR3, and CCR5) have been developed in mammalian lineage as a survival mechanism to maximise protection against invading pathogens[181, 182]. However, after duplication, each chemokine receptor gene can have many copies that can each develop independently and take on specialised functions[183]. For instance, CCR2 and CCR3, which both evolve from a common ancestral CC receptor, attract different leukocyte subsets. CCR2 regulates monocyte and DC migration, whereas CCR3 is responsible for eosinophil recruitment.

1.3 The chemokine system in homeostasis and immunity

It is becoming clear that chemokine signalling is essential for orchestrating cell migration and directing them to specific locations throughout the body, where they promote organ development, maintain tissue homeostasis, and generate innate and adaptive immune responses. During homeostasis, the circulating immune cells in the blood and their positioning within the tissue are critical for allowing them to act as a sensor for infection[119, 187]. As previously stated, their activation begins after PRRs identify PAMPs (e.g., LPS), which, in turn, induce the local release of inflammatory cytokines and chemokines. This induction further recruits innate immune cells that express specific chemokine receptors to the site

of infection and damage, where their ligands are found. Chemokines and their receptors also guide antigen-presenting cells to the secondary lymphoid tissues, where they interact with T- and B-cells to initiate the adaptive immune response[119, 187].

1.3.1 Chemokines in homeostasis

The CXCL12/CXCR4/ACKR3 axis drives homing of neural progenitors in the developing brain during homeostasis[188, 189]. In the bone marrow, interactions between CXCL12 and CXCR4 are essential for both the maintenance of HSCs and the development of immune cells. CXCL12, which is produced by reticular cells, binds to CXCR4 on HSCs and retains them in BM niches[190]. CXCL12/CXCR4 interactions continue to be crucial for BM retention and the normal development of other immune progenitor cells, such as B cells[139, 145]. CXCR4 is downregulated during neutrophil maturation, allowing mature neutrophils to circulate in the periphery while being kept in the BM by CXCR4 signals[191]. Therefore, blocking CXCL12/CXCR4 signalling leads to abnormal neutrophil mobilisation into circulation[192]. While its blockade leads to only a slight increase in monocyte release into the blood as their egress from BM largely depends on CCR2 signalling[193]. Dyer et al. showed that CCR2 is nonredundantly necessary for monocytes to exit the bone marrow and enter the resting circulation[194]. In addition, several other homeostatic chemokines and their receptors are constitutively produced in SLOs to maintain their architecture. For instance, in the B -cells follicles, DCs produce CXCL13, which recruits B-cells via CXCR5, but the positioning of the B-cells in the marginal zone of the spleen depends on CXCR7 signalling[119, 135]. In the T-cells area, CCL19, CCL21 and CXCL12 control the recruitment and positioning of T-cells and DCs via CCR7 and

CXCR4, respectively[119, 135]. Overall, homeostatic chemokine signalling is critical for leukocyte recruitment to sustain tissue development and their precise localisation to be ready for an immune response triggered by damage or a pathogen.

1.3.2 Chemokines in innate immunity

Upon inflammation, resident immune cells such as mast cells and macrophages immediately release inflammatory cytokines and chemokines to promote leukocyte recruitment. For example, mast cells have been shown to release CXCL1 and CXCL2- containing granules, which induce early neutrophil recruitment[195]. In response to these chemokines, neutrophils upregulate CXCR2 expression to exit from BM into circulation[196]. Like the homeostatic state, inflammatory monocytes migrate early to the site of infection in response to CCR2 signalling, where they differentiate into either macrophages or DCs depending on the environmental cytokines[194, 197]. A recent study found that some inflammatory monocytes in the blood that express CCR2 also increase CCR1 expression, which might enhance their ability to cross the endothelium[198]. Once they have entered inflamed areas, CCR1 expression is further induced and maintained during differentiation into macrophages[198]. However, eosinophils express CCR3 to migrate to the periphery in response to different chemokines, such as eotaxins (CCL11, CCL24, CCL26)[119, 194]. NK cells also express chemokine receptors to migrate to inflammatory sites; these include CCR5 in the Toxoplasma gondii mouse model[199] and CXCR3 in hepatitis and cardiac transplant models[200]. As a result, their activation triggers the release of a variety of cytokines, including IFNγ and TNFα, which promote DC maturation and induce an adaptive immune response[187].

1.3.3 Chemokines in adaptive immunity

As is well known, the adaptive immune response is initiated when immature DCs are exposed to innate immune stimuli, which then modulates the expression of chemokine receptors to induce their migration to the lymphoid tissue. Their maturation results in downregulating CCR1, CCR5 and CCR6 and enhancing CCR7 expression to facilitate their migration toward the afferent lymphatic vessels following CCL21 gradients and subsequent migration to the T-cell area[187, 201]. Each subset of activated CD4+ T-helper cells expresses different chemokine receptors to direct their migration into inflammatory sites. For example, Th1 cells mostly express CCR5, and CXCR3, whereas Th2 cells express CCR4 and CCR8[202, 203]. Activation of T follicular helper cells decreases CCR7 and increases CXCR5 to mediate their migration toward the B-cell area following CXCL13 gradients produced by follicular stromal cells[203, 204]. For CD8+ T cells to be effectively primed, chemokines and their receptors also drive CD8+ T cells toward DCs licensed by CD4+Th cells. For instance, T-helper cells and DCs interact to produce CCL4 and CCL3, which then attract naive $CD8^+$ T-cells to the $CD4^+$ Th cell/DC area via CCR5[205]. As a result, this maintains the interactions between $CD8^+$ T cells and $CD4^+$ Th cell licensed DCs, which is crucial for the optimal response from memory $CD8^+$ T cells.

1.4 Chemokine system in disease

As described above, chemokine signalling is essential for orchestrating the migration of leukocytes into inflamed sites to mediate immune responses. However, in turn, excessive or aberrant production of chemokines can drive continued infiltration of cells, resulting in organ or tissue damage. Several chemokines and their corresponding receptors are considered important

mediators in the development of chronic inflammatory diseases, including autoimmune diseases and cancer. Therefore, their ability to drive inflammation has highlighted them as promising therapeutic targets[135, 206, 207]. This section will mainly focus on the role of CC-chemokines and their receptors in the pathogenesis of major inflammatory diseases.

1.4.1 Autoimmune diseases

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterised by sustained leukocyte infiltration, including neutrophils, monocytes, macrophages, and lymphocytes, into synovial tissue, eventually leading to joint destruction[208, 209]. Multiple chemokines have been implicated in different stages of RA. For example, in early arthritis, CCL4, CXCL4, CXCL7 and CXCL13 were all expressed, whereas CCL3 and CCL9 were released at later stages[209-211]. In addition, other chemokines, including CCL2, CCL5, CCL15 and CCL23 and their receptors CCR2, CCR5 and CCR1, have been implicated in the pathogenesis of RA[212-214]. They are mostly associated with macrophage/monocyte recruitment, whose number in inflamed joints is considered a diagnostic marker for assessing RA disease severity. CCR5 expression was also implicated in the infiltration of Th1 cells into rheumatoid joints, where its ligands were abundantly expressed[215]. Therefore, multiple antagonists targeting these chemokine receptors have been tested in clinical trials for rheumatoid arthritis; however, only CCR1 blockade was able to reduce the severity of the disease in RA patients[216, 217]. For instance, RA patients who received a CCR1 antagonist every eight hours for a week had fewer macrophages and CCR1-positive cells in their synovial tissue[218]. The Medina-Ruiz et al. study, which shows that inflammatory monocytes quickly increase CCR1 expression inside the

inflammatory site and maintain its expression during their differentiation into macrophages, may point to a crucial role for CCR1 in the intra-tissue migration of these cells and provide an explanation as to why only CCR1 blockade had positive effects[198].

In multiple sclerosis (MS), the infiltration of leukocytes, mainly T-cells, monocytes, and macrophages, into the brain destroys the myelin sheath, leading to neuron demyelination and axonal damage[219, 220]. CXCR3, CCR5 and CCR6 were responsible for Th-cell recruitment into the CNS, where their ligands were highly expressed in active MS lesions[221, 222]. Also, higher expression of CCR5 and CCR1 was detected on monocytes and activated microglia in the brain of MS patients[223]. Furthermore, their ligands, CCL3, CCL4 and CCL5, were upregulated at different stages of the disease development, including the progressive MS plaque stage[224, 225]. Therefore, blocking CCR1 via administration of a CCR1 antagonist reduced the incidence of experimental allergic encephalomyelitis (EAE), suggesting a role of CCR1 in MS[207, 226]. In addition, CCL2 was significantly expressed in the active lesions of MS patients[207, 223]. Binding to its receptor CCR2 was associated with monocyte and T-cell recruitment to the brain, whereas blocking CCR2/CCL2 signalling reduced the development of EAE and the clinical symptoms of the disease[227, 228]. Several other chemokines and their receptors, including CCL19, CCL21/CCR7, CXCL16/CXCR6 and CXCL1, CXCL2/CXCR2, have also been implicated in the development of MS pathology[229, 230].

1.4.2 Viral infection

Both CCR5 and CXCR4 serve as co-receptors for the cellular entry of the human immunodeficiency virus type 1 (HIV-1). For HIV-1 infection, viral envelop

glycoprotein (gp120) binds to the primary receptor CD4 on the target cells and then to the co-receptor CCR5 or CXCR4. CCR5 is responsible for the R5 viral strains, mostly involved in early HIV-1 infection and disease transmission[231-233]. Individuals homozygous for the CCR5Δ32 mutation have been associated with natural resistance to HIV-1 infection, whereas those heterozygous for this mutation for CCR5Δ32 have lower viral load and delayed disease progression toward AIDS[234, 235]. Therefore, several antagonists for targeting CCR5 in HIV-1 infection have been developed, and maraviroc was licenced for clinical use [236]. CXCR4 is a co-receptor for X4-viral strains, which are predominant in the later stages of HIV-1 infection, mainly during disease progression. Some HIV-1 variants are dual strains, termed R5-X4, which can use both CCR5 and CXCR4 for cellular entry[28, 232].

In contrast, CCR5 plays a protective role in West Nile Virus (WNV) infection. For instance, CCR5 deficient mice infected with WNV could not control virus replication in CNS due to decreased leukocyte infiltration into the brain, impaired virus clearance and reduced mice survival[237, 238]. Furthermore, a meta-analysis study on WNV-positive cohort patients revealed a higher probability of symptomatic infection in those who are homozygous CCR5Δ32 carriers[239]. Other chemokine receptors with their cognate ligands, including CCR2 and CCR1, are highly expressed in the infected brain of the WNV mice model[240]. Additionally, WNV-infected mice lacking CXCR3 or its ligand CXCL10 displayed increased viral burden and mortality that is related to a decrease in $CD8^+$ T cell recruitment to the brain[241, 242].

1.4.3 Bacterial and parasitic infections

In the animal model of hepatic failure induced by Propionibacterium acnes infection, CCL17 has been seen to be responsible for the recruitment of CCR4 expressing $CD4^+$ T cells into the liver leading to massive liver injury[243]. Therefore, CCL17 neutralisation prevented mice from fatal liver failure[243]. However, in people with Staphylococcus aureus infection, CCL2 released from certain neutrophil subsets, known as PMN-II, has been observed to stimulate M2 macrophages and decrease host defence[244]. Also, CCL2-deficient mice displayed resistance to Leishmania major parasite infection[245]. But Listeria monocytogenes infection was more likely to occur in $CCR2^{-/-}$ mice [246]. CCR5 was found to direct the recruitment of naturally occurring $CD4^+CD25^+$ regulatory T-cells to L.major infected sites, where they promote parasitic survival and persistence of infection[247]. Loss of CCR1 significantly enhanced cytokine production and bacterial clearance in a septic mouse model[248]. CCR2 antibody in mice challenged with Streptococcus pneumonia progressively impaired pneumococcal lung clearance but prevented the formation of chronic irreversible bronchiolitis obliterans[249]. Also, CCR4-deficient mice had a delayed innate immune response to Mycobacterium bovis infection as well as diminished late-stage inflammation[250].

Furthermore, CCL1, CCL3, CXCL1, CXCL2, CXCL9 and CXCL10 are upregulated in patients with active pulmonary tuberculosis compared to latent tuberculosis and healthy controls[251]. CCL5 is also increased in children with pneumococcal pneumonia compared to pneumonia caused by other pathogens[252]. Chemokines and their receptors contribute significantly to bacterial infection and may serve as indicators for disease severity.

1.4.4 Cancer

Chemokines and their receptors play an essential role in shaping the tumour microenvironment (TME) by recruiting the immune cells to the local tumour sites, where they promote tumorigenesis. For example, CCL2 in colorectal cancer induced pro-tumour myeloid cell recruitment[253, 254]. These cells are further differentiated into mature tumour-associated macrophages (TAM) with characteristics of M2-polarised macrophages. M2-macrophages have well-documented tumour-promoting functions, including supporting tumour cell proliferation, suppression of anti-tumour response by releasing IL10 and TGFβ and remodelling of extracellular matrix[255, 256]. CCL5 is also found to induce the infiltration of intra-tumour Th2 lymphocytes, which are considered a poor prognostic sign[257]. In ovarian cancer, CCL28 and CCL22 expression are upregulated, which leads to the recruitment of $CCR4^{+}CCR10^{+}$Tregs[258, 259].

1.4.4.1 Angiogenesis

The chemokine system is implicated in tumour angiogenesis by regulating the development of new vascular networks around the tumour, facilitating cancer cell survival, proliferation, invasion, and metastasis[255, 256]. Targeting the ELR+ chemokines CXCL1, CXCL2, and CXCL3, in melanoma reduced angiogenesis and tumour growth[260, 261]. A study describes how CCL2 and its receptor CCR2 contribute to tumour survival and angiogenesis[262]. Showing that CCL2 has an autocrine effect on the proliferation and survival of CCR2+ cancer cells and a paracrine effect on the recruitment of CCR2+ macrophages[262]. This shows that by modulating TAMs, CC-chemokines indirectly contribute to angiogenesis. The angiogenic factor TGFβ, produced by such pro-angiogenic cells, also stimulates the synthesis of vascular endothelial growth factor (VEGF). The VEGF induces a

positive feedback loop that further increases the synthesis of chemokines[263, 264]. The angiogenic effects of CCL5 have also been demonstrated in vivo and in vitro[265]. Therefore, blocking CCR1 or CCR5 reduces CCL5-induced vascular development[265].

1.4.4.2 Metastasis

Metastasis is the main factor in about 90% of cancer-related deaths. Tumour cells spread from the initial site through the bloodstream and move to distant organs throughout this process. Numerous studies have suggested that the main cause of metastasis is chemokines and their receptors. By overexpressing chemokine receptors, tumour cells can use the chemokine pathways to direct their migration to another area to form metastases[123, 256, 266]. For example, high levels of CCL9 and CCL15 in colon cancer resulted in attracting $CCR1^{+}$ immature myeloid cells (iMCs) and promoted the metastasis of colon cancer cells to the liver[267]. This result was further supported by Rodero et al. study [268], who found that liver metastasis was significantly decreased in CCR1 knockout mice compared to control mice and that this inhibition was associated with a decrease in monocyte infiltration. CCL5 is also found to be induce in breast cancer cells to facilitate their dissemination to the liver and lungs[269]. Overexpression of CCL2 and its receptor CCR2 have been indicated in liver, breast, pancreatic and colon cancers. Therefore, blocking the CCR2\CCL2 signalling in hepatocellular carcinoma (HCC) prevented tumour metastasis to the liver and activated anti-tumour CD8+ T cells[270]. CCR3 and its ligand CCL11 are implicated in cutaneous T-cell Lymphoma (CTCL) enhance tumour cell survival and dissemination by establishing a Th2-dominant TME[271]. Other chemokine receptors contribute to cancer growth and metastasis, including CXCR4 in 24 metastatic cancers, CCR7 in

lymph nodes metastasis, CCR9 and CCR10 in melanoma metastasis, CXCR5 in head and neck cancer, and CCR5 in Hodgkin's lymphoma and prostate cancer[135, 272].

1.4.5 Other inflammatory diseases

Atherosclerosis is a major cause of cardiovascular disease leading to myocardial infarction or stroke. It is characterised by excessive lipid, immune cell, and cell debris accumulation in the arterial wall and subsequent formation of fibrous plaques. However, more frequently, these plaques burst, causing thrombus formation[273, 274]. In the experimental mouse model of atherosclerosis, apolipoprotein E-deficient mice (ApoE-/-), circulating monocyte subsets use CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques, where they develop into macrophages[275]. Differentiated macrophages will take up the modified lipid in the vessel wall and become foam cells, which in turn release pro-inflammatory mediators leading to the lesion's growth[273, 274]. Histological analysis of atherosclerotic plaque size in $ApoE^{-/-}$ mice shows it to be reduced in the absence of CCR2[276]. This reduction is associated with fewer macrophages accumulating at the lesion sites[276]. CCL5 is strongly detected in early atherosclerotic lesions[207, 277]. Therefore, blocking CCL5 signalling via the administration of CCR1/CCR5 antagonist Met-RANTES increased atherosclerotic plaque stability and reduced inflammation in $ApoE^{-/-}$ mice[207, 277]. CXCR3 and its ligands CXCL9, CXCL10 and CXCL11 are increased in human atherosclerotic lesions contributing to T-cell recruitment into the vessel wall[278]. CXCR3 deficiency in $ApoE^{-/-}$ mice was associated with a reduction in lesion formation that is correlated with increases in T-reg numbers and IL-10 expression[279].

Furthermore, asthma and chronic obstructive pulmonary disease (COPD) are characterised by chronic infiltration of leukocytes into the airway and lung

tissue, leading to severe airway inflammation and obstruction. They are both considered to be the most common lung diseases[207, 280]. In asthma, Th2-cells, eosinophils, and mast cells play a pathogenic role. CCR1, CCR3, CXCR1, CXCR3 and CXCR4 are responsible for mast cell infiltration into the airway of asthmatic patients, where their ligands CCL5, CCL11, CXCL8, CXCL10 and CXCL12 are highly expressed[207, 281]. CCR3 and its ligands CCL5 and CCL11 mainly contributed to the airway recruitment of eosinophils in asthmatic patients[207, 281]. In COPD, macrophages, neutrophils and CD8+ T-cells are involved in disease progression[282, 283]. CCL2/CCR2 signalling mediated the recruitment of monocyte-derived macrophages into the airway epithelium in COPD[282, 283]. The expression of CCL2 is high in bronchoalveolar lavage fluid and lungs of patients with COPD[207, 282, 283].CCR5, CCR6, CXCR2 and CXCR3 have also been implicated in disease pathology[280, 283].

1.5 Therapeutic targeting of the chemokine system and its failure

Studies above have shown the major role of chemokines and their receptors in driving the pathogenesis of several diseases, ranging from asthmatic inflammation to cancer metastasis. It is, therefore, not surprising that they become attractive targets for drug discovery. However, thus far, the FDA has only successfully approved two chemokine receptor-targeting drugs, and none of them is used to treat inflammatory or autoimmune diseases. The first one is Maraviroc, approved in 2007 for blocking CCR5 in HIV-1 infection. The second one is Mozobil™ or AMD3100, initially developed to treat HIV and then approved at the end of 2008 to target CXCR4 for hematopoietic stem cell mobilisation[284, 285].

Drug target	Disease	Company	Drug name	Clinical phase	Statues
CCR5	HIV infection	Pfizer	Maraviroc	Approved	Launched
CXCR4	Hematopoietic stem cell mobilisation	Genzyme	Plerixafor (Mozobil/AMD31)	Approved	Launched
CCR1	MS/psoriasis/ endometriosis	Berlex/Schering	BX471	II	Terminated (No efficacy)
CCR1	COPD	AstraZeneca	AZD4818	II	Terminated (No efficacy)
CCR1	RA/MS	Millennium	MLN3701/ MLN3897	II	Terminated (No efficacy)
CCR1	RA	Pfizer	CP481-715	II	Terminated (No efficacy)
CCR2	RA/MS	Merck	MK-0812	II	Terminated (No efficacy)
CCR2	MS/Arthrosclerosis	Millennium	MLN-1202	II	Terminated (No efficacy)
CCR2	MS	ChemoCentryx	CCX140	I	Terminated (No efficacy)
CCR3	Asthma/allergic rhinitis	GlaxoSmithKline	GSK766994	II	Terminated (No efficacy)
CCR3	Asthma	Bristol-Meyers Squibb	BMS-639623	I	Terminated (No efficacy)
CCR5	HIV infection	GlaxoSmithKline	Aplaviroc	III	Terminated (Toxicity)
CCR5	RA	Pfizer	Maraviroc	I	Terminated (No efficacy)

Figure 1-4 Failure of inflammatory chemokine receptor antagonists. Despite the success in developing of CCR5 and CCR4 drugs, most antagonists that target inflammatory chemokine receptor in autoimmune and inflammatory diseases were not approved and failed to enter the market. Data adapted from “Chemokine receptor drugs launched, in clinical trials or terminated” by Solari et al.[286] and “Clinical trials reported for chemokine receptor antagonists” by Horuk[287].

1.5.1 Reasons for failed clinical trials

The failure in clinical trials has frequently been linked to the chemokine/receptor network's complexity, where one chemokine ligand can bind several different receptors, and each receptor can recognise multiple ligands (Figure 1-5)[285, 288]. Thus, the chemokine system contains apparent redundancy and promiscuity and represents a serious block to developing antagonists that inhibit a specific chemokine receptor[284-286]. However, it has been suggested that the system in vivo is mostly under spatio-temporal control to ensure fine tuning of leukocyte responses to different inflammatory stimuli[289, 290].

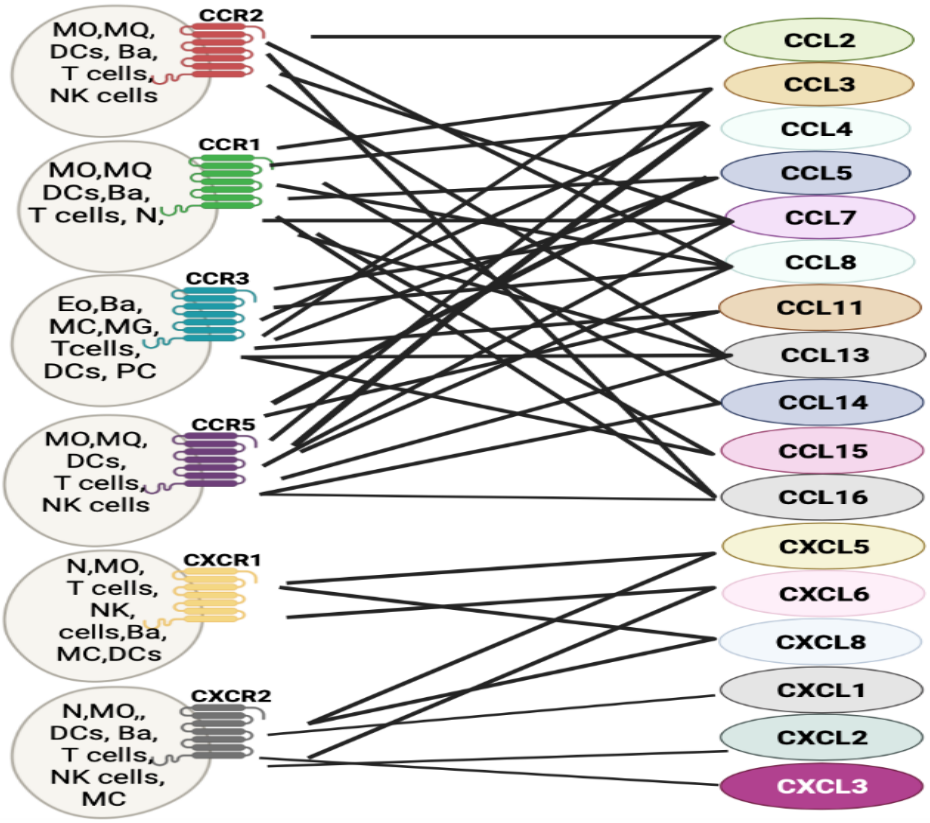

Figure 1-5 Apparent redundancy and promiscuity in chemokine/receptor network. The chemokine system is remarkably redundant and promiscuous, with different cells expressing different receptors, and each receptor can bind many ligands. Data adapted from "Chemokines" Griffith et al.[119] and "Chemokine receptor specificity for ligands and leukocytes" by Bachelerie et al.[291].

The failure of the clinical studies could have been due to other factors outside the chemokine system's redundancy. For instance, BX471, a CCR1 inhibitor

and the first antagonist to enter clinical trials, failed to provide any signs of clinical benefit in multiple sclerosis phase II clinical studies. Loss of species cross-reactivity was the cause of this failure[292]. In comparison to humans, BX471 showed a 2-log decline in affinity for rat CCR1, necessitating a large dose to exert enough action against rat CCR1[287, 293]. Therefore, this may make it more difficult to interpret any positive response shown in rodent disease, as there may be a chance for off-target effects due to cross-reactivity with other GPCRs[287, 293]. This was also the case with compound 1, a potent inhibitor of human CCR1, which interfered with other GPCRs like adenosine A3 and dopamine D receptors[294]. This compound's further development was therefore terminated since the effective outcomes were mediated by the influence on dopamine responses rather than CCR1 inhibition, where they can reduce T cell function and Th1 cytokine secretions that are associated with the pathophysiology of MS[295]. In phase II RA, CCR1 antagonists CP-481,715 and MLN3897 also failed to meet the clinical endpoint, primarily because of an inadequate dose[289].

Likewise, several antagonists were developed to block CCR2 in rheumatoid arthritis, which is the main receptor expressed on circulating monocytes, and monocytes/macrophages in inflamed joints[208]. However, the outcomes of clinical trials targeting CCR2 and its chemokine ligand CCL2 had disappointing results[208, 296]. For instance, phase II trials of the CCR2 antagonist MK0812 did not show efficacy in treating both RA and MS[289]. In fact, in RA clinical trial, the group that received CCR2 antagonists performed poorly compared to the placebo group[289]. MK0812 antagonist has been found to lack specificity for CCR2 as it has a comparable ability to inhibit CCR5[285, 289]. In experimental arthritis, Doodes et al. found evidence for the role of CCR5 in the recruitment of regulatory T cells into inflamed synovium, which led to the reduction of inflammation[297].

This shows the drug's failure was caused by its effect on CCR5, which, in turn, inhibited cells necessary for regulating the disease.

1.5.2 Considerations when targeting the chemokine system

In complex diseases like rheumatoid arthritis, targeting one chemokine receptor may not be sufficient as multiple receptors have been implicated in the pathology of such disease, including CCR1, CCR2, CCR5, CXCR3 and CXCR2[298]. Similarly, multiple sclerosis is a heterogeneous disease with at least four distinct types of demyelination: antibody-mediated, macrophage-mediated, distal oligodendroglopathy and primary oligodendrocyte damage[299]. Each of them includes different leukocyte subsets that possibly have been recruited using different chemokine receptors[300]. Therefore, targeting multiple chemokine receptors might provide a much better chance for the successful development of anti-inflammatory therapy. However, to effectively target inflammatory chemokine receptors, a deeper understanding of their complex biology is required to define whether leukocytes express single or combined receptors to migrate to the inflammatory sites. Also, the discrepancies between human and murine biology should be considered, where the chemokine system is among the top eight rapidly evolving gene families, as is the case for most genes involved in immunity and host defence[124, 186]. Due to such rapid evolution, some chemokine genes can exist in one species but can be absent, or lack a functional equivalent, in another reflecting the differences in disease phenotypes[124, 186].

1.6 Inflammatory CC-chemokine receptors

1.6.1 CCR1

CCR1 was the first CC-chemokine receptor identified by gene cloning in 1993[301-303]. It is widely expressed by several hematopoietic cells, including neutrophils, monocytes, B and T lymphocytes, NK cells and $CD34^+$ bone marrow cells[302, 304]. Human CCR1 and mouse CCR1 share 80% homology[286]. CCR1 is mostly expressed on monocytes in both humans and mice, despite earlier thoughts that CCR1 was expressed on neutrophils in mice. Dyer et al. clearly demonstrated that none of CCR1, CCR2, CCR3, and CCR5 were involved in the migration of neutrophils to resting or acutely inflamed tissues[194]. CCL3 is the most potent ligand for both human and mouse CCR1, but CCR1 is highly promiscuous and binds to other chemokines. The additional cognate ligands for CCR1 are CCL5, CCL6, CCL7, CCL8, CCL9, CCL13, CCL14, CCL15, CCL16, and CCL23[301, 302, 304].

No developmental defects or lethality were observed in mice with CCR1 deletion, and there were no alterations in the histology of the bone marrow, lymphoid organs, peripheral blood counts, or clearance of spontaneous infection[305]. However, in pathology, the receptor has been implicated in the recruitment of different immune cells to sites of inflammation. Lionakis et al. found that 60% of the leukocytes failed to mobilise into $CCR1^{-/-}$ injured kidneys compared to WT control in the later stages of invasive candidiasis[306]. This reduction also improved renal function and mice survival without affecting fungal burden, demonstrating the significance of CCR1 in developing severe inflammation[306]. CCR1 expression was also correlated with the exacerbation of respiratory inflammation through increased recruitment of T-cells to the lung and lymph node, suggesting that CCR1 is a potential target for alleviating airway hyperreactivity[307]. Targeting CCR1 in colorectal cancer decreased the

formation of liver metastases and the accumulation of myeloid progenitor cells[267]. In lung cancer, CCR1/CCL5-mediated signalling also promotes tumour cell invasion and metastasis[308]. These results imply that CCR1 targeting is a potential antimetastatic treatment for different cancer types.

1.6.2 CCR2

CCR2 is the main chemokine receptor for inflammatory monocyte mobilisation from the bone to the circulation in both resting and inflamed conditions[194, 309, 310]. Its ligand CCL2 was the first characterised chemoattractant for monocytes[302]. Consequently, mice lacking CCR2 had normal development but were unable to attract leukocytes, particularly monocytes, to the peritoneum after receiving thioglycolate[311]. Several other ligands can activate CCR2, including CCL7, CCL8, CCL13, CCL12 and CCL16. The expression of CCR2 has been detected on activated $CD8^+$ T cells during viral infection and mediated their migration to the infected site[312]. Endothelial cells may also express CCR2, which allows them to migrate to the wound area and might facilitate their proliferation and angiogenesis[313]. In NK cells, CCR2 expression is strongly correlated with their tumoricidal activity[314]. It is also found on the surface of other cell types, including both subsets of $CD4^+$ T cells (Th1/Th2 cells)[315, 316], γδT cells[317], B cells[318], microglia[319], Tregs[320], basophils[321] and stem cells[322].

Strong evidence of the role that CCR2 plays in causing the pathophysiology of several diseases, including multiple sclerosis, atherosclerosis, and type 2 diabetes, has been provided by genetic deletion or antibody blockage of CCR2, which lowers disease symptoms in animal models. For instance, in CCR2 deficient mice, the development of EAE and the invasion of monocytes and T cells into the

CNS were inhibited[227, 228]. Similar to this, the deletion of CCR2 or CCL2 in atherosclerosis reduced the recruitment of such cells to the artery wall and plaque development[323, 324]. Also, inhibiting CCR2/CCL2 signalling increased lung graft survival and reduced the risk of acute transplant rejection in both CCR2-/- mice and WT mice treated with CCL2-neutralising antibodies[325]. This prolongation was associated with a significant reduction in the bronchial recruitment of mononuclear phagocytes after tracheal transplantation and led to the attenuation of chronic bronchitis[325]. CCR2 has also been implicated in cancer progression, where over 50% of metastatic renal cell carcinoma was $CCR2^{+}$[326]. High CCR2 mRNA expression in pancreatic cancer promoted tumour development and metastasis[327]. Therefore, mice deficient in CCR2 had a stronger immune response to tumours by switching pro-tumour Th2 cell infiltration into anti-tumour Th1 cells[328]. However, CCR2 has been shown to regulate NK cell migration toward metastatic lesions in the lung, suggesting that it may have a particular role in this process. To be clear, ACKR2 KO mice showed an increase in CCR2 expression by NK cells, which enhanced their migration into tumour sites, improved their tumoricidal activity, and prevented metastasis[314].

1.6.3 CCR3

CCR3 is expressed mainly on eosinophils and on other immune cells, including basophils, mast cells and Th2 cells[302]. The ligands for CCR3 are CCL5, CCL7, CCL11, CCL13, CCL15, CCL24, and CCL26[329]. CCR3-deficient mice develop normally but show impaired basal eosinophil migration to the resting gut[194] and skin[330]. CCR3-/- mice showed dramatically reduced eosinophil recruitment to the lung in a model of airway hyperresponsiveness (AHR), with the majority of cells trapped in the subendothelial area and unable to pass out into the lung

parenchyma[330]. However, lack of CCR3 did not affect the number of eosinophils in the lung, and thymus under homeostasis, suggesting that other chemokine attractants might be essential for their extravasation from the bloodstream to resting tissues[194, 330]. Other studies identified a similar defect of eosinophil recruitment in the $CCR3^{-/-}$ mice with ovalbumin (OVA)-induced skin inflammation[331] and OVA-induced allergic asthma[332]. In addition, high expression of CCR3 was detected in airway epithelial cells and airway smooth muscle cells of asthmatic lung patients compared to non-asthmatic controls, indicating that the biological effects of CCR3 in airways might extend beyond leukocyte migration[333, 334]. CCR3 expression has also been reported to be significantly increased at the mRNA and protein levels in the skin and bronchial mucosa of patients with atopic dermatitis[335] and asthma[336], respectively. Therefore, these findings indicate that CCR3 has an important role in the pathology of allergic diseases, particularly asthma, and could be a promising drug target.

1.6.4 CCR5

CCR5 is expressed on activated and memory Th1 cells and many other cell types, including CD8+ T cells, monocytes, macrophages, NK cells, and immature DCs[231, 337]. In lymphocytes, CCR5 is not only important to induce their migration toward chemokine gradients but is also found to behave as a costimulatory molecule[337, 338]. For instance, CCR5 (and CXCR4) are involved in the formation of stable immunological synapses between T cells and antigen-presenting cells by extending the duration of their interaction and providing costimulatory signals for efficient T-cell activation[339]. CCR5 mediates its activity by binding to CCL3, CCL4, and CCL5[231, 337]. Mice deficient in CCR5 had

no developmental defect[340]. In contrast, using the Listeria monocytogenes infection model, $CCR5^{-/-}$ mice exhibited a reduction in the clearance capacity of the infection with a partial defect in macrophage function[340]. Subsequent studies showed that CCR5 expression increases as monocytes differentiate into macrophages, indicating its role in macrophage recruitment[198, 341, 342]. CCR5-deficient mice were also found to have defective bone marrow repair due to impaired osteoclast differentiation and osteoblast maturation[343]. Another study showed that the absence of CCR5 increased the severity of brain damage in experimental stroke[344].

The role of CCR5 and the potential effects of CCR5Δ32 mutation in autoimmune and several inflammatory diseases have been intensively studied, especially after being identified as a major co-receptor for HIV infection. In gastric cancer, CCR5/CCL5 mediated signalling was found to promote the immunosuppressive tumour microenvironment and induce cancer cell proliferation and metastasis[345]. Therefore, targeting CCR5 with Maraviroc in breast cancer reduced regulatory T cell recruitment and lowered tumour metastasis to the lung[346]. Moreover, CCR5Δ32 polymorphism was shown to play a protective role in juvenile idiopathic arthritis[347], heart diseases[348], systematic lupus erythematous[349], type 2 diabetes mellites[350], Sjogren syndrome[351], and childhood asthma[352]. In MS, homozygous carriers for CCR5Δ32 developed the disease but had a reduction in the clinical symptoms, suggesting that CCR5 antagonists might ameliorate disease activity in patients with MS[353].

1.7 Aims

Accumulating evidence has demonstrated the role of the inflammatory chemokine receptors CCR1, CCR2, CCR3 and CCR5 (hereafter referred to as iCCRs) in the development of most inflammatory and immune pathologies, making them possible therapeutic targets. Unfortunately, the apparent complexity of the chemokine system has limited our understanding of how these receptors control the inflammatory response and has hindered all the efforts to target them in inflammatory diseases. Therefore, a deep understanding of the individual and combined roles of the inflammatory chemokine receptors is required to develop successful anti-inflammatory drugs.

Targeted deletion of a single inflammatory receptor in many diseases always resulted in mild phenotypic changes, indicating that other inflammatory receptors might compensate for the role of the deleted one. Generating mice with double receptor deficiency would be ideal for testing this hypothesis. However, these inflammatory chemokine receptors CCR1, CCR2, CCR3, and CCR5 are tightly clustered within a genomic region of 170kb on chromosome 9 in mice. This genomic proximity makes the chance of chromosomal recombination very low and generating multi-receptor deficient mice to study their combinatorial function is almost impossible. To overcome this problem, the Chemokine Research Group has generated a novel iCCR fluorescent reporter mouse (iREP). iREP mice express a distinct fluorescent protein for each of these chemokine receptors and allow us to directly visualise changes in iCCRs expression on the cell surface.

This project aims to investigate the role of the inflammatory chemokine receptors, iCCRs 1, 2, 3 and 5, in inflammation. The aims of this study will be accomplished through the following approaches.

1. Determine the expression of iCCRs at mRNA and protein levels under several inflammatory conditions using TLR ligands and inflammatory cytokines.
2. Analyse the individual and combinatorial expression of iCCRs in monocytes and macrophages under sustained inflammation using the in-vivo subcutaneous implant mini pump model.
3. Transcriptomic analysis of characteristic changes in Ly6Chi inflammatory monocytes expressing different iCCR combination under sustained inflammation.

Chapter 2 Materials and Methods

2.1 Mice

All animal experiment was performed under a UK Home Office Project Licence (PP6655603) and in compliance with animal care and welfare protocols approved by the University of Glasgow. All animal strains were maintained under specific pathogen free conditions at the at Beatson Institute for Cancer Research, Glasgow. Mice undergoing surgical procedure were allowed to acclimatise for 7 days after transfer to their new environment. Postoperative assessment was carried out by daily monitoring of their body weight and health. Once experiments were completed, mice were euthanised by carbon dioxide asphyxiation or cervical dislocation. All mice used in these experiments were females and aged between 8-12 weeks.

2.1.1 C57BL/6 mice

Wild-type mice were purchased from Charles River or bred in house. all experiments were performed on age- matched females.

2.1.2 iCCR-reporter (REP) mice

iCCR-REP mice were developed on a C57BL/6 background by Dr. Laura Medina-Ruiz. This strain possesses a bacterial artificial chromosome (BAC) where each of iCCR coding sequence has been replaced with genes encoding for fluorescent proteins. The BAC was randomly inserted into the genome, and sequencing analysis revealed that the BAC integrations happened in the same area of chromosome 16. These integrations happened in a section of the genome containing non-coding DNA, far from the normal iCCR locus, suggesting that no genes were disrupted during the transfection process. Thus, these reporter mice should express both the inflammatory chemokine receptors and the fluorescent

protein under the same conditions. Each fluorescent protein has a distinct emission spectrum which should be detectable by microscopy and flow cytometry. These reporters were CCR1-Clover, CCR2-mRuby2, CCR3- mTagBFP2, CCR5-iRFP682.

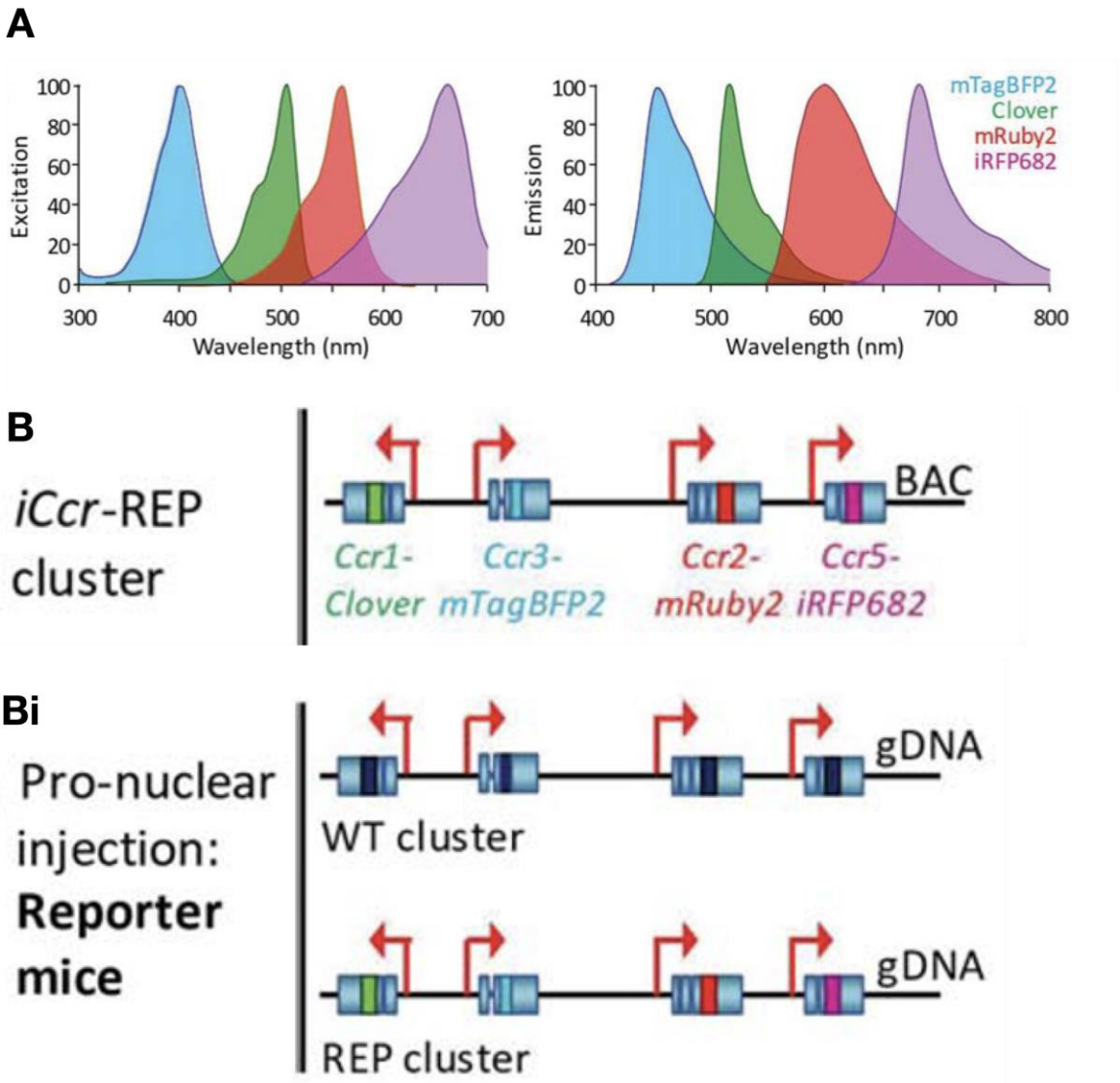

Figure 2-1 Generation of iCCR-reporter mice. A) Histograms show fluorescent reporter proteins that were selected to generate iCCR-reporter mice based on their discrete excitation and emission spectra: Clover represents the expression of CCR1 in green, mRuby2 for CCR2 in red, mTagBFP2 for CCR3 in blue, and IRFP682 for CCR5 in purple. Schematic diagrams illustrate B) the BAC structure where the iCCR locus was targeted and replaced with different fluorescent proteins, and Bi) transgenic iCCR-REP mice were generated through the insertion of the iCCR-REP BAC via pro-nuclear injection. Adapted from “Generation of the reporter mice” by Medina-Ruiz et al.[198].

2.2 In-Vivo Model of Inflammation

2.2.1 Implantation of cytokine-loaded subcutaneous osmotic pumps

To study chronic inflammation in blood and bone marrow, a subcutaneous osmotic pumps were implanted to provide continuous release of cytokines into the circulation. Mice were anaesthetised using isoflurane inhalation and were given a subcutaneous injection of 100 µl of 1 mg/ml carprofen (Rimadyl) for analgesia before the procedure was started. The surgery was carried out by trained and licensed group members; Dr. Laura-Medina Ruizs and Dr. Julie-Myrtille Bourgognon and performed under aseptic technique. A small pocket (incision) was made under dorsal skin to insert the sterile pump (Charles River, ALZET® Osmotic Pumps, model 2001) loaded with a cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) or vehicle PBS. Then, the skin incision was closed with 2-3 wound clips (Finescience,12022-09). Infusion of cytokine cocktail or the PBS was maintained for 7 days. After the infusion period, mice were culled with rising CO2 concentration and blood, BM and membrane surrounding the pump were extracted for analysis.

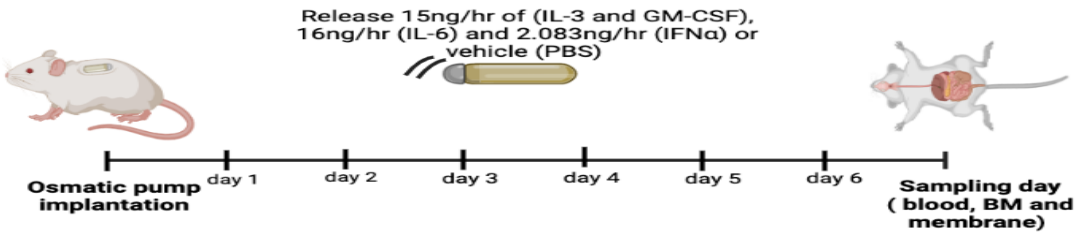

Figure 2-2 Subcutaneous Osmotic Pump implantation. Diagram describing the outline of the minipump model. Pump releases 1ul of the loaded cytokine cocktail per hour for 7 days. The concentration of cytokine cocktail was selected based on the literature, and the final infused concentration indicated above on the diagram. The pump delivers the loaded mediators into the circulation to induce acute inflammation that help to study cell trafficking during inflammatory response.

2.3 Tissue Culture Method

2.3.1 Isolating Bone Marrow Cells

Femur and tibias were collected from culled mice and excess tissue was removed. Then bones were processed under a sterile hood and directly dipped in 70% Ethanol for 1 minute to remove any bacterial contamination. The femur and tibia were flushed out to collect bone marrow cells using out using 5ml of RPMI-1640 (Sigma) in a 5ml syringe with a 23G needle. The cell suspension was filtered through a 70 µm strainer to prevent clumping and centrifuged at 400g for 5 minutes at 4C. The pellet was resuspended with 1ml of ACK lysis buffer (Gibco, A10492-01) for 1 minute to remove RBCs, then topped up with 9 ml PBS (Gibco, 14190-094) to stop the lysis reaction. Cells were centrifuged at 400g for 5 minutes and went through another round of washing steps with PBS to ensure the lysis buffer was all removed. Then a single cell suspension was made to count cells and resuspend them according to the subsequent experimental requirements.

2.3.2 L929 conditioned media- source of CSF-1

L929 CM (conditioned media) was obtained from L929 cells were kindly provided by Dr.Helen Taylor, University of Edinburgh. Cells were cultured to confluency in T225 flasks (CytoOne, Starlabs) and maintained in DMEM/F12 with Glutamax (1:1) medium (Gibco, 31331) supplemented with 1% Primocin (InvivoGen, Ant-pm-2) and 10% Heat Inactivated FBS (Invitrogen 10270106, 56C for 30min). Cells were first cultured in a T75 vented flask containing 20 ml of media. Once confluent, cells were detached using 2ml of Trypsin/EDTA 0.05% (Invitrogen 25300-054), incubated at 37°C for 2 minutes and centrifuged at 300g for 5 minutes. The resuspended pellet was split into 5 new T75 vented flasks and left

to grow until cells became confluent. For each T225 flask, 9 million cells were seeded in 90ml of media (100,000 cells/ml). Cells were then allowed to grow undisturbed for 4 days. Then, the medium was changed, and cells were left for another 7 days. After a week, the medium containing CSF-1 was collected, filtered using 0.22μm Millipore sterile vacuum (EMD, Millipore) and aliquoted for freezing at -80°C to be used later as a source of CSF-1 for macrophage differentiation.

2.3.3 Bone Marrow Derived Macrophages

Bone marrow cells were isolated as described before. Cells were resuspended at 1x106 cells/ml in modified GMEM (GIBCO 11710035) supplemented with 15% L929 CM, 10% FBS (GIBCO 10270106), 1% NEAA (GIBCO 11140050), 1% L-glutamine (GIBCO 35050061), 1mM sodium pyruvate (GIBCO 11360070), 1mM of β-Mercaptoethanol (GIBCO 31350010) and 1% Primocin (Invivogen Ant-pm-2). Then, cells were cultured by adding 10ml of cell suspension in 10 CM petri dish and incubated. Media was carefully changed on day 4 to remove cell debris and undifferentiated cells, washed with 1x PBS and replaced with a 10 ml fresh media. On day 5, differentiated macrophages were collected, using TrypLe Select (Gibco A1217701) as detachment enzyme, and re-plated in 6-well plates for stimulation.

2.3.4 In-vitro Bone Marrow Derived Macrophages (BMDM) Stimulation

Differentiated macrophages were treated with various cytokines and TLR agonists. Cells were plated in triplicate in 6 well plates at 1.2x106 cells/well and grown overnight or different time points; 4h,8h,12h or 24h, in the presence of the following stimuli using the concentration indicated below.

Table 2-1 Cytokines and TLR-Agonists*

Cytokines				*TLR-agonists*			
Cytokines	*Working Concentration*	*Supplier*	*CAT. Number*	*TLR-agonists*	*Working Concentration*	*Supplier*	*CAT. Number*
IL-3	10ng/ml	Peprotech	213-13	Pam3CSK4	150ng/ml	InvivoGen	tlrl-pms
IL-4	20ng/ml	Peprotech	214-14	HKLM	1.00E+07cells/ ml	InvivoGen	tlrl-hklm
IL-5	20ng/ml	Peprotech	215-15	Poly(I:C) (HMW)	1µg/ml	InvivoGen	tlrl-pic
IL-6	50ng/ml	Peprotech	216-16	Poly(I:C) (LMW)	5µg/ml	InvivoGen	tlrl-picw
IL-10	20ng/ml	Peprotech	210-10	LPS-EK	5µg/ml	InvivoGen	tlrl-eklps
IFNα	25ng/ml	BioLegand	752802	LPS-O127:B8	100ng/ml	Sigma-Aldarich	L45-16
IFNγ	100ng/ml	Peprotech	315-05	FLA-ST	5µg/ml	InvivoGen	tlrl-stfla
TGFß	20ng/ml	R&D systems	7666-MB-005	FSL-1	50ng/ml	InvivoGen	tlrl-fsl
GM-CSF	20ng/ml	Miltenyi Biotech	130-095-742	ssRNA40	2µg/ml	InvivoGen	tlrl-lrna40

*List of concentrations, suppliers and catalogue numbers of reagents used to stimulate In-Vitro bone marrow derived macrophages. Pam3CSK4, synthetic triacylated lipopeptide; HKLM, heat-killed bacteria; Poly(I:C) (HMW) and Poly(I:C) (LMW), Polyinosinic-polycytidylic acid high and low molecular weight, respectively; FLA-ST , flagellin from S. typhimurium; FSL-1, synthetic diacylated lipoprotein; ssRNA40, single-stranded RNA.

2.3.5 HEK-293 cells expressed different iCCR

Human embryonic kidney (HEK)-293 cells, transfected to express each one of the fluorescent reporter proteins expressed by the iCCR-Reporter strain, were provided by Dr. Laura Medina-Ruiz. These cells were used instead of antibodies as single controls to set up voltages and compensation on flow cytometry. Each HEK-293 cryo-vial was immediately thawed, and cells were transferred to a 50ml falcon tube. 10ml of DMEM/F-12, GlutaMAXTM modified medium (Gibco, 31331028) with 10% Heat Inactivated FBS (Invitrogen 10270106, 56C for 30min), and 1% Primocin (InvivoGen, Ant-pm-2) were gently added, and then cell suspension was centrifuged at 300g for 5 minutes. The pellet was resuspended in 10ml of culture medium and allowed to recover until confluency in a T75 vented flask. When cells were approximately 80% confluent, they were detached using 2ml of Trypsin/EDTA 0.05% (Invitrogen 25300-054) for 2 minutes at 37°C and centrifuged for 5 minutes at 300g after the enzyme was deactivated by the medium. The pellet was used to seed new T75 vented flasks, and cells were grown to confluency once more. This was necessary as fluorescent reporter protein expression by HEK cell is very low

after defrosting and increases only after a week of culture. Cells were then detached after becoming confluent once more, and then they were washed with 10 ml PBS (Gibco, 14190-094) and centrifuged. The pellet was resuspended in 1ml Fixation Buffer (Biolegend, 420801) for 20 minutes at RT. After being fixed, cells were washed in PBS and resuspended in 1 ml of FACS buffer to be stored at 4°C [PBS (Gibco, 14190-094) + 2mM EDTA (Invitrogen, 15575038) + 2% FBS (Invitrogen, 10270106)]. Some of the cell pellet was gently resuspended in 1ml of FBS, followed by 2ml of FBS+20% DMSO, and then aliquoted into cryo-vials to create a stock of HEK-293 cells. Cells were then stored at -80 for at least one night and then transferred to liquid nitrogen.

2.4 Molecular Methods

2.4.1 RNA Extraction from cells

Bench surface, pipettes and tubes were all cleaned with RNaseZap® before starting RNA extraction to reduce RNA degradation from any environmental contamination. BMDM after overnight or time point (4h, 8h, 12h, or 24h) stimulation were disrupted using buffer RLT and homogenised using the QIAshredder system (Qiagen, 79654). Then, the homogenised lysate was applied to a RNeasy Mini spin column and the total RNA was extracted using the RNeasy Mini Kit (Qiagen, 74104), including the optional step of on-column DNase digestion using RNAse-free DNase Set (Qiagen,79254). To completely remove any DNA residues, DNase treatment was done using DNA-free™ DNA Removal Kit (Invitrogen, AM1906) according to the manufacturer's instructions. Finally, total RNA was

eluted in 30ul RNase free water and RNA concentration was quantified using the NanoDrop 1000 Spectrophotometer (Thermo Scientific). RNA samples were directly stored at -80°C until needed.

2.4.2 cDNA Generation

RNA was reverse transcribed using High-Capacity RNA to cDNA Kit (Applied Biosystems). RNA samples were first thawed on ice, then 1ug of total RNA was used to generate cDNA, according to the manufacturer's instructions. The following thermal cycle conditions were applied; 37°C (60min), 95°C (5min), and 4(∝), and the reaction was run on Veriti™ 96-well Thermal Cycler (Applied Biosystems, Model #9902). Finally, cDNA was stored at -20°C for subsequent analysis.

2.4.3 Generating Standard for qPCR

Standard (STD) primers used in this study are listed in table 2-2. Each of the STD primer pairs was amplified in a single 50ul PCR tube using a Platinum™ Taq DNA Polymerase kit (Invitrogen) to generate standards with known copy numbers of each target gene. All steps were carried out in accordance with the manufacturer's instructions, and after 2 minutes of an initial denaturation step at 94°C, the reaction mixtures were run through 35 thermal cycles. Each cycle consisted of the following three steps:

- A denaturation step at 94°C for 15 seconds
- An annealing step at the calculated Tm (melting temperature) of each primer pair for 30 seconds
- An elongation step at 72°C for 2.5 minutes

After 35 cycles, there was a last elongation step for 5 minutes at 72°C. While the reaction was taking place, 1% of agarose gel in 1x TAE buffer was prepared by heating a flask containing 100 ml of TAE buffer and 1g of agarose powder in the microwave for around two minutes or until the agarose was completely dissolved (swirling every 30 sec to avoid boiling). 1ul of ethidium bromide was added once the mixture had cooled and then slowly poured into a tray to avoid bubbles. A comb was then placed, and the gel was left to sit at RT for 45 minutes to solidify. Once thermal cycles were completed, PCR products were run in electrophoresis for 45 minutes at 110 volts after being stained with 10ul of 6x loading dye (BioLabs, B7025S) and visualised under UV light (AlphaImagers™, Alpha Innotech). The PCR products were purified using PureLink™ PCR Purification Kit (Invitrogen) as per the manufacturer's instructions after specificity was verified by observing a single amplicon of the expected size compared to a 1Kb DNA ladder. The concentration of the purified products was determined before they were diluted 100-fold and stored at -20°C using the Nanodrop (Thermo Scientific). Finally, the formula below was used to determine how many molecules were present in each standard.

$$\text{Number of copies} = \frac{\text{concentration } \left(\frac{\text{g}}{\mu\text{l}}\right)}{Molecular\ weight\ of\ dsDNA\ \left(\frac{g}{mol}\right)} x\ Avogadro's\ constant$$

$$* Molecular\ weight\ of\ dsDNA\ \left(\frac{g}{mol}\right) = number\ of\ primer's\ bp\ x\ average\ weight\ of\ sDNA\ bp$$

Table 2-2 List of the STD primer sequences used in this study.

Gene	Forward Standard	Reverse Standard
CCR1	5’ TCT AGT TGG TCC ACA GAG AGG 3’	5’ CAA TCT TAG CTT CCA TGC CTG 3’
CCR2	5’ ACCACAGAATCAAAGGAAATGG 3’	5’ GTTGCCCACAAAACCAAAGA 3’
CCR3	5’ CCT ATG CTT TAC CAC CAC CCA T 3’	5’ CCA CCT GGG AAC GTG TTG TT 3’
CCR5	5’ GGC CAA CAA TTG CTT TAA CCT 3’	5’ GAA GTC CTC ATA CTC AGC CTG G 3’
Tbp	5’ GAG TTG CTT GCT CTG TGC TG 3’	5’ ATA CTG GAA AGG CGG AAT GT 3’

2.4.4 Quantitative PCR

Each cDNA sample was analysed using Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR analyser in quadruplicate on 384 well-plates. For all qPCR analysis, the final reaction volume of each well was 10µl, and the reaction setup contained; 5µl of PerfeCta®Sybr®Green master mix (VWR™ 733-1386), 1µl cDNA, 0.15µl primer mix (1:1 of 100µM stock) and 4µl nuclease-free water. The thermal conditions were set up as follow; denaturation and polymerase activation at 95°C (30sec) followed by 40 thermal cycles for cDNA amplification at 95°C (4sec) and 60°C (25sec). The expression level of inflammatory chemokine receptors was quantified by generating standard curves for each one of the genes analysed (using serial dilutions of cDNA) and normalised to the housekeeping gene, Tbp. Primers used are listed in table 2-3.

Table 2-3 List of qPCR primers' sequence used in this study.

Gene	Forward Primer	Reverse Primer
CCR1	5' CGG CTT TGA CCT TGT TCT CA 3'	5' GCC CTC ATT TCC CCT ACA A 3'
CCR2	5' TCA GTT CAT CCA CGG CAT ACT 3'	5' TGA CAA GGC TCA CCA TCA TC 3'
CCR3	5' ACC TTC GGC TCT TTT TCC AC 3'	5' TGT TCT TTC CAT TTT CTC ACC A 3'
CCR5	5' GGC CAA CAA TTG CTT TAA CCT 3'	5' AGC AAA CAC AGC ATG GAC AA 3'
Tbp	5' TGC TGT TGG TGA TTG TTG GT 3'	5' AAC TGG CTT GTG TGG GAA AG 3'

2.5 Protein Analysis

2.5.1 Tissue Processing for Staining

2.5.1.1 Bone Marrow (BM)

To collect bone marrow cells, the same protocol for isolating bone marrow cells was followed. Briefly, tibias and femurs were cut on both ends, and the bone marrow was flushed out using a 5ml syringe with 5ml of RPMI 1640 (Sigma). The cell suspension was filtered with a 70um strainer, and red blood cells were removed with ACK lysis buffer incubation. The pellet was resuspended 150µl of PBS to proceed with antibody staining.

2.5.1.2 Blood

Blood samples were extracted from portal vein by inserting a 1ml syringe with a 23G needle soaked in 0.5M EDTA (Invitrogen, 15575-038) into the inferior vena cava. Then blood was collected and placed in a 15ml falcon tube containing 100 µL of 0.5M EDTA to prevent blood clotting. Samples were centrifuged at 400g for 5 minutes at RT then supernatant was discarded. RBCs were lysed using 1ml of ACK lysis buffer (Gibco, A10492-01) and incubated for 5 minutes. The lysis reaction was stopped by adding 0.5ml of PBS and the samples were centrifuged again. The samples needed another RBC lysis for 2 minutes to make sure the pellet was white,

and all RBCs were lysed. The remaining leukocytes were then resuspended PBS (Gibco, 14190-094) and processed for antibody staining.

2.5.1.3 Minipump Surrounding Membrane

The membrane surrounding the minipump was isolated and collected into a 2ml Eppendorf tube containing 1 mL of HBSS (Gibco, 24020-091). The membrane samples were then transferred to a new 2ml Eppendorf tube containing 1 mL of HBSS with 13 Wünsch units of Liberase per ml (Roche) after being weighted to do absolute counts later for data analysis. The membrane samples were digested at 37˚C for 1 hour in a thermoshaker (ThermoFisher) at 1000 rpm. To inactivate the Liberase 20µl of FBS was added. Then, the membrane cells were smashed and filtered through a 70 µm strainer into a 50 ml falcon tube and washed in PBS (Gibco, 14190-094). Filtered samples were centrifuged at 400g for 5 minutes. The pellet was resuspended in 150µl of PBS and processed for antibody staining.

2.5.2 Flow Cytometry Staining

Various tissue samples prepared as described above (Blood, BM and membrane) were stained by adding 100µl of fixable viability dye eFluor 506 (eBioscience, 65086618) at 1/1000 dilution in PBS. Cells were incubated for 20 minutes at 4˚C. Cells were then centrifuged at 400g for 5 minutes and washed twice with FACS buffer [PBS (Gibco, 14190-094) + 2mM EDTA (Invitrogen, 15575038) + 2% FBS (Invitrogen, 10270106)]. The supernatant was discarded, and cells were stained with appropriate antibodies cocktail containing Fc Block (Macs Miltenyi Biotech) at a 1/100 concentration in FACS buffer or Brilliant Stain Buffer (BD Bioscience) for 20 minutes at 4˚C. Cells were washed twice again with FACS buffer, centrifuged at 400g for 5 minutes and resuspended in 150µl of FACS buffer

and either analysed on the same day or fixed with 100µl Fixation Buffer (Biolegend, 420801) for 20 minutes at 4°C. Once fixed, cells were washed twice with FACS buffer and resuspended in 150µl of FACS buffer for analysis the next day. Samples were filtered through a nylon mesh before being analysed to remove any clumps that could clog the machine.

Table 2-4 Flow Cytometry Antibodies*.

Antibody	Fluorophore	Clone	Supplier	CAT. Number
Fixable Viability dye	eFluor 506		eBioscience	65-0866-18
CD45	PerCP-Cy5.5	30-F11	eBioscience	45-0451-82
CD11b	APC-Cy7	M1/70	Biolegend	101226
SiglecF	BV711	E50-2440	BD Bioscience	740764
F480	BV785	BM8	Biolegend	123141
Ly6C	AF700	HK1.4	Biolegend	128024
CD11c	BUV737	HL3	BD Bioscience	612796
MHCII	BV605	M5/114	Biolegend	107639
Ly6G	BUV395	1A8	BD Bioscience	563978
CD19	PE-Cy7	ID3	eBioscience	25-0193-82

*List of antibody targets used to stain BM, blood, and membrane cells along with supplier, clone, catalogue number, and conjugated fluorophore for each one.

2.5.3 Flow cytometry Analysis

The Fortessa (BD Bioscience) cytometer was used for every flow cytometry acquisition, and the data were analysed using the FlowJo software. Voltage compensation was carried out on the stained samples using UltraCompeBeads (Bioscience, 01-2222-42) as single-stained controls. Fluorescence Minus One controls were used to in order to identify the positive populations. Using a standard gating strategy, samples were first selected for the cell gate based on FSC (Forward scatter) and SSC (side scatter). Viability stain was then used to assess

which cells were still alive. Negative cells for the stain were chosen, and single cells were subsequently gated based on FSC-H and FSC-A.

2.6 Statistical Analysis

All statistical analysis was performed using Prism GraphPad software. For data with a normal distribution, a one-way ANOVA was used. For data that were not normally distributed, either a non-parametric one-way ANOVA, using the Kruskal-Wallis test or a Mann-Whitney test was used, as appropriate.

Chapter 3 In-vitro Regulation of iCCR Receptor Expression in Bone Marrow Derived Macrophages

3.1 Overview

The expression of the inflammatory chemokine receptors CCR1, CCR2, CCR3 and CCR5 is regulated during cellular differentiation or inflammation. Monocytes strongly express CCR2, and as they are recruited into or within inflamed tissue and differentiate, their expression of CCR2 decreases[354, 355]. This change in chemokine receptor expression has also been reported on other leukocytes. For example, naïve T cells fluctuate the expression of CCR5 during differentiation into memory or effector cells[356]. However, determining how these inflammatory chemokine receptors are regulated, and how they influence cell trafficking during immune responses has not been studied in detail. Therefore, first, we examined whether the activation of in vitro bone marrow-derived macrophages via TLR agonists and some inflammatory cytokines could modulate iCCR receptor expression. Then, with an iCCR fluorescent reporter strain (iREP), we examined the surface expression of iCCRs. This strain expresses spectrally distinct fluorescent proteins for each of CCRs 1, 2, 3, and 5 and allows us to directly visualise changes in iCCR expression in the cell.

Macrophages were selected as a cell type for studying the expression of iCCRs. A protocol to generate BM-derived macrophages was chosen because it offers high yields and purity of differentiated macrophages and because they are known to express three of the four iCCRs 1, 2, and 5 at high levels[198]. Thus, allowing us to determine if these receptors are redundantly expressed or only expressed under certain inflammatory conditions, such as bacterial or viral infection.

3.2 iCCR expression in BMDM under inflammatory conditions

Bone marrow cells were isolated from WT C57BL/6 (9-12 weeks old) mice to determine macrophage expression of iCCRs in vitro under several inflammatory conditions. Cells were then cultured in the presence of CSF-1 to induce macrophage differentiation. Light microscopy was used each day to monitor the progression of cell differentiation. Differentiated macrophages were identified by their increased confluency and changing morphology into spindle-shaped cells and becoming more adherent at day 5 compared to day 3 (Figure 3-1). On day 5, BMDMs were detached using detachment enzyme TrypLe Select (See Materials and Methods, sections 2.3.3 and 2.3.4), replated in 6 well plates, and allowed to adhere for 3 hours before cells were stimulated with a range of TLR ligands and pro-inflammatory and anti-inflammatory cytokines. After stimulation overnight, BMDMs were collected for further analysis (Figure 3-1).

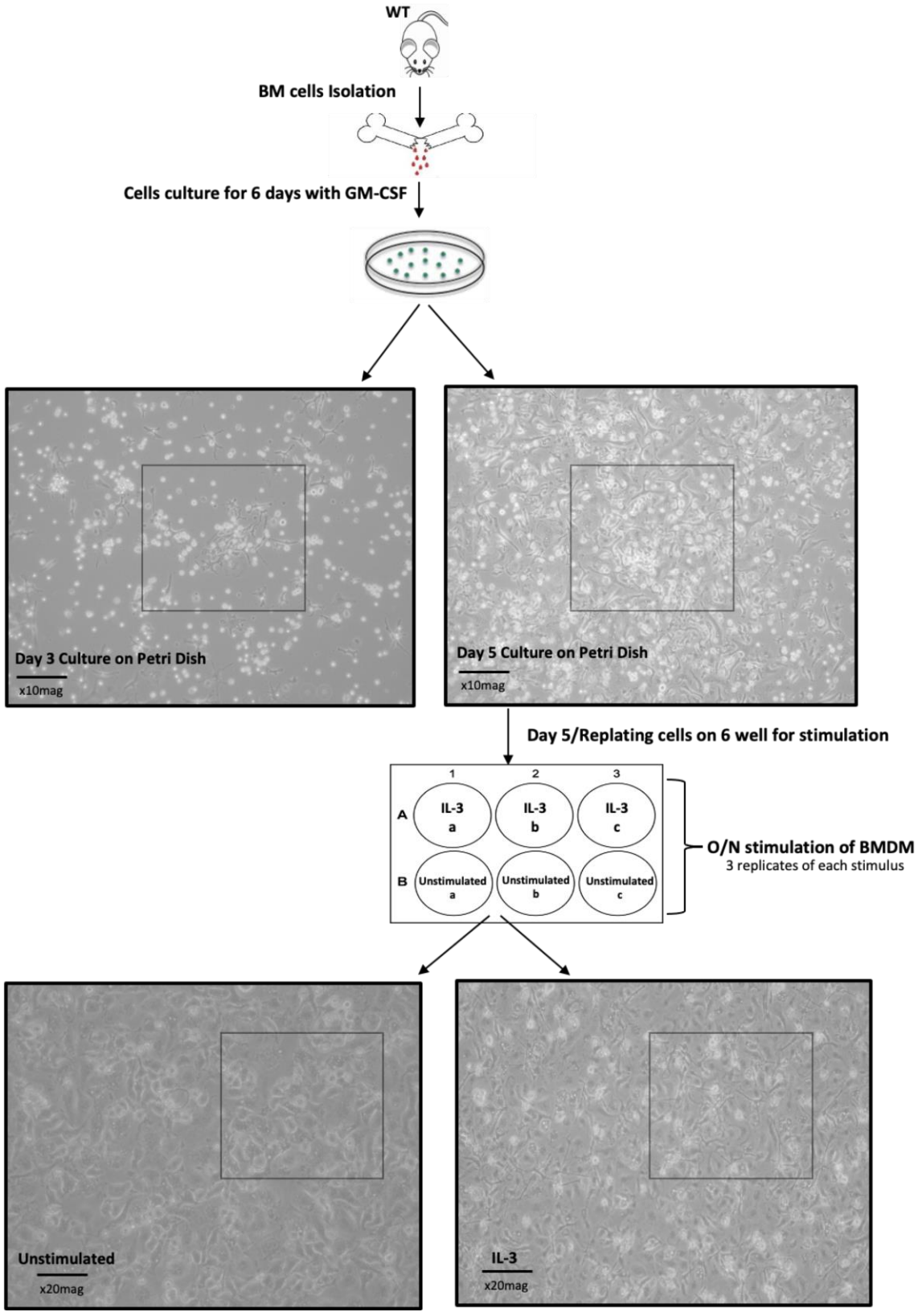

Figure 3-1 Diagram summarising BMDM differentiation and stimulation protocol. Bone marrow cells were obtained from the tibia and femur of mice then cells were cultured in GMEM media containing CSF-1 for macrophage differentiation. On day 5, differentiated macrophages were collected and then replated in triplicate in 6 well plates for stimulation. Cells were detached and processed after stimulation for subsequent analysis. Macrophage colonies are highlighted in the square to show their increased confluence on day 5 compared to day 3 and how stimulated cells become more elongated and spindle-shaped than unstimulated cells**.**

3.2.1 The effect of ON stimulation by cytokines on iCCR mRNA expression level by BMDM

After BMDMs were stimulated overnight with the cytokines shown in Figure 3-2, we examined the mRNA expression levels of each inflammatory chemokine receptor, CCR1, CCR2, CCR3, and CCR5. By using qPCR, the expression of these receptor genes was quantified and expressed as fold change compared to unstimulated cells (Figure 3-2).

3.2.1.1 The effect of pro-inflammatory cytokines

Following overnight treatment of BMDM, neither IL-4 nor IL-5 had any significant effects on the expression of CCR1 and CCR5 in comparison to the unstimulated control cells (Figure 3-2 A,D). Also, GM-CSF did not change CCR5 expression, but it increased CCR1 expression by around 6-fold compared to the unstimulated control cells (Figure 3-2 A,D). IL-3 and IL-6 significantly increased CCR1 expression by approximately 4-fold compared to the unstimulated control cells. However, CCR5 expression only slightly increased in response to IL-6 and IL-3 stimulation, but this was not enough to be statistically significant when compared to the unstimulated control cells (Figure 3-2 A,D).

The pro-inflammatory cytokine IFNα, which is typically induced in response to viral infection, was the only stimulatory agent that significantly increased CCR5 expression in BMDM compared to the unstimulated control cells, with an increase of about 4.5-fold (Figure 3-2 D). IFNγ, in contrast, did not significantly increase CCR5 expression compared to the unstimulated control cells. However, CCR1 mRNA expression was not altered significantly compared to the unstimulated control cells following either IFNα or INFγ treatment of BMDM (Figure 3-2 A).

Regarding the expression of CCR2 and CCR3, there were no detectable differences between the BMDM stimulated with pro-inflammatory cytokines and

the non-stimulated control cells (Figure 3-2 B,C). Overall, IFNα can act as a stimulus for the induction of CCR5 expression at transcriptional levels, whereas GM-CSF, IL-3, and IL-6 are inducers for CCR1 mRNA expression in BMDM.

3.2.1.2 The effect of anti-inflammatory cytokines

To assess the effect of anti-inflammatory cytokines on iCCR transcriptional levels, BMDMs were stimulated overnight with IL-10 and TGF-β. The results shown in Figure 3-2 A and D demonstrate that neither IL-10 nor TGF-β had any detectable effect on the mRNA expression of CCR1 and CCR5 when compared to the unstimulated control cells. Also, the expression of CCR2 and CCR3 remained unchanged in BMDM following overnight treatment with either agent (Figure 3-2 B,C).

Together, these data support our earlier findings that CCR2 and CCR3 expression in BMDM is not significantly regulated after cytokine activation. Furthermore, neither the transcription of CCR1 nor CCR5 in BMDM are altered in response to anti-inflammatory cytokines.

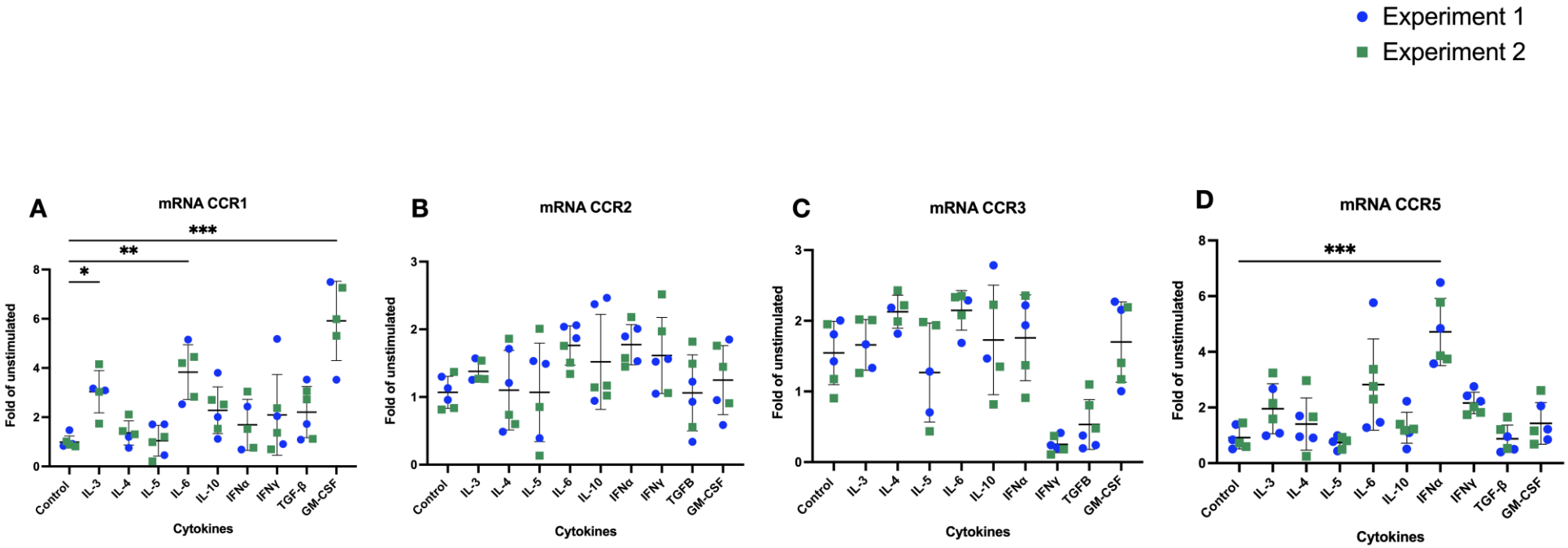

Figure 3-2 Effect of cytokines on iCCR mRNA levels in bone marrow-derived macrophages. Graphs show the change in mRNA expression levels of A) CCR1, B) CCR2, C) CCR3 and D) CCR5 after ON stimulation with cytokines indicated on the X-axis. Results are normalised to Tbp, and mRNA levels are expressed as fold change compared to the non-stimulated cells. *P <0.05, **P <.01, ***P <.001, One-way ANOVA, non-parametric test. Results are shown as Mean ± SD of 2 independent experiments.

3.2.2 The effect of ON stimulation by TLR agonists on iCCR mRNA expression level by BMDM

To evaluate how the activation of TLR signalling using synthetic PAMPs derived from bacteria and viruses will influence the expression of each iCCR, BMDMs were also treated with variety of TLR-agonists that mimicked bacterial or viral components (Detailed in Figure 3-3). Following overnight stimulation, iCCR mRNA expression was quantified by qPCR and expressed as fold change compared to unstimulated cells.

3.2.2.1 The effect of bacterial-derived TLR agonists

We found from the qPCR analysis that the synthetic bacterial lipopeptide Pam3CSK4, which selectively triggers TLR1/2 heterodimer, significantly increased CCR1 expression by 2.5-fold compared to the unstimulated control cells (Figure 3-3 A). A similar effect was observed after stimulating TLR4 on BMDM with gram-negative bacterial lipopolysaccharide (LPS) from two different strains of Escherichia coli, K12 and 0127: B8 (Figure 3-3 A). The gram-positive intracellular bacterium heat-killed listeria monocytogenes (HKLM), a ligand for TLR2, marginally increased CCR1 expression; however, this increase was not statistically significant (Figure 3-3 A). Mycoplasma lipopeptide fibroblast stimulating ligand-1 (FSL-1), a TLR2/6 ligand, upregulated CCR1 by approximately 3-fold compared to the unstimulated control cells (Figure 3-3 A). In contrast to the CCR1 results, we could not detect any significant induction of CCR5 expression by BMDM after stimulating cells with bacterial-derived TLR agonists (Figure 3-3 D). Also, there were no significant differences in CCR2 and CCR3 expression between the stimulated BMDM and the unstimulated control cells (Figure 3-3 B,C).

Thus, a significant induction of CCR1 transcription occurs as a result of the activation of TLR1/2, TLR2/6, and TLR4 signals by bacterial components in BMDMs.

3.2.2.2 The effect of viral-derived TLR agonists

However, stimulation of BMDM with viral-derived TLR agonists, double-stranded RNA (dsRNA), Poly I:C (HMW) and (LMW), which activate TLR3, only increased CCR5 expression by around a 4-fold compared to the unstimulated control cells, with no detectable effects on CCR1 expression (Figure 3-3 A,D). Neither CCR1 nor CCR5 expression significantly changed in response to TLR7 activation by viral synthetic ssRNA (Figure 3-3 A,D). As previously, treatment of BMDM with viral-derived TLR agonists did not result in any detectable differences for CCR2 and CCR3 expression compared to the unstimulated control cells (Figure 3-3 B,C). This would imply that CCR2 and CCR3 are not regulated in mature macrophages.

In summary, the results shown in Figure 3-3 suggest that CCR1 and CCR5 expression fluctuates depending on the inflammatory condition, where CCR1 often responds to bacterial infection, whereas CCR5 is a viral response. However, CCR2 and CCR3 are not significantly induced in macrophages in response to inflammation.

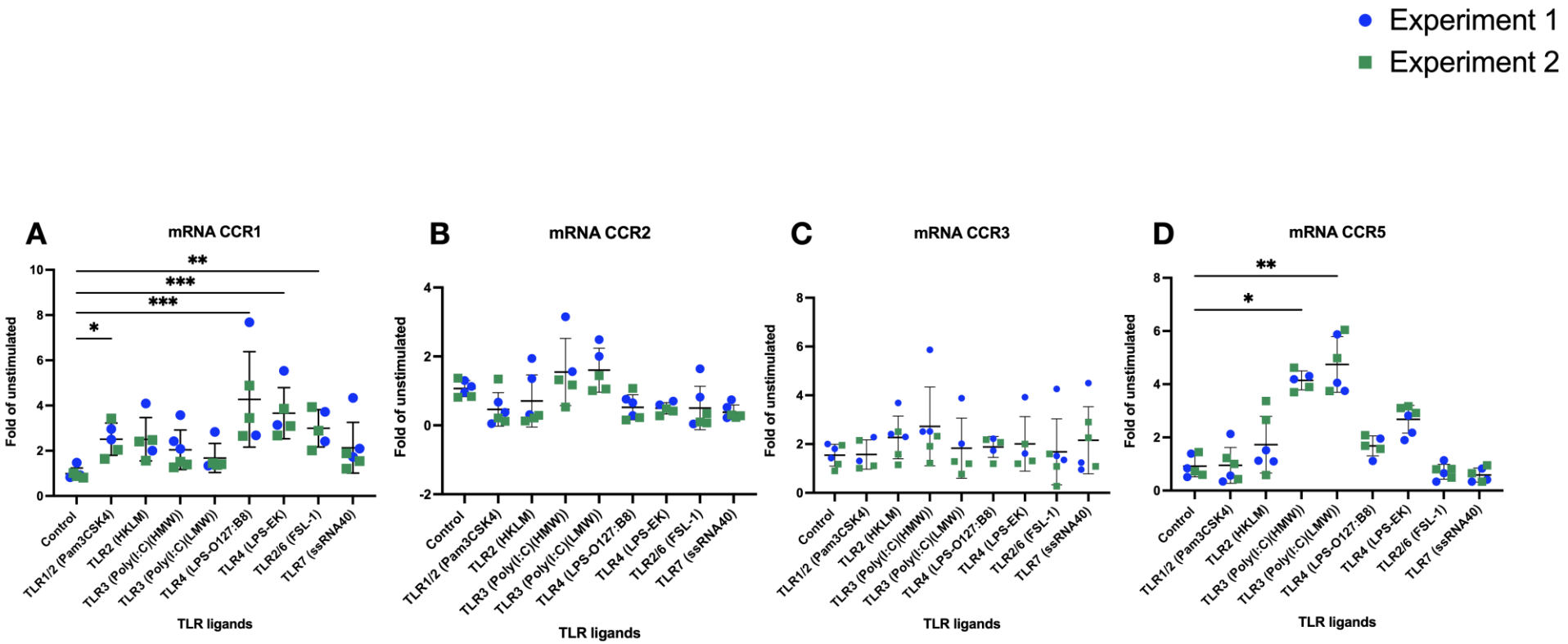

Figure 3-3 Effect of TLR-agonists on iCCR mRNA levels in bone marrow-derived macrophages. Graphs show the change in mRNA expression levels of A) CCR1, B) CCR2, C) CCR3 and D) CCR5 after ON stimulation with cytokines and TLR-agonists indicated on the X-axis. Results are normalised to Tbp and mRNA levels are expressed as fold change compared to the non-stimulated cells. *P <0.05, **P <.01, ***P <.001, One-way ANOVA, non-parametric test. Results are shown as Mean ± SD of 2 independent experiment**.**

3.3 Analysis of CCR1 and CCR5 expression in BMDM under inflammatory conditions at different time points

Next, a time point experiment was carried out to determine if the effects of cytokines and TLR agonists are direct or secondary to the initial stimulus. CCR2 and CCR3 were not included in this experiment since BMDMs did not show any detectable changes in their expression in response to any of the tested agents. To examine the temporal aspects of CCR1 and CCR5 transcriptional induction, stimuli that significantly enhanced the mRNA levels of CCR1 and CCR5 in the previous overnight experiment were selected. Therefore, BMDMs were stimulated with a selected panel of cytokines and TLR ligands for 4, 8, 12, and 24 hours as specified in the following figures.

3.3.1 The effect of cytokines on CCR1 expression in BMDM

3.3.1.1 IL-3

Results from qPCR analysis in Figure 3-4, A showed that treatment of BMDM with IL-3 started to induce CCR1 mRNA expression at 4 hours. However, this increase was not statistically significant when compared to the unstimulated control cells. CCR1 expression was further induced and significantly increased at 8 hours compared to the unstimulated control cells by around 7-fold. At 12 hours, its expression decreased to a 4-fold increase and then increased by around 6-fold at 24 hours compared to the unstimulated control cells (Figure 3-4, A).

Therefore, IL-3 rapidly induces an increase in CCR1 expression, and the expression stays elevated up to 24 hours later.

3.3.1.2 IL-6

Similar to the effect of IL-3, qPCR analysis showed that IL-6 stimulation significantly enhanced CCR1 mRNA expression in BMDM after 8 hours; however, this induction was less strong than seen with IL-3 and only represented an approximately 3.5-fold induction compared to the unstimulated control cells (Figure 3-4, B). After that, CCR1 expression remained unchanged at 12 hours, showing a comparable increase to 8 hours of around 3.5-fold. However, it did not reach its maximum induction until 24 hours, when it increased by roughly 7.5-fold in comparison to the unstimulated control cells (Figure 3-4, B).

Therefore, IL-6 increased CCR1 expression more gradually than IL-3, but by 24 hours, both IL-3 and IL-6 showed high CCR1 expression compared to unstimulated control cells.

3.3.1.3 IFNα

IFNα had a barely detectable effect on CCR1 mRNA expression in BMDM, which increased by around 2.5-fold at 4 hours compared to the unstimulated control cells (Figure 3-4, C). At 8 hours, a similar increase was seen, after which CCR1 expression began to decline at 12 hours, reaching approximately baseline levels. Finally, at 24 hours, we detected around a 3-fold increase in CCR1 expression compared to the unstimulated control cells (Figure 3-4, C).

Thus, INFα also induces a peak response after 24 hours of stimulation, but the increase in CCR1 expression is less than what was seen for IL-6 (~2.5-fold increase vs ~7.5-fold increase).

3.3.1.4 GM-CSF

GM-CSF stimulation of BMDM led to a similar upregulation of CCR1 mRNA expression to that observed for IL-3 by inducing it at 4 hours by about 3.3-fold and reaching statistically significant levels at 8 hours by about a 7-fold increase in comparison to the unstimulated control cells (Figure 3-4, D). At 12 hours, CCR1 expression in BMDM was reduced to a 4-fold increase before increasing by about 6.3-fold at 24 hours compared to the unstimulated control cells (Figure 3-4, D).

Thus, CCR1 expression is affected similarly by GM-CSF and IL-3, peaking at 8 hours and remaining elevated at 12 and 24 hours.

3.3.1.5 Combination

BMDMs were also treated with a combination of LPS, IL6, IL3, and GM-CSF to examine how a combination of these stimuli would impact CCR1 expression. This resulted in CCR1 upregulation by around 7-fold at 8 hours, 8.5-fold at 12 hours, and a peak induction of roughly 17-fold at 24 hours as compared to the unstimulated control cells (Figure 3-4, E).

In summary, the results shown in Figure 3-4 demonstrate that GM-CSF and IL-3 can act as direct transcriptional inducers of CCR1 by starting their action at 4 hours, and that combining these cytokines with IL-6 and LPS results in an additive increase in CCR1 mRNA levels.

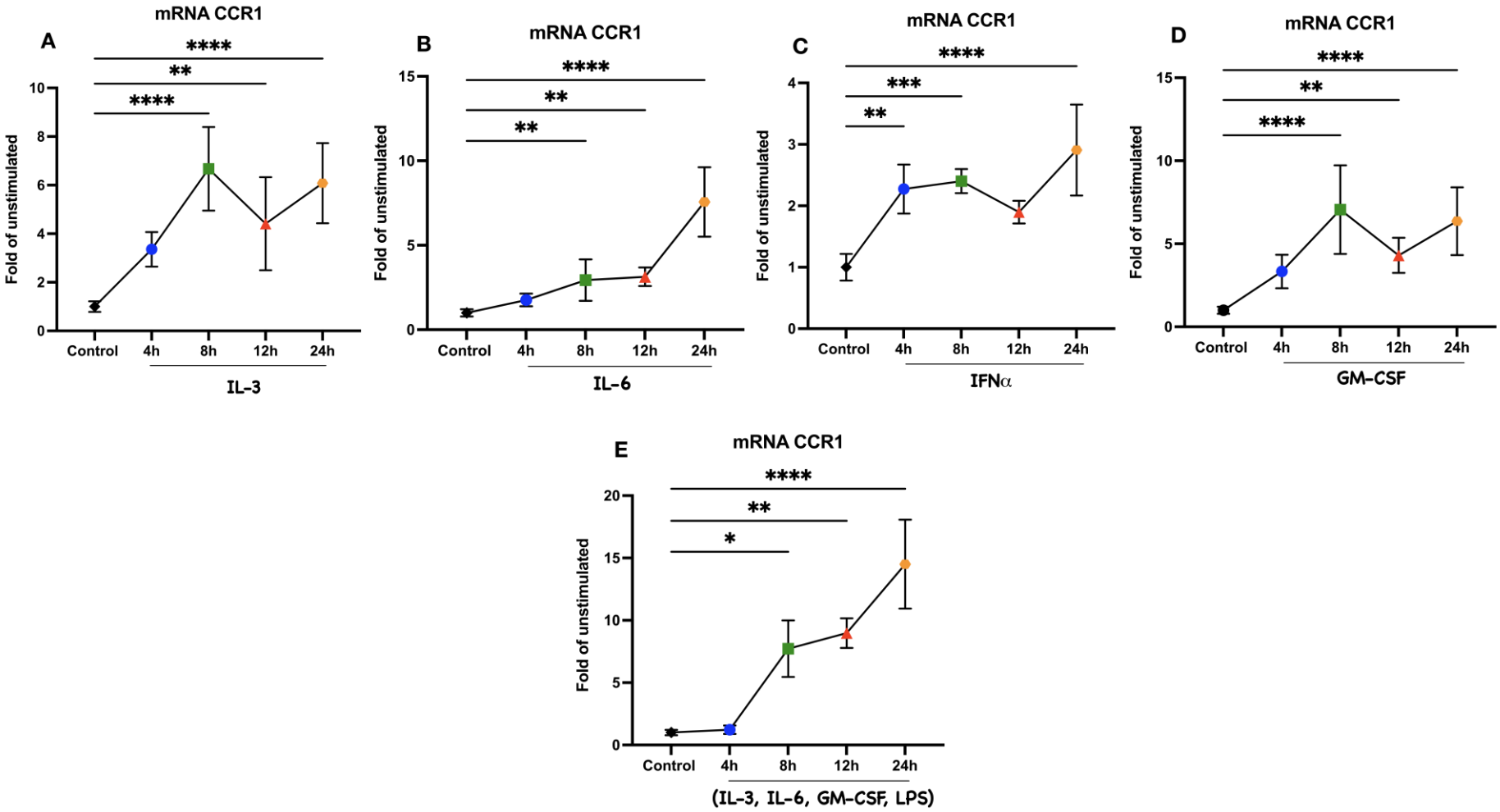

Figure 3-4 Effect of Cytokines on CCR1 mRNA levels in BMDM at different time points. Graphs show the expression of CCR1 at 4h, 8h, 12h, and 24h in BMDM treated with A) IL-3, B) IL-6, C) IFNα, D) GM-CSF, and E) combination of IL-3, IL-6, GM-CSF, and LPS. Results are normalised to Tbp and mRNA levels are expressed as fold change compared to the non-stimulate BMDM. *P <0.05, **P <.01, ***P <.001, and ****P <.0001, One-way ANOVA, non-parametric test. Results are shown as Mean ± SD of 3 independent experiments.

3.3.2 The effect of TLR agonists on CCR1 expression in BMDM

3.3.2.1 Bacterial-derived TLR agonists

3.3.2.1.1 TLR1/2

After 8 hours of treatment with synthetic bacterial lipopeptide Pam3CSK4, a TLR1/2 ligand, the CCR1 mRNA levels in BMDM reached a plateau, showing a statistically significant increase of about 3.5-fold compared to unstimulated control cells (Figure 3-5, A). However, by 24 hours, this plateau began to fade gradually, resulting in a 2.6-fold increase that was no longer statistically significant when compared to the unstimulated control cells (Figure 3-5, A).

3.3.2.1.2 TLR4

A tendency toward decreased CCR1 mRNA expression as early as 4 hours was seen after stimulation of BMDM with the TLR4 ligand, LPS, from two different strains of E. coli, 0127:B8 and K12, as shown in Figure 3-5 B and C, respectively. However, this was not significantly different from the unstimulated control cells. LPS from both E. coli strains increased CCR1 mRNA expression in a similar manner as compared to the unstimulated control cells, starting at roughly 3.3-fold at 8 hours, increasing to 4.2 and 3.7, respectively, at 12 hours, and peaking at about 4.6 and 4.2, respectively, at 24 hours (Figure 3-5, B, C).

3.3.2.1.3 TLR5

Even though bacterial flagellin (FLA-ST), a TLR5 ligand, tended to decrease CCR1 expression after 4 hours, its stimulatory effect demonstrated the highest induction of CCR1 mRNA levels, increasing by around 11 times at 24 hours in comparison to the unstimulated control (Figure 3-5 D). Its effect on CCR1 expression with other TLR agonists began at 8 hours with around a 2.5-fold

increase, but it doubled at 12 hours with a 4.5-fold increase and peaked at 24 hours compared to the unstimulated control cells (Figure 3-5 D).

3.3.2.1.4 TLR2/6

TLR6 activation by mycoplasma lipoprotein had no detectable effect on CCR1 expression in BMDMs at 4 hours as compared to the unstimulated control cells (Figure 3-5 E). As expected, CCR1 mRNA expression significantly increased at 8 hours; however, this was a minor induction of about 2-fold, which then remained at almost the same levels for 12 hours before peaking at 24 hours by about 2.7-fold in comparison to the unstimulated control cells (Figure 3-5 E).

Overall, the results outlined in Figure 3-5 show that CCR1 transcription levels in BMDM significantly and rapidly increase when TLRs are activated with PAMP derived from bacteria.

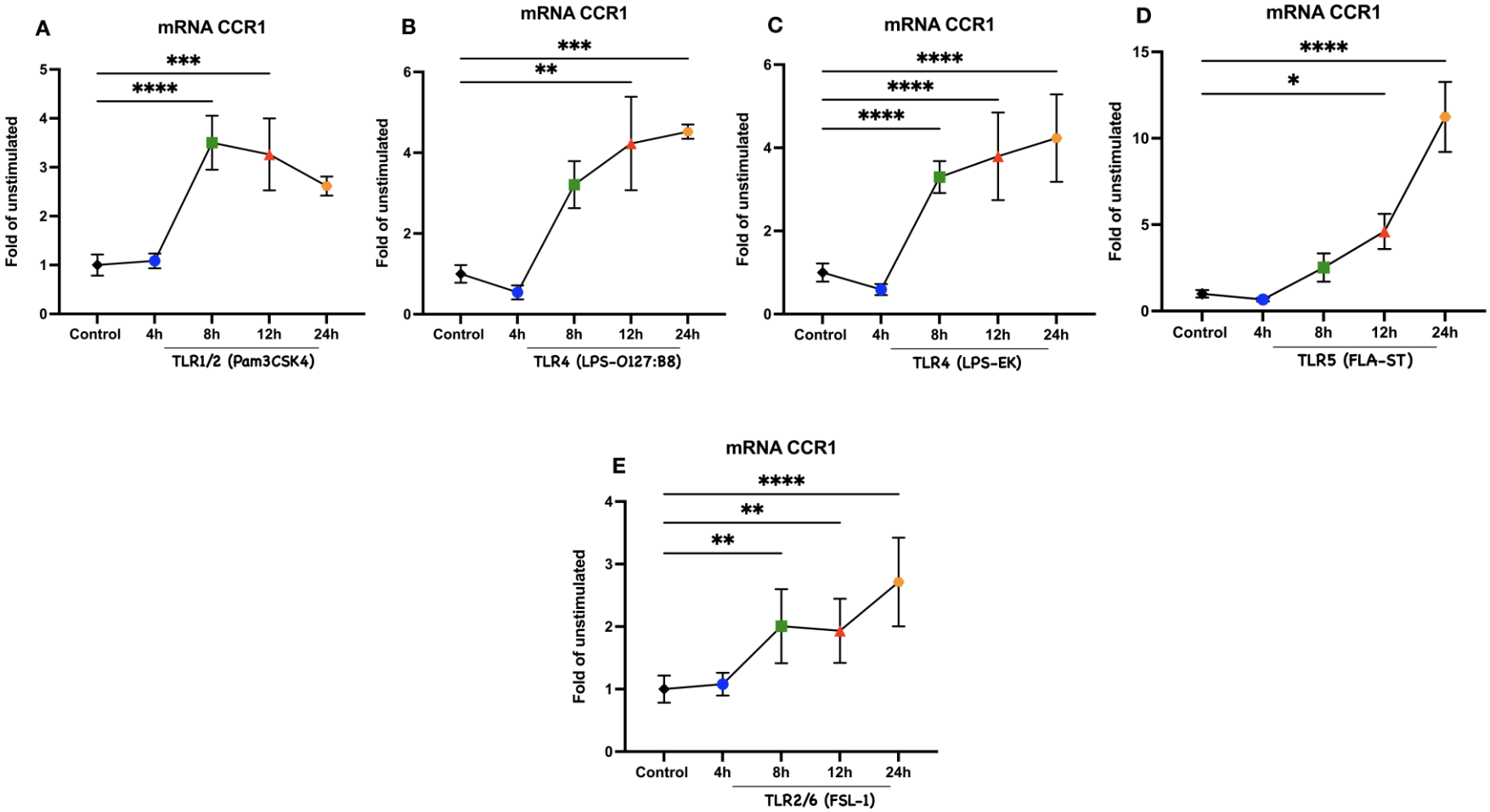

Figure 3-5 Effect of bacterial-derived TLR agonists on CCR1 mRNA levels in BMDM at different time points. Graphs show the expression of CCR1 at 4h, 8h, 12h, and 24h in BMDM treated with agonists for A) TLR1/2, B and C) TLR4, D) TLR5, and E) TLR2/6. Results are normalised to Tbp and mRNA levels are expressed as fold change compared to the non-stimulate BMDM. *P <0.05, **P <.01, ***P <.001, and ****P <.0001, One-way ANOVA, non-parametric test. Results are shown as Mean ± SD of 3 independent experiments.

3.3.2.2 Viral-derived TLR agonists

3.3.2.2.1 TLR3

BMDM in response to Poly I:C (HMW) and (LMW) at 4 hours did not significantly reduce CCR1 expression, but it did show a tendency toward lower expression when compared to the unstimulated control cells, which is similar to what was observed for TLR4 and TLR5 agonists (Figure 3-6 A, B). At 8 hours, Poly I:C (HMW) and (LMW) began to trigger CCR1 mRNA expression, but their effects were not statistically significant compared to the unstimulated control cells (Figure 3-6 A, B). However, they significantly increased CCR1 expression at 12 hours by around 2-fold in comparison to the unstimulated control cells, peaking at 24 hours by roughly a 2.3- and 2.5-fold increase for Poly I:C (HMW) and (LMW), respectively (Figure 3-6 A, B).

Thus, viral-derived TLR3 is not highly effective in regulating CCR1 transcript levels in BMDMs.

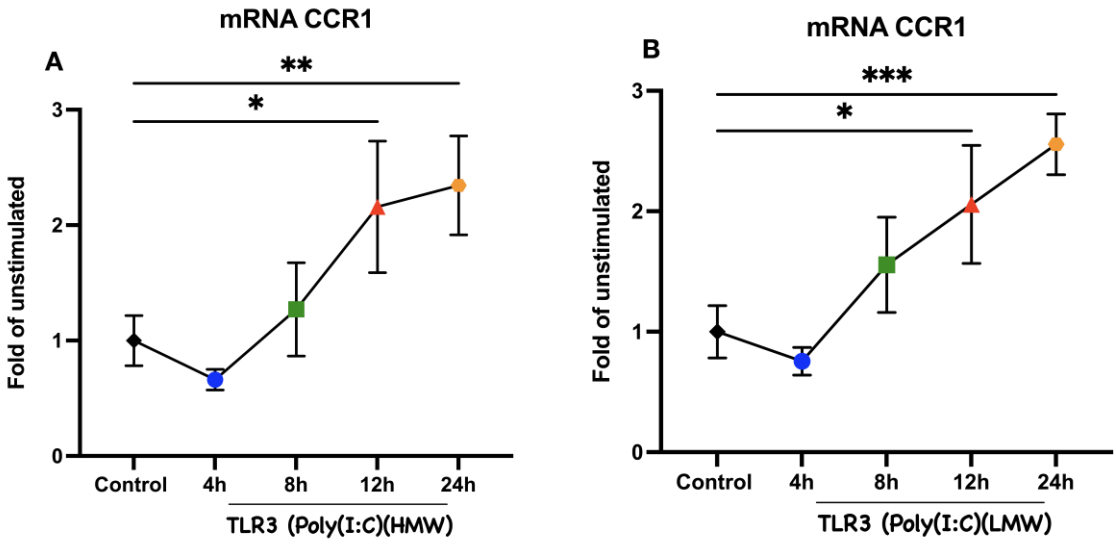

Figure 3-6 Effect of viral-derived TLR3 agonists on CCR1 mRNA levels in BMDM at different time points. Graphs show the expression of CCR1 at 4h, 8h, 12h, and 24h in BMDM treated with TLR3 agonist Poly I:C A) HMW, and B) LMW. Results are normalised to Tbp and mRNA levels are expressed as fold change compared to the non-stimulate BMDM. *P <0.05, **P <.01, and ***P <.001, One-way ANOVA, non-parametric test. Results are shown as Mean ± SD of 3 independent experiments.

3.3.3 The effect of cytokines on CCR5 expression in BMDM

3.3.3.1 IL-3

qPCR analysis showed that IL-3 stimulation of BMDM resulted in a significant increase in CCR5 mRNA expression levels by approximately 2.6-fold compared to unstimulated control cells after 4 hours (Figure 3-7 A). The highest effect of IL-3 on CCR5 expression was at 8 hours, when it increased by almost 3.5-fold compared to the unstimulated control cells (Figure 3-7 A). After that, CCR5 expression remained stable until 24 hours, showing an almost 2.6-fold increase compared to control cells, with only a slight decrease at 12 hours, reaching a 2-fold increase (Figure 3-7 A).

Therefore, these results suggest that CCR5 expression rapidly peaks around 8 hours after IL-3 stimulation and then remains constant until 24 hours.

3.3.3.2 IL-6

Figure 3-7 B shows that after IL-6 treatment, CCR5 transcription significantly increased in BMDM by about 2.3-fold at 8 hours compared to the unstimulated control cells and then slightly increased to 2.7-fold at 12 hours and peaking at 24 hours at roughly 4.2-fold induction.

Therefore, unlike the response to IL-3, CCR5 transcriptional levels in BMDMs kept increasing as time progressed in response to IL-6.

3.3.3.3 IFNα

After 4 hours of IFNα stimulation, CCR5 mRNA expression in BMDM was slightly but not significantly increased by around 2-fold (Figure 3-7 C). However, compared to the unstimulated control cells, IFNα significantly induced CCR5 transcription at 8 hours by around a 4-fold increase, followed by increases of 4.6-

fold at 12 hours, and reached its peak induction at 24 hours with around a 6.5-fold increase (Figure 3-7 C).

Therefore, the response to IFNα is similar to IL-6, with a progressive increase as time increases.

3.3.3.4 GM-CSF

A significant increase of 2.5-fold in CCR5 mRNA expression levels was observed in BMDMs after 4 hours of GM-CSF treatment (Figure 3-7 D). At 8 hours, a similar induction was seen when compared to the unstimulated control cells. However, at 12 hours, this induction reduced to around a 1.9-fold increase before increasing again to 2.5-fold at 24 hours (Figure 3-7 D).

Thus, the response to GM-CSF was similar to that of IL-3, with a peak observed at 8 hours and stable expression thereafter.

3.3.3.5 Combination

In response to the combination treatment of IL-3, IL-6, GM-CSF, and LPS, CCR5 mRNA expression in BMDM was slightly increased. As shown in figure 3-7 E, CCR5 transcriptional levels at 4 hours did not show any additional and detectable induction beyond what had been observed with each of these stimuli alone. Combining these stimuli, however, resulted in a significant 5-fold increase in CCR5 transcription after 8 hours; this increase then dropped to a 4-fold increase at 12 hours; and lastly, it peaked at 24 hours with a roughly 5.5-fold increase in comparison to the unstimulated control cells (Figure 3-7 E).

These results in Figure 3-7, taken together, suggest that IL-3 directly and rapidly increases CCR5 transcriptional levels starting at 4 hours, but to a lesser extent than was seen for CCR1 expression. When combined IL-3 and GM-CSF with

IL-6 and LPS, no further or additive effects on the expression of CCR5 were seen. INFα has the highest regulatory effect on CCR5 transcriptional levels in BMDMs.

3.3.4 The effect of TLR agonists on CCR5 expression in BM

3.3.4.1 Bacterial-derived TLR agonists

3.3.4.1.1 TLR1/2

TLR1/2 ligand bacterial lipopeptide significantly increased CCR5 mRNA expression at 4 hours by about 1.9-fold in comparison to the unstimulated control cells; nevertheless, this is a relatively small induction (Figure 3-8 A). Longer stimulation for 8, 12, and 24 hours resulted in no further induction; rather, it returned CCR5 transcriptional levels to baseline as compared to the unstimulated control cells (Figure 3-8 A).

3.3.4.1.2 TLR4

CCR5 mRNA levels in BMDM were similarly affected by the activation of TLR4 by LPS from two different E. coli strains, 0127: B8 and K12, as both demonstrated a significant increase in CCR5 transcription within 12 hours by about 3.8-fold compared to the unstimulated control cells and triggered a further induction at 24 hours by about 4.3- and 4.5-fold, respectively (Figure 3-8 B, C).

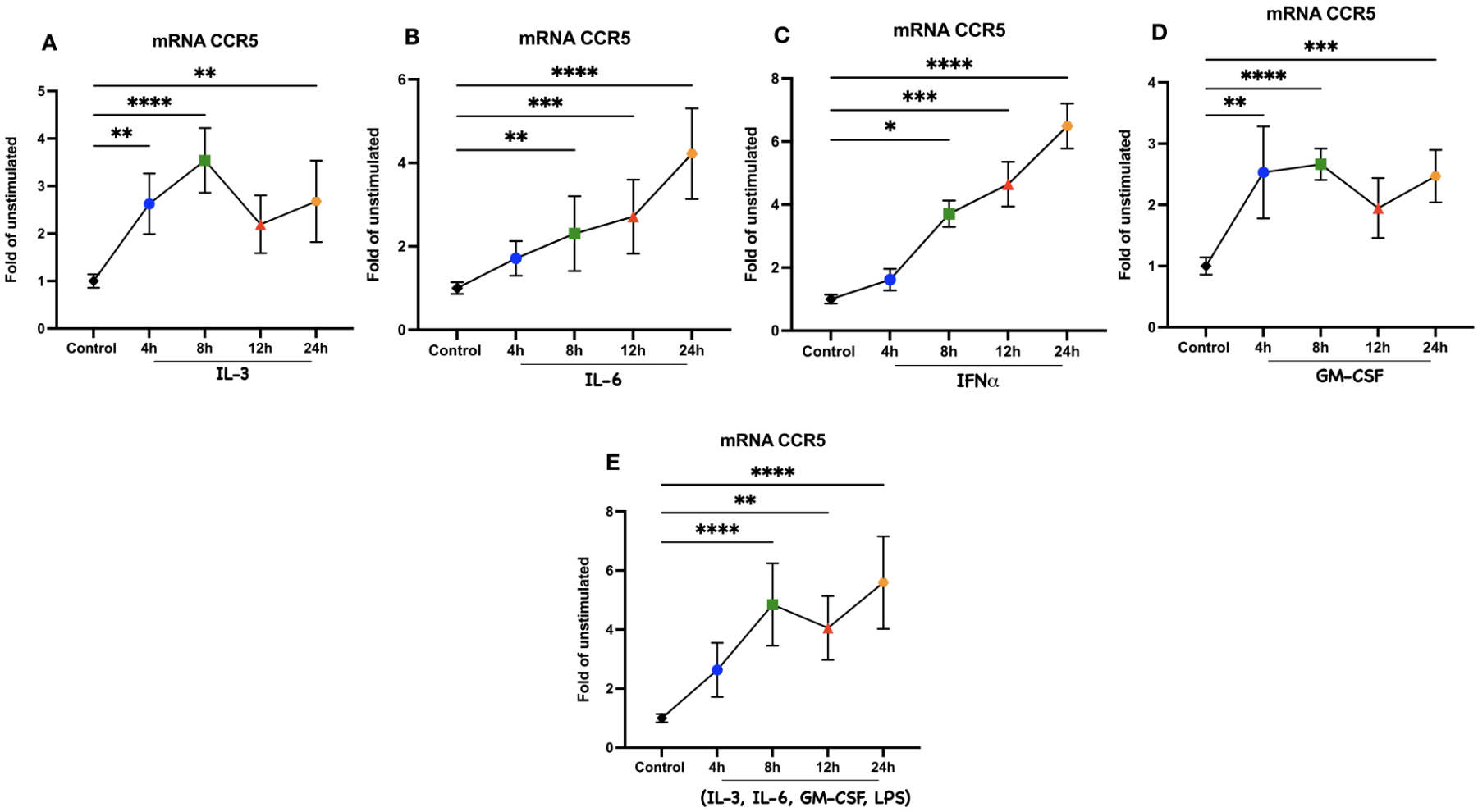

Figure 3-7 Effect of Cytokines on CCR5 mRNA levels in BMDM at different time points. Graphs show the expression of CCR5 at 4h, 8h, 12h, and 24h in BMDM treated with A) IL-3, B) IL-6, C) IFNα, D) GM-CSF, and E) combination of IL-3, IL-6, GM-CSF, and LPS. Results are normalised to Tbp and mRNA levels are expressed as fold change compared to the non-stimulate BMDM. *P <0.05, **P <.01, ***P <.001, and ****P <.0001, One-way ANOVA, non-parametric test. Results are shown as Mean ± SD of 3 independent experiments.

3.3.4.1.3 TLR5

Similarly, BMDMs showed a substantial increase in CCR5 mRNA expression at 12 hours following stimulation with the TLR5 ligand bacterial flagellin (FLA-ST) by about 3-fold compared to the unstimulated control cells (Figure 3-8 D). After 24 hours of stimulation, this induction was slightly increased to 3.5-fold in comparison to the unstimulated control cells (Figure 3-8 D).

3.3.4.1.4 TLR2/6

In response to TLR6 activation by mycoplasma lipoprotein, CCR5 mRNA levels in BMDM showed a tendency to decrease at 8 hours, even though this did not reach statistical significance compared to the unstimulated control cells (Figure 3-8 E). Then, at 12 and 24 hours, CCR5 transcription was statistically significantly higher by roughly 1.7-fold when compared to the unstimulated control cells (Figure 3-8 E). These differences, however, are very small and, together with those shown after TLR1/2 activation, suggest that activation of TLR2 had almost no effect on the expression of CCR5 mRNA in BMDMs (Figure 3-8 A, E).

In summary, the results shown in Figure 3-8 show that LPS and flagellin activation of TLR4 and TLR5, respectively, promote induction in CCR5 transcriptional levels in BMDM. However, this induction takes longer than what was observed for CCR1, it might imply that BMDMs wave the expression of CCR1 and CCR5 in response to bacterial-derived TLR by raising CCR1 first, then CCR5.

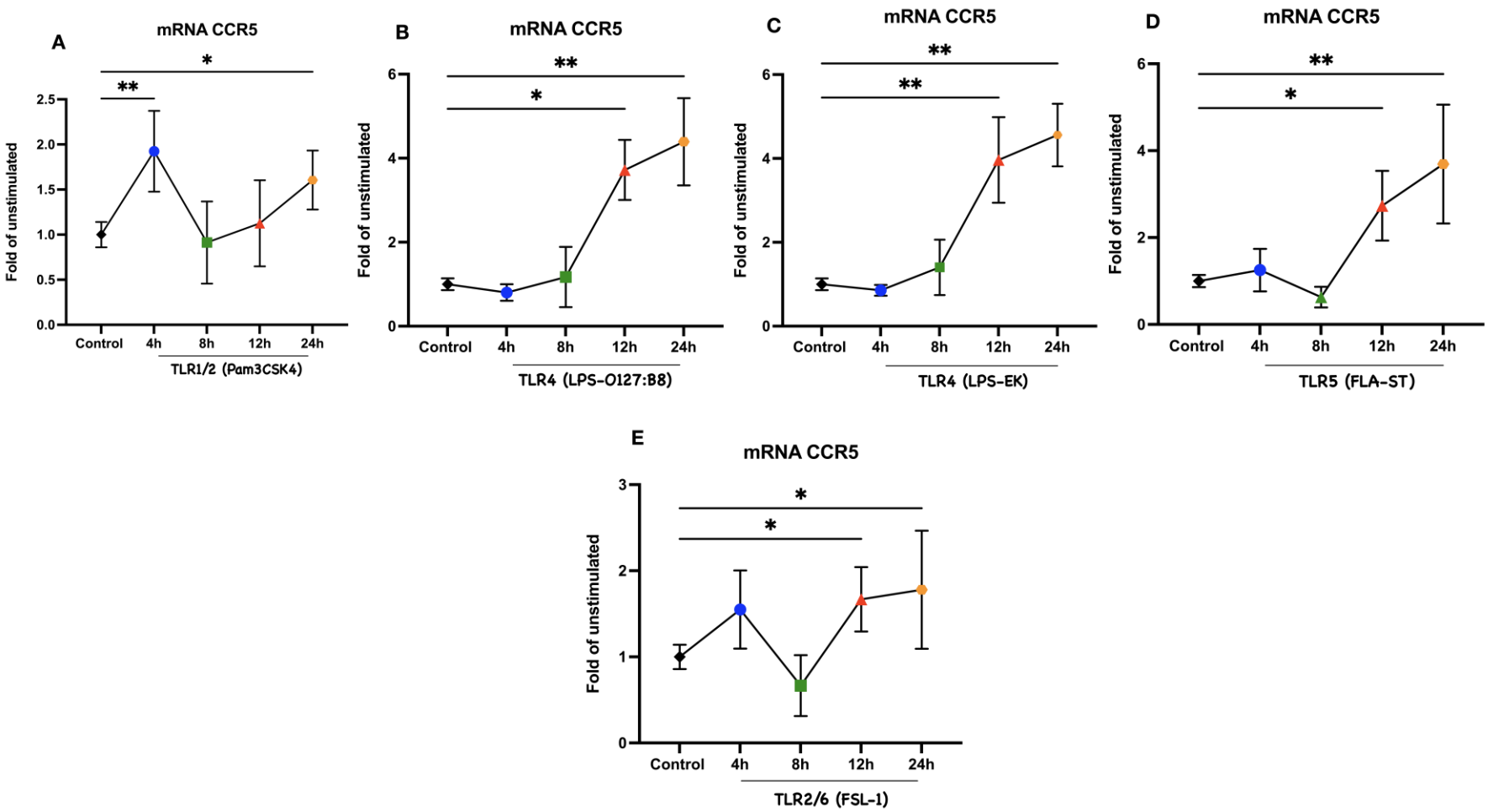

Figure 3-8 Effect of bacterial-derived TLR agonists on CCR5 mRNA levels in BMDM at different time points. Graphs show the expression of CCR5 at 4h, 8h, 12h, and 24h in BMDM treated with agonists for A) TLR1/2, B and C) TLR4, D) TLR5, and E) TLR2/6. Results are normalised to Tbp and mRNA levels are expressed as fold change compared to the non-stimulate BMDM. *P <0.05, **P <.01, ***P <.001, and ****P <.0001, One-way ANOVA, non-parametric test. Results are shown as Mean ± SD of 3 independent experiments.

3.1.1.1 Viral-derived TLR agonists

3.3.4.1.5 TLR3

After 8 hours of treatment with viral-derived Poly I:C (HMW) and (LMW), CCR5 mRNA expression in BMDM significantly increased as expected by almost 3-fold when compared to the unstimulated control cells (Figure 3-9 A, B). In addition, Poly I: C (HMW) and (LMW) both increased CCR5 transcriptional levels by about 4.1 and 4.6, respectively, at 12 hours, and these increases peaked at 24 hours with 5.5- and 6-fold increases, respectively, in comparison to the unstimulated control cells (Figure 3-9 A, B).

Overall, the results presented in Figure 3-9 support what we previously noticed in the first experiment, where BMDMs were activated overnight. Both experiments demonstrate that BMDMs primarily increase CCR5 transcriptional levels in response to viral stimulation. A further point to note is that CCR5 transcriptional levels were higher than those observed following stimulation with bacterial-derived TLR agonists.

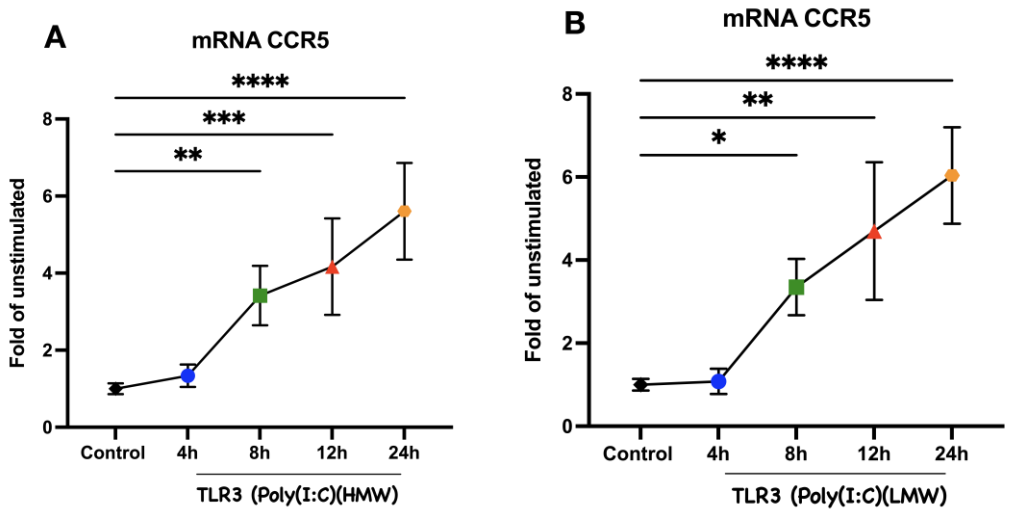

Figure 3-9 Effect of viral-derived TLR3 agonists on CCR5 mRNA levels in BMDM at different time points. Graphs show the expression of CCR5 at 4h, 8h, 12h, and 24h in BMDM treated with TLR3 agonist Poly I:C A) HMW, and B) LMW. Results are normalised to Tbp and mRNA levels are expressed as fold change compared to the non-stimulate BMDM. **P <.01, ***P <.001, and ****P <.0001, One-way ANOVA, non-parametric test. Results are shown as Mean ± SD of 3 independent experiments.

3.4 iCCR protein expression in BMDM under inflammatory conditions after ON stimulation

The next step was to determine whether the observed induction in CCR1 and CCR5 mRNA levels in response to cytokines and TLR-ligands also reflects induction at the protein level. Therefore, bone marrow cells were isolated from both iCCR reporter strain (iREP) and WT mice. The fluorescent reporter proteins were Clover for CCR1 in green, mRuby2 for CCR2 in red, mTagBFP2 for CCR3 in blue, IRFP682 for CCR5 in purple and WT mice were used as a control for background autofluorescence. On day 5, differentiated macrophages were stimulated overnight with the same cytokines and TLR ligands used before, but this time, cells were collected and analysed by flow cytometry. Microscopic images in Figure 3-10 demonstrated that bone marrow cells isolated from iREP were normally differentiated and did not differ in appearance from WT cells, as previously shown in Figure 3-1.

In-vitro differentiated BMDMs were identified by their expression of two extracellular markers, CD11b and F480. Figure 3-10 shows that cells were initially gated on SSC-A and FSC-A to eliminate any debris before selecting differentiated BMDM. As this gate did not completely remove all dead cells, live cells were gated using a live/dead cell marker. After that, doublets were excluded. The remaining cells were gated on CD11b and F480 to select differentiated BMDM as $CD11b^+$ $F480^+$, and finally, the surface expression of each iCCR as defined by reporter expression was analysed.

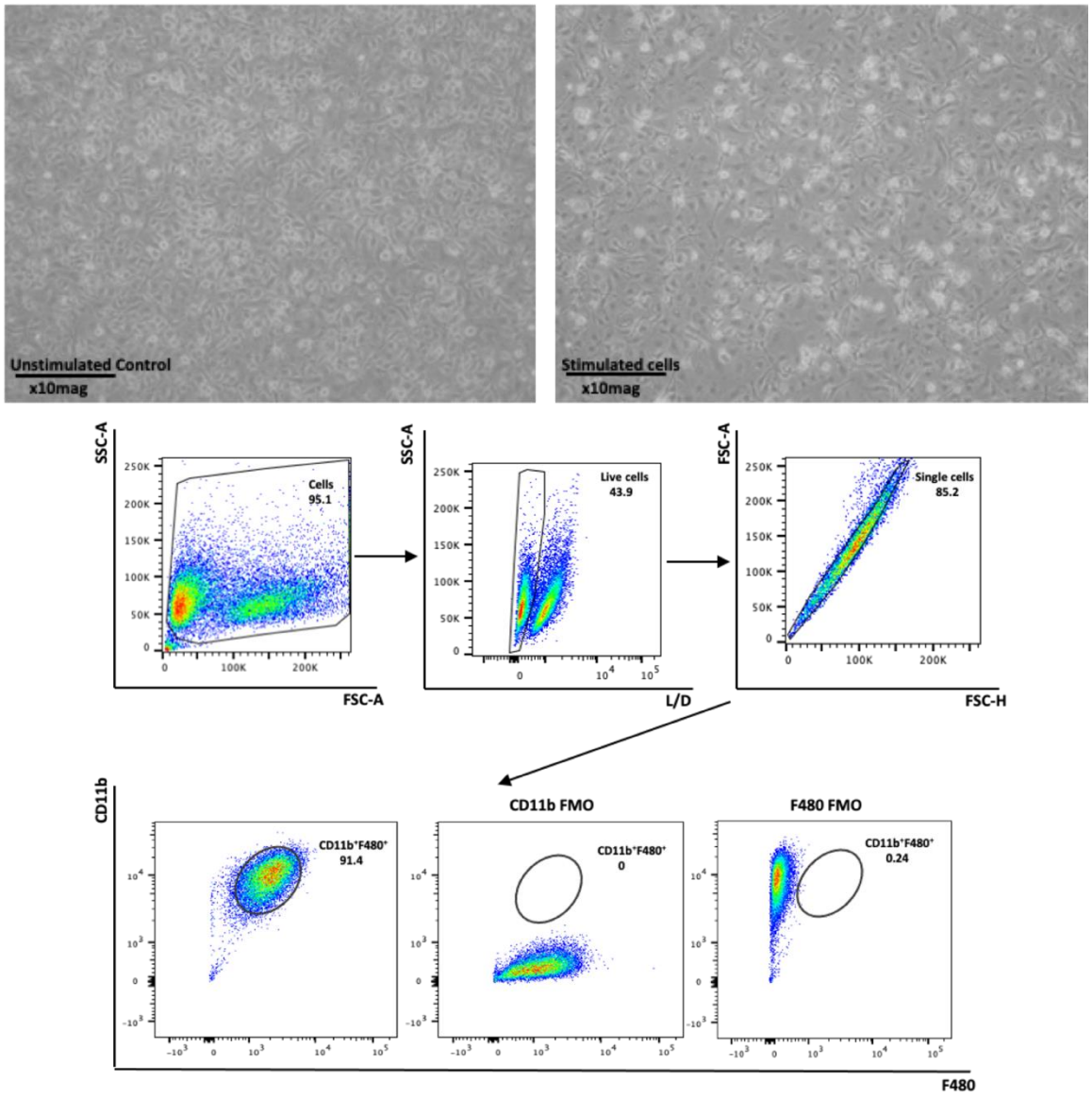

Figure 3-10 Gating strategies for analysis of ICCR expression on BMDM after ON stimulation. Bright-field images at (x10) magnification of reporter BMDM show typical macrophage differentiation and morphology. Flow cytometric plots showing the gating strategies were used to identify in-vitro differentiated macrophages by first gating on cells using FSC-A and SSC-A. Followed by identifying of live cells that stained negative for the live-dead marker. Single cells were then gated to remove cell clumps before assessing surface phenotype. Positive gate for CD11b and F480 was compared to FMO controls to remove autofluorescence cells.

3.4.1 The effect of cytokines on CCR1 reporter expression level by BMDM

Flow-cytometric analysis shown in Figure 3-11 A and B demonstrated a slight increase in CCR1 reporter expression following an overnight treatment with IL-3 with about 46% of $CD11b^{+}$ $F480^{+}$ BMDM positive for Clover/CCR1 compared to 31% of unstimulated cells. These cells treated with IL-3 also had significantly higher

mean fluorescence intensities (MFI) for Clover/CCR1 expression compared to the unstimulated control (Figure 3-11 C). Similar results were observed after GM-CSF treatment, with around 48% of $CD11b^+$ $F480^+$ BMDM expressing Clover/CCR1 and MFI showing a 1.25-fold increase in CCR1 reporter levels compared to the unstimulated cells (Figure 3-11 A,B,C). Approximately 37% of $CD11b^+$ $F480^+$ BMDM expressed Clover/CCR1 after IL-6 stimulation, and when compared to the unstimulated control, there was a significant induction in MFI that was nearly identical to the GMCSF effect (Figure 3-11 A,B,C). FACS plots in Figure 3-11 A showed that IFNα had no effect on Clover/CCR1 expression and statistical analysis also failed to detect any significant changes in CCR1 reporter expression in $CD11b^+$ $F480^+$ BMDM (Figure 3-11 B,C). Combining IL-3, GM-CSF, IL-6, and ILPS increased the percentage of $CD11b^+$ $F480^+$ BMDM expressing Clover/CCR1 to about 45% compared to 31% of unstimulated cells and MFI for CCR1 reporter levels slightly increased by 1.4-fold in comparison to unstimulated cells (Figure 3-11 B,C). However, this induction does not reflect an additive increase compared to what we have shown for the individual effect of each of these stimuli.

Overall, the findings in Figure 3-11 indicate that GM-CSF or IL-3 are both effective inducers of $CD11b^+$ $F480^+$ BMDM to express the CCR1 reporter. IL-6 also positively affects CCR1 reporter levels, whereas IFNα did not induce CCR1 reporter expression in CD11b+ $F480^+$ BMDM.

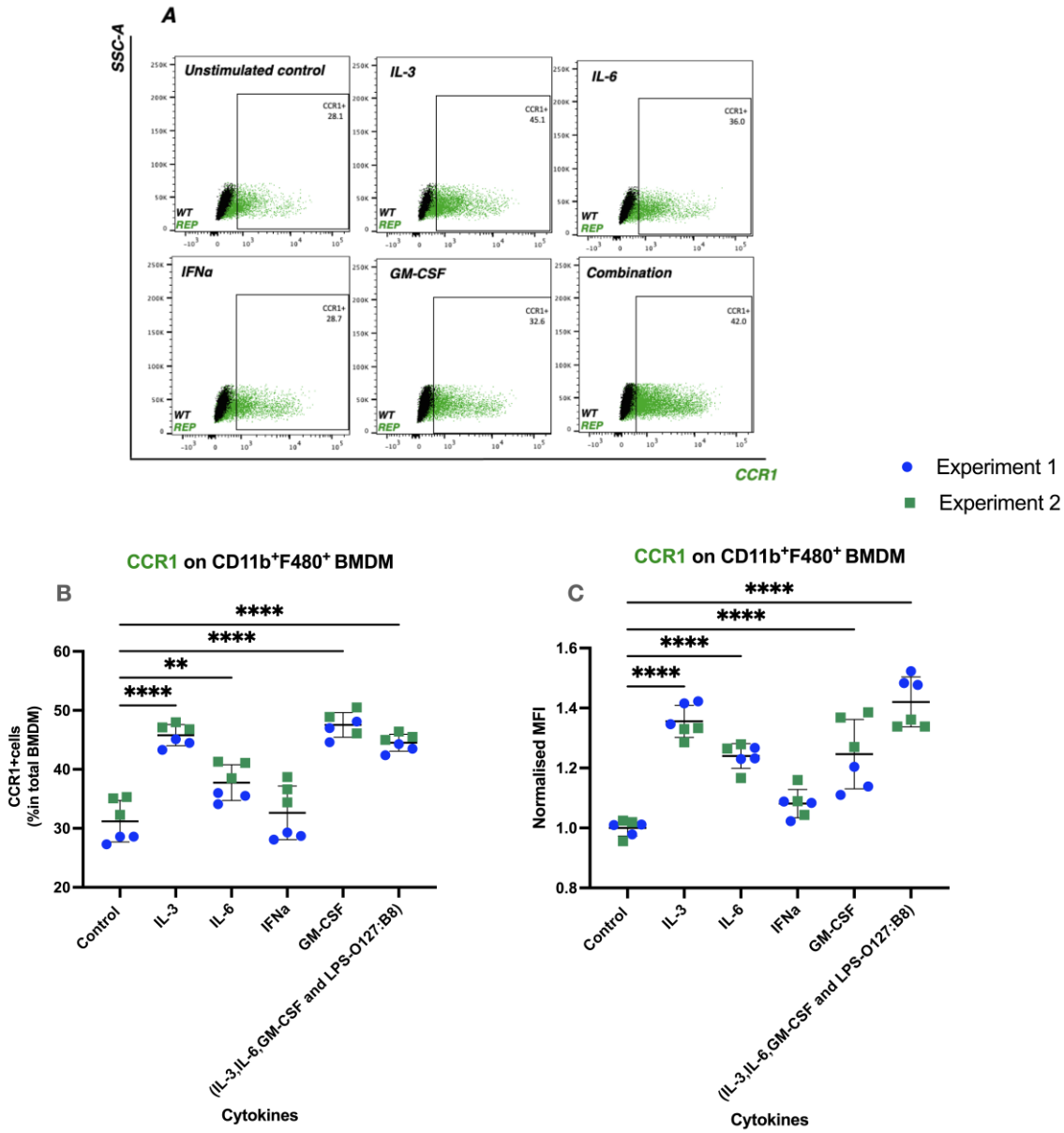

Figure 3-11 Effect of cytokines on CCR1 reporter expression in BMDM. BM cells were isolated from reporter and WT mice. Cells were then allowed to differentiate in culture for 5 days. On day 5, BMDM were replated in triplicate in 6 well plates and stimulated with different cytokines ON. BMDM were then detached and analysed for expression of the iCCR reporters. A) flow cytometric analysis of CCR1-Clover expression on CD11b$^+$F480$^+$BMDM from unstimulated and stimulated cells. Graphs show B) quantification and C) mean fluorescent intensity (MFI) of CCR1-Clover expression on CD11b$^+$F480$^+$BMDM. The florescent receptor CCR1/clover is shown in green and positive expression was identified using non-fluorescent WT in black. Results are expressed as fold change compared to the non-stimulated control. **P <.01, and ****P <.0001, One-way ANOVA. Results are shown as Mean ± SD of 2 independent experiments.

3.4.2 The effect of TLR-agonists on CCR1 reporter expression level by BMDM

The expression of CCR1 reporter in BMDM was then assessed after they were treated overnight with TLR agonists. Flow cytometric analysis showed that the TLR1/2 ligand, bacterial lipopeptide, significantly increased Clover/CCR1

expression in CD11b$^+$ F480$^+$ BMDM, with roughly 45% of these cells expressing Clover/CCR1 compared to 31% of unstimulated cells (Figure 3-12 A,B). Also, compared to the unstimulated control, bacterial lipopeptide enhanced Clover/CCR1 MFI by almost 1.4 times (Figure 3-12 C). In contrast, neither Poly I:C (HMW) nor (LMW), TLR3 ligands, significantly affected the expression of Clover/CCR1 by CD11b$^+$ F480$^+$ BMDM (Figure 3-12 A,B,C). Clover/CCR1 markedly increased in CD11b$^+$ F480$^+$ BMDM after stimulation with LPS from both E. coli strains compared to the unstimulated control, with roughly 43% of cells expressing Clover/CCR1 (Figure 3-12 A,B). These cells also increased MFI for Clover/CCR1 in response to LPS by 1.3-fold compared to the unstimulated control (Figure 3-12 C). A TLR5, ligand bacterial flagellin (FLA-ST), triggered about 47% of CD11b$^+$ F480$^+$ BMDM to express Clover/CCR1, which was statistically significant when compared to the unstimulated control (Figure 3-12 A,B). These cells also had higher MFI levels for Clover/CCR1 by roughly 1.5 times more than the unstimulated control (Figure 3-12 C). Mycoplasma lipopeptide fibroblast stimulating ligand-1 (FSL-1), a TLR2/6 ligand, showed a slight tendency toward higher Clover/CCR1 positive CD11b$^+$ F480$^+$ BMDM; however, this was not statistically significant, and there were no differences in MFI compared to the unstimulated control (Figure 3-12 A,B,C).

All of these findings show that the BMDM CCR1 reporter expression is increased in response to TLR-activated signalling, especially when bacterial PAMPs are present.

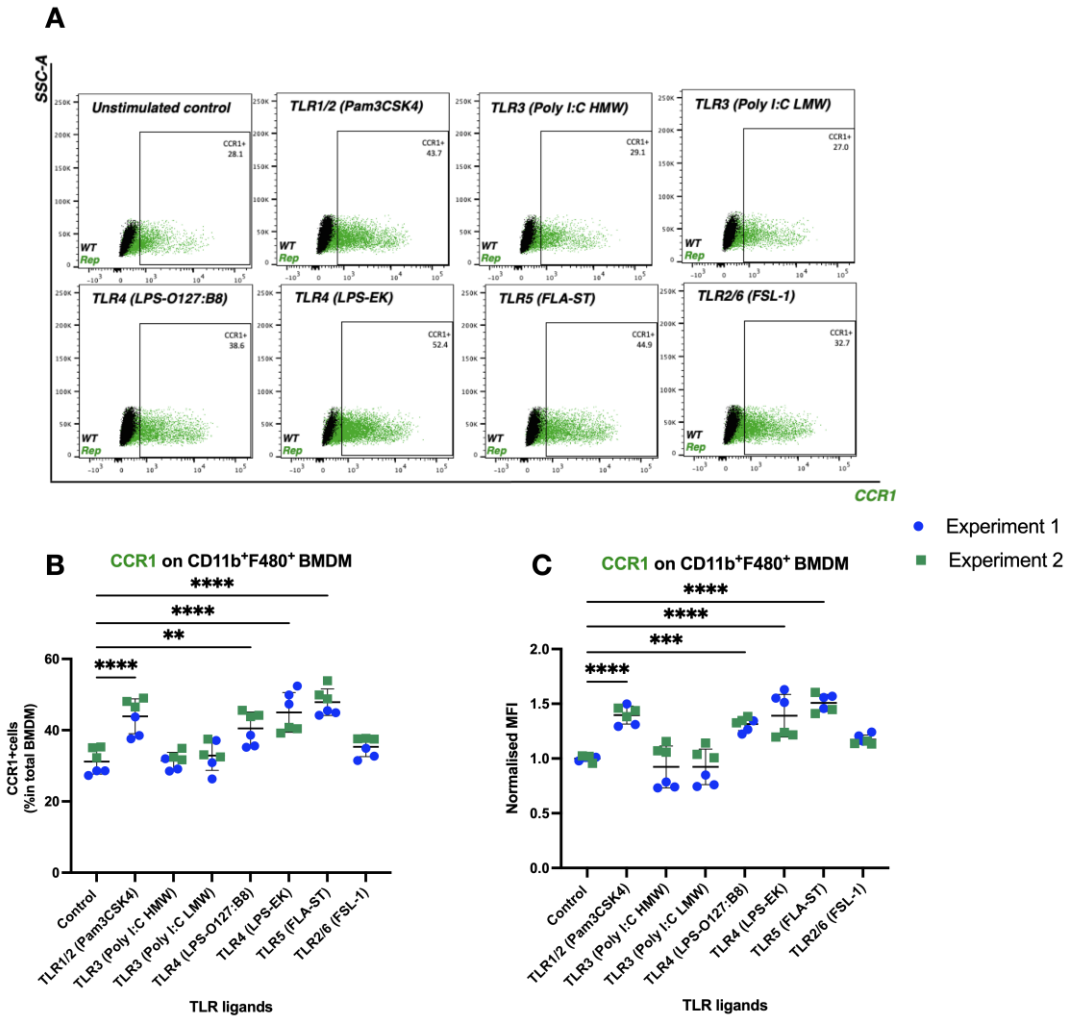

Figure 3-12 Effect of TLR-agonists on CCR1 reporter expression in BMDM. BM cells were isolated from reporter and WT mice. Cells were then allowed to differentiate in culture for 5 days. On day 5, BMDM were replated in triplicate in 6 well plates and stimulated with different TLR-agonists ON. BMDM were then detached and analysed for expression of the iCCR reporters. A) flow cytometric analysis of CCR1-Clover expression on CD11b+F480+BMDM from unstimulated and stimulated cells. Graphs show B) quantification and C) mean fluorescent intensity (MFI) of CCR1-Clover expression on $CD11b^{+}F480^{+}$BMDM. The florescent receptor CCR1/clover is shown in green and positive expression was identified using non-fluorescent WT in black. Results are expressed as fold change compared to the non-stimulated control. **P <.01, ***P <.001, and ****P <.0001, One-way ANOVA. Results are shown as Mean ± SD of 2 independent experiments.

3.4.3 The effect of cytokines on CCR5 reporter expression level by BMDM

Surface expression of CCR5 in BMDM was also assessed following overnight cytokine stimulation. Flow cytometric results showed that in response to IL-3, 13% of $CD11b^{+}$ $F480^{+}$ BMDM were positive for iRFP682/CCR5, with an enhanced MFI for iRFP682/CCR5 that was about 1.3 times greater than the unstimulated control (Figure 3-13 A,B,C). A similar induction was observed following IL6 stimulation,

with around 12% of CD11b$^+$ F480$^+$ BMDM expressing iRFP682/CCR5 compared to about 7% of the unstimulated control (Figure 3-1 A,B). However, this positive effect of IL-6 was not accompanied by an increase in iRFP682/CCR5 MFI in CD11b$^+$ F480$^+$ BMDM (Figure 3-13 C). Despite a trend toward more iRFP682/CCR5-positive CD11b$^+$ F480$^+$ BMDM in response to GM-CSF, the data indicated no statistically significant difference from the unstimulated control, and MFI remained unchanged (Figure 3-13 A,B,C). Combining cytokine stimulation with IL-3, GM-CSF, IL-6, and LPS led to slightly higher iRFP682/CCR5 MFI levels by about 1.4-fold increases in comparison to the unstimulated control (Figure 3-13 C); however, it did not demonstrate a significant upregulation in the proportion of CD11b$^+$ F480$^+$ expressing iRFP682/CCR5 (Figure 3-13 A,B). In contrast to what we previously observed at CCR5 transcript levels, stimulation with the antiviral cytokine INFα did not enhance CD11b$^+$ F480$^+$ BMDM expression of iRFP682/CCR5 or its MFI in comparison to the unstimulated control (Figure 3-13 A,B,C).

Our findings indicate that the direct increase in CCR5 mRNA levels caused by IL-3 is also accompanied by an increase in CCR5 reporter levels in BMDM, and when IL-3 is combined with GM-CSF, IL-6 and LPS, they have no additive effect on CCR5 reporter expressing BMDM.

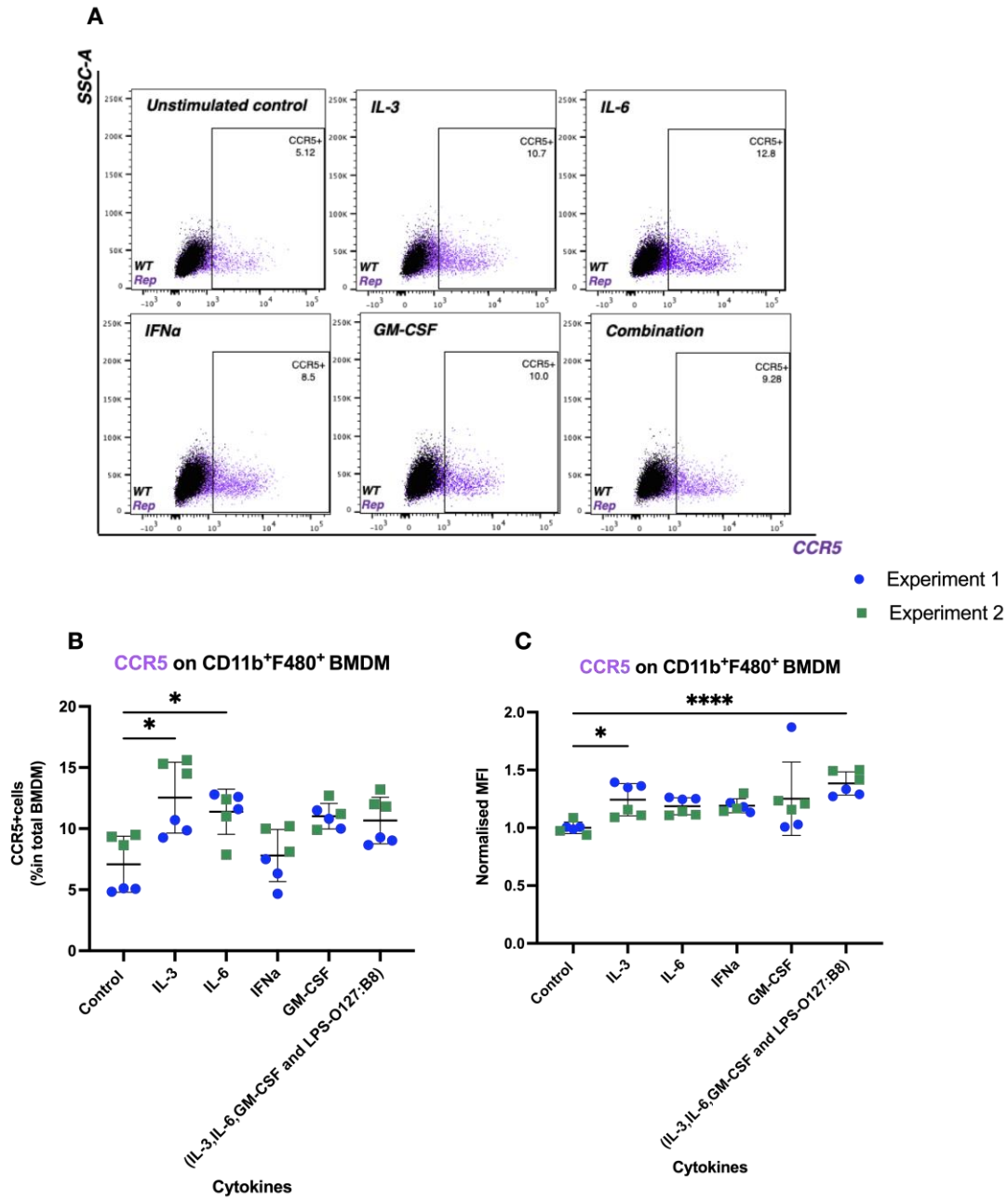

Figure 3-13 Effect of cytokines on CCR5 reporter expression in BMDM. BM cells were isolated from reporter and WT mice. Cells were then allowed to differentiate in culture for 5 days. On day 5, BMDM were replated in triplicate in 6 well plates and stimulated with different cytokines ON. BMDM were then detached and analysed for expression of the iCCR reporters. A) flow cytometric analysis of CCR5-iRFP682 expression on CD11b+F480+BMDM from unstimulated and stimulated cells. Graphs show B) quantification and C) mean fluorescent intensity (MFI) of CCR5-iRFP682 expression on $CD11b^{+}F480^{+}$BMDM. The florescent receptor CCR5-iRFP682 is shown in purple and positive expression was identified using non-fluorescent WT in black. Results are expressed as fold change compared to the non-stimulated control. *P <0.05, and ****P <.0001, One-way ANOVA, non-parametric test. Results are shown as Mean ± SD of 2 independent experiments.

3.4.4 The effect of TLR-agonists on CCR5 reporter expression level by BMDM

Expression of CCR5 was next assessed after BMDM were stimulated with TLR agonists overnight. Flow cytometric analysis indicated that none of the activated

TLR signals markedly changed iRFP682/CCR5 expression compared to the unstimulated control (Figure 3-14 A,B,C). However, the TLR1/2 ligand, bacterial lipopeptide, increased the number of iRFP682/CCR5-positive $CD11b^+$ $F480^+$ BMDM and MFI for iRFP682/CCR5 in this population, but not to the level where it would statistically differ from the unstimulated control (Figure 3-14 A,B,C). Similar results were observed following stimulation of BMDM with both TLR3 ligands, Poly I:C (HMW) and (LMW), as the percentage of $CD11b^+$ $F480^+$ BMDM expressing iRFP682/CCR5 increased in response to TLR3 activation, but this increase was not statistically different from the unstimulated control (Figure 3-14 A,B). However, Figure 3-14 C demonstrated that in $CD11b^+$ $F480^+$ BMDM, TLR3 agonists neither had an effect nor showed a tendency to enhance MFI for iRFP682/CCR5 in $CD11b^+$ $F480^+$ BMDM.

Additionally, there were no significant differences in iRFP682/CCR5 expression between the unstimulated and stimulated BMDM with LPS or bacterial flagellin (FLA-ST) (Figure 3-14 A,B,C). Our previous findings from the time points experiment using these stimuli found that their effects on CCR5 mRNA levels started to be detectable after 12 hours and peaked at 24 hours. Therefore, these results would suggest that they might require more than overnight stimulation to replicate the transcriptional upregulation of CCR5 expression to protein levels in $CD11b^+$ $F480^+$ BMDM. Again, there were no significant differences in iRFP682/CCR5 expressing $CD11b^+$ $F480^+$ BMDM and its MFI after mycoplasma lipoprotein triggered TLR2/6 signalling (Figure 3-14 A,B). However, the pattern of the MFI data in Figure 3-14 C revealed a slight increase compared to the unstimulated control.

Overall, these results are consistent with the time-point experiment that previously showed that TLR agonists appear to indirectly regulate CCR5 transcription induction, which happens more slowly than CCR1 transcription

induction. This would suggest that macrophages respond to TLR signals by upregulating CCR1 first and CCR5 second and that an overnight stimulation is insufficient to cause an effect at the protein level for CCR5. This would explain why, following an overnight TLR-agonist treatment, we could not find any significant changes in the expression of CCR5.

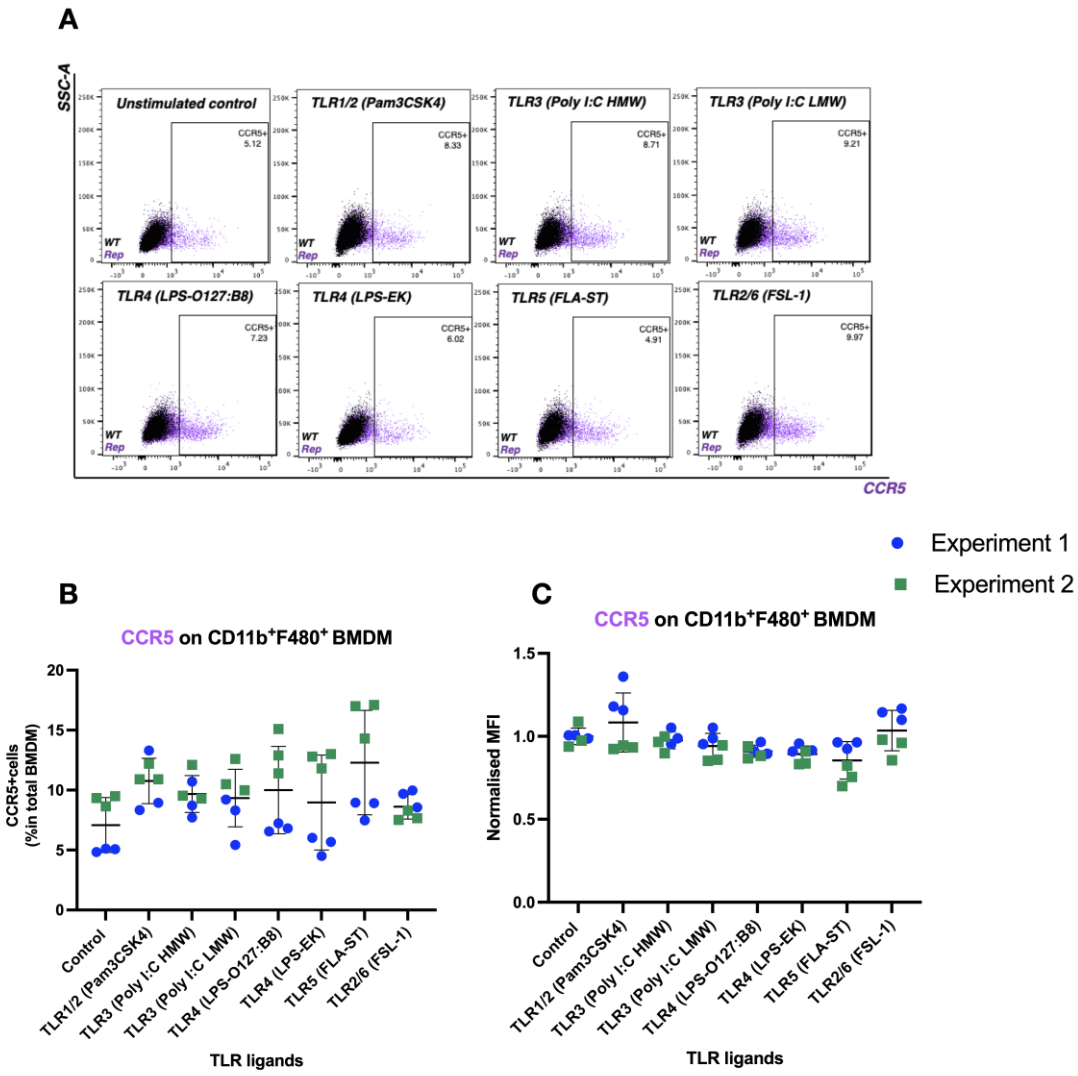

Figure 3-14 Effect of TLR-agonists on CCR5 reporter expression in BMDM. BM cells were isolated from reporter and WT mice. Cells were then allowed to differentiate in culture for 5 days. On day 5, BMDM were replated in triplicate in 6 well plates and stimulated with different TLR-agonists ON. BMDM were then detached and analysed for expression of the iCCR reporters. A) flow cytometric analysis of CCR5-iRFP682 expression on CD11b+F480+BMDM from unstimulated and stimulated cells. Graphs show B) quantification and C) mean fluorescent intensity (MFI) of CCR5-iRFP682 expression on CD11b^{+}F480^{+}BMDM. The florescent receptor CCR5-iRFP682 is shown in purple and positive expression was identified using non-fluorescent WT in black. Results are expressed as fold change compared to the non-stimulated control. One-way ANOVA, non-parametric test. Results are shown as Mean ± SD of 2 independent experiments.

3.4.5 The effect of cytokines and TLR-agonists on CCR2 reporter expression level by BMDM

We next examined CCR2 reporter expression in CD11b+ F480+ BMDM after overnight stimulation with cytokines and TLR agonists. The results of flow cytometric analysis in Figure 3-15 confirmed our earlier findings from transcript analysis of CCR2 expression in BMDM following exposure to these stimuli, demonstrating that neither cytokines nor TLR agonists had any marked effects on the surface expression of mRuby2/CCR2 in CD11b^{+} F480^{+} BMDM or on its level of intensity in these cells. These results confirmed that the expression of inflammatory chemokine receptor CCR2 is not regulated in mature macrophages by the tested agents.

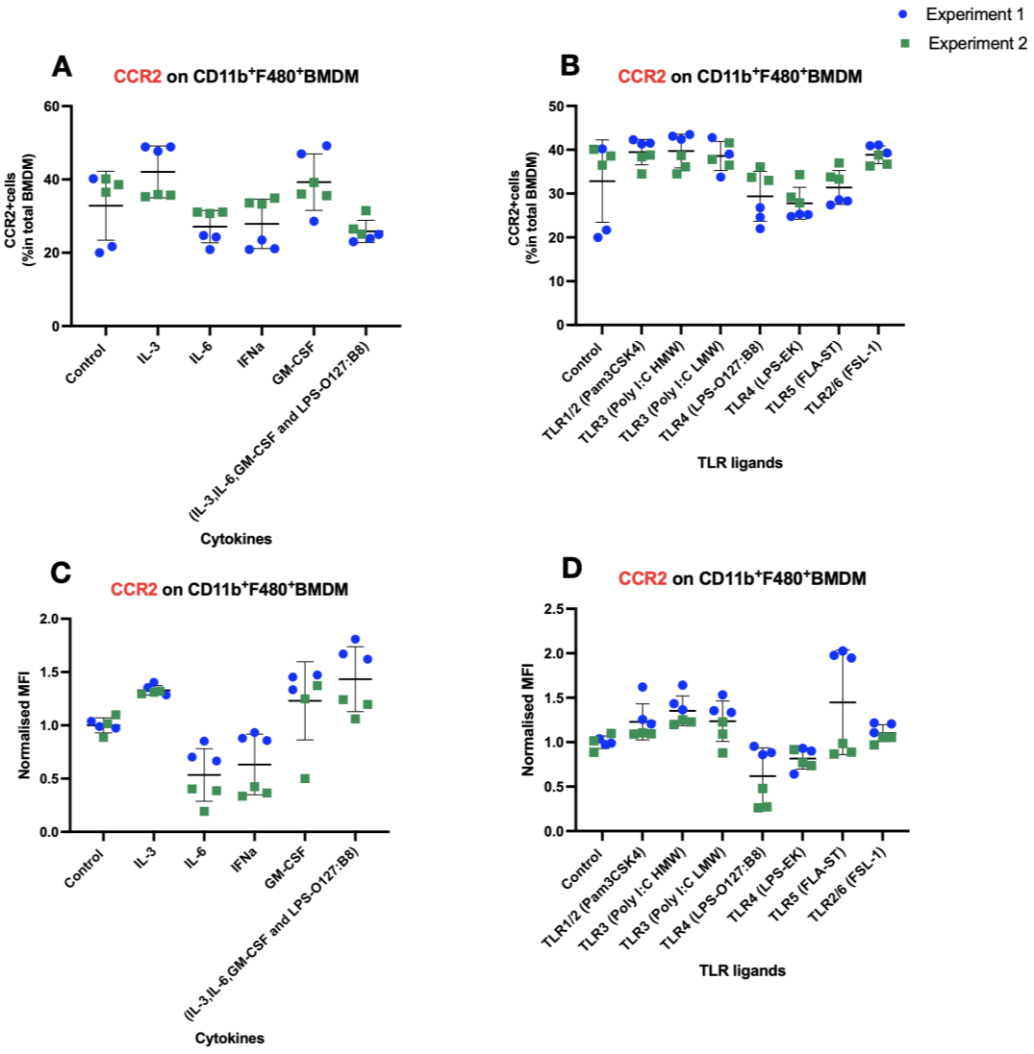

Figure 3-15 Effect of cytokines and TLR-agonists on CCR2 reporter expression in BMDM. Reporter CCR2/mRuby2 expression in BMDM after ON was analysed. Quantification of CCR2/mRuby2 percentage expression in $CD11b^{+}F480^{+}$BMDM stimulated with A) cytokines and B) TLR- agonists. CD11b+F480+BMDM rates of CCR2/mRuby2 expression level as MFI for treated cells with C) cytokines and D) TLR-agonists. Results are expressed as fold change compared to the non-stimulated control. One-way ANOVA, non-parametric test. Results are shown as Mean ± SD of 2 independent experiments.

3.4.6 The effect of cytokines and TLR-agonists on CCR3 reporter expression level by BMDM

Similar results were obtained when mTagBFP2/CCR3 expression in $CD11b^{+}$ $F480^{+}$ BMDM was examined using iCCR REP mice. However, unlike other iCCRs, CCR3 was barely expressed, with around ~3% of macrophages expressing it at rest (Figure 3-16 A, C). Compared to unstimulated cells, $CD11b^{+}$ $F480^{+}$ BMDM did not change their surface expression of mTagBFP2/CCR3 in response to cytokines and TLR agonists stimulation (Figure 3-16). As a result, the data shown in Figure 3-16 suggest that mature macrophages during an inflammatory response do not mainly regulate the expression of CCR3 as CCR2 reporters in response to the tested agents.

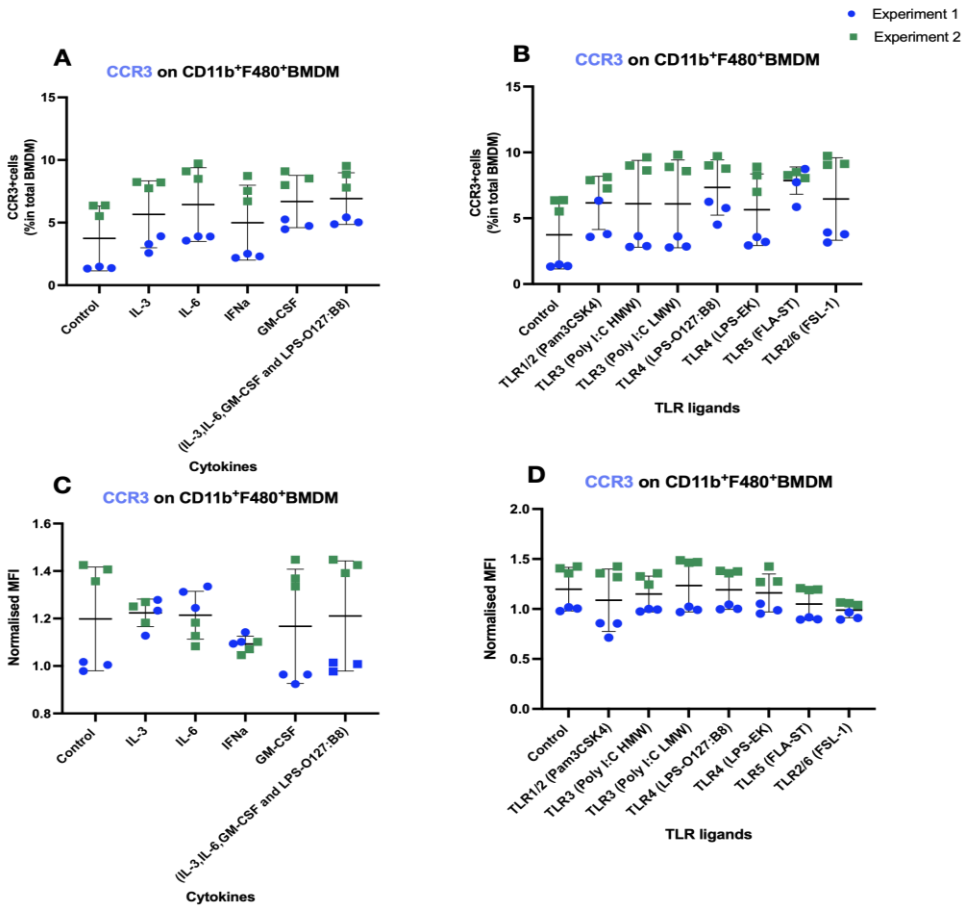

Figure 3-16 Effect of cytokines and TLR-agonists on CCR3 reporter expression in BMDM. Reporter CCR2/mRuby2 expression in BMDM after ON stimulation was analysed. Quantification of CCR3-mTagBFP2 percentage expression in CD11b$^+$F480$^+$BMDM stimulated with A) cytokines and B) TLR- agonists. CD11b$^+$F480$^+$BMDM rates of CCR3-mTagBFP2 expression level as MFI for treated cells with C) cytokines and D) TLR-agonists. Results are expressed as fold change compared to the non-stimulated control. One-way ANOVA, non-parametric test. Results are shown as Mean ± SD of 2 independent experiments.

3.5 Discussion and conclusion

The inflammatory iCCR receptors are mainly involved in orchestrating non-neutrophilic myeloid cell recruitment at rest and during inflammation[194, 198]. CCR2 is well known for its critical role in monocyte egress from the bone marrow and migration into infected sites[194, 357]. However, several studies have found its expression to be dramatically downregulated as human monocytes differentiate in vitro into macrophages[355, 358]. Our group recently published data to support the hypothesis that classical inflammatory monocytes gradually express CCR1 and CCR5 as they develop into macrophages, highlighting the

functional significance of these two chemokine receptors in macrophages[198]. This is in line with earlier studies by Kaufmann et al., who found that as monocytes differentiate into macrophages, they increase their chemotactic responses to CCL3, a ligand for CCR1, and CCR5 while reducing their responses to a CCR2 ligand, CCL2[355]. Therefore, all these findings suggest that CCR1 and CCR5 are two inflammatory chemokine receptors involved in the localization and function of tissue macrophages. Therefore, we were interested to learn more about the regulatory mechanisms that control their expression and whether each has a specific function in a particular biological context.

We cultured bone marrow-derived macrophages and stimulated them with a range of cytokines, as well as TLR agonists, that mimic viral and bacterial pathogens, to study how these stimuli can affect the expression of the iCCRs in macrophages. Our results suggest that IL-3 and GM-CSF can act as selective transcriptional inducers of CCR1 expression in bone marrow-derived macrophages. This induction is also accompanied by an increase in both the number of macrophages expressing CCR1 reporter and its expression level in cells. In contrast to Jarmin et al.[359] who demonstrated that IL-3 and GM-CSF could regulate CCR1 and its ligand CCL-3 but did not alter CCR5 expression in BMDM after 4 hours of stimulation, our data showed that both of these stimuli directly increased CCR5 mRNA levels at 4 hours, but to a lesser extent than CCR1. This discrepancy may be explained by several factors, including that our experimental design differed from the one used in the previous work in which we treated BMDM on day 5 rather than day 7. The fact that the transcription upregulation we observed was small, peaked 4–8 hours after stimulation, and then eventually stabilised suggests that these cytokines have limited effects on CCR5 expression and do not progress over time or as BMDMs age. This might help to explain why they did not observe a

difference when they examined the expression in older macrophages. This may also explain why we did not observe any detectable changes in CCR5 triggered by these cytokines in our first experiment following overnight stimulation. Also, GM-CSF concentration between our study and the previous one is different (20 ug/ml versus 10ug/ml), indicating that the effect we saw is concentration- and time-dependent.

In this study, we report that CCR1 transcriptional and reporter protein levels in BMDMs are increased in response to activation of both TLR1/2 (by synthetic bacterial lipopeptide, Pam3CSK4) and TLR4 (by LPS purified from E. coli K12 and 0127: B8). On the other hand, a study by Parker et al.[360] showed that activation of TLR2 or TLR4 downregulated the expression of CCR1 in a human monocytic cell line. This discrepancy in the results might indicate that the regulation of CCR1 by these TLR agonists on murine macrophages differs from that observed on human monocytes; however, our findings are consistent with a study by Medina-Ruiz et al.[198] who found CCR1 expression is increased in circulating monocytes in response to carrageenan, which acts through TLR4 like LPS.

It has been reported by Mian et al.[361] according to the type of cell, different innate immune responses are triggered by different lengths of dsRNA (poly I:C), as High molecular weight (HMW) induced higher cytokine amounts such as TNFß and protection against viral replication in fibroblasts and low molecular weight (LMW) in myeloid cells. For this reason, in this experiment, BMDMs were treated with both forms of Poly I: Cs: (HMW), with a length of 1.5 kb to 8 kb, and (LMW), with a length of 0.2 to 1 kb, to assess the activation efficiency of TLR3 and their effect on iCCRs expression in BMDMs. Identical results were observed following stimulation of BMDM with both TLR3 ligands, Poly I:C (HMW) and (LMW), with CCR5 mRNA roughly reaching a 7-fold significant increase at 24 hours

compared to unstimulated cells and both demonstrating a similar trend toward induction of CCR5 at protein levels in $CD11b^+$ $F480^+$ BMDM. The Main et al.[361] study used a different cell type and molecular length for Poly I: Cs, which could explain these discrepancies in the results. For instance, the RAW264.7 macrophage cell line was used and might respond to, or detect RNA viruses, differently from the primary BMDM. Poly I: Cs (HMW) had a molecular length of over 5 kb, whereas, in the present study, the average size ranged from 1.5 kb to 8 kb. We cannot verify whether it is greater than 5 kb because we did not measure the actual size. CCR5 mRNA levels also increase when LPS triggers TLR4 at 12 hours and peak at 24 hours by around 3- to 4-fold compared to the unstimulated cells. It is interesting to note that a study has shown that TLR3 and TLR4 have a significant antiviral role through the activation of interferon regulatory factor 3 (IRF3) in their signalling cascade[362]. Further study showed that TLR4 deficient mice increased susceptibility to respiratory syncytial virus (RSV) infection [363]. Therefore, our results suggest that activation of either TLR3 or TLR4 enhances CCR5 expression in BMDMs to mediate the antiviral immune response. IFNα also has a similar positive effect on CCR5 expression in BMDM. This result is consistent with study of Stoddart et al.[364], who found that IFNα induces CCR5 expression on human T cells, which suggests that a similar role could be played by IFNα on murine macrophages.

These findings demonstrate that macrophages differentially regulate CCR1 and CCR5 expression in response to inflammatory cytokines and pathogen-activated TLRs. More specifically, our data show that GM-CSF and IL-3 are efficient inducers of CCR1 by raising its transcriptional and protein levels and activating TLR signalling in macrophages by the synthetic bacterial components similarly increased CCR1 expression. On the other hand, macrophages exposed to TLR3

agonists derived from viruses and antiviral cytokines IFNα had the highest levels of CCR5 transcriptional induction. CCR2 and CCR3 are not significantly differentially regulated in macrophages, which may be explained by the fact that these chemokine receptors are highly expressed in monocytes and eosinophils, respectively, but not in these mature macrophages. Therefore, activated macrophages exhibit higher levels of CCR1 and CCR5 expression, with CCR1 possibly being further enhanced in response to bacterial infections and CCR5 possibly increased in response to viral infections.

Chapter 4 In-vivo Regulation of iCCR Receptors

4.1 Overview

We have shown in the previous chapter that the transcription of CCR1 and CCR5 fluctuates according to the inflammatory conditions. While CCR2 is one of the most expressed iCCRs (expressed by up to 40% of $CD11b^+$ $F480^+$ BMDMs), its expression does not appear to fluctuate and remains stable. CCR3 expression also does not appear to fluctuate in response to various inflammatory stimuli, but in contrast with CCR2, CCR3 is barely expressed by $CD11b^+$ $F480^+$ BMDMs (only ~7% positive).

After assessing iCCR expression by BMDM in vitro, we wanted to see if we could replicate these finding in vivo. Importantly, the in vitro experiments described were performed on fully differentiated macrophages. As mentioned previously, iCCR expression has been shown to fluctuate according to the differentiating status of the cell of interest. For example, monocytes have been shown to downregulate CCR2 and upregulate CCR5 as they turn into fully differentiated macrophages[354, 355, 358], and this is a process we would not have been able to detect in our in vitro conditions.

In this chapter, the expression of iCCRs on monocytes was assessed to determine how their expression changes over time in a model of acute inflammation, starting at the point at which monocytes leave the bone marrow and enter the bloodstream up until they infiltrate the inflamed areas and undergo macrophage differentiation.

In this model, iREP mice were implanted with a subcutaneous osmotic pump loaded with IL-3, IL-6, GM-CSF and IFNα or PBS (control) to provide continuous release of inflammatory cytokines into the circulation and simulate the condition the leukocytes would find in an inflamed animal. These cytokines were selected

because they either induce a significant increase in CCR1 and CCR5 transcription and protein levels, as shown in the previous chapter, or a trend towards that.

4.2 A mouse model of inflammation using subcutaneous osmotic mini pump implantation

As shown in Figure 4-1 A, osmotic pumps loaded with IL-3, IL-6, GM-CSF, and IFNα, or with vehicle (PBS), were implanted for up to 7 days under the dorsal skin of both iREP and WT mice to assess the effects of systemic inflammation on iCCR reporter expression on monocytes, and monocyte-derived macrophages. WT mice served as a negative control, and their cells were used as a reference to determine the positive fluorescence in the iREPs.

The continuous release of these inflammatory mediators into the bloodstream for 7 days caused a systemic inflammatory response that was reflected in higher spleen weight in mice exposed to the cytokine cocktail than in control mice treated with vehicle (PBS), indicating that induction of systemic inflammation was successful, and the mini-pump implant functioned as anticipated (Figure 4-1 Bi).

Another indication of inflammation was an increase in the number of circulating classical inflammatory monocytes ($CD11b^{+}$ $ly6c^{hi}$), as seen in FACS plots and graphs in Figure 4-1 Bii, as well as other leukocyte subsets like neutrophils ($CD11b^{+}$ $Ly6G^{hi}$), B-cells ($CD11b^{-}$ $CD19^{+}$), eosinophils ($CD11b^{+}$ $SiglecF^{+}$), and DCs ($CD11b^{+}$ $CD11c^{+}$ $MHCII^{hi}$) when compared to (PBS) control mice (Figure 4-1 Biii).

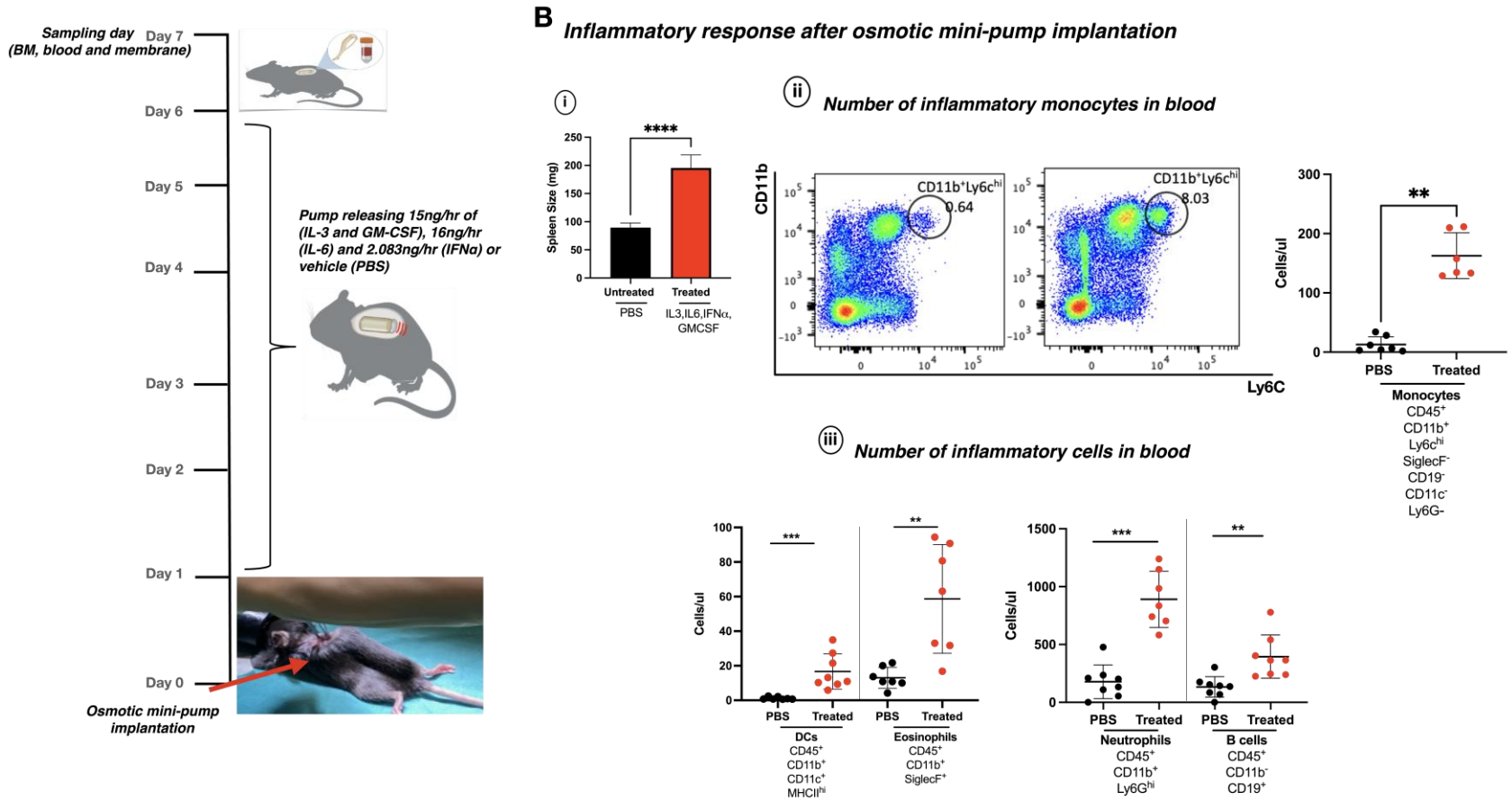

Figure 4-1 Subcutaneous osmotic mini pump implantation. A) Schematic diagram represents the outline of the minipump model. The osmotic pumps were surgically implanted to deliver the loaded mediators indicated above into the circulation for 7 days to induce sustained inflammation help for studying cell trafficking during the inflammatory response. After that time, mice were culled and then BM, blood and membrane surrounding the minipump were extracted for flowcytometric analysis. B) graphs show the outcomes of systemic inflammation induced in response to the minipump implantation i) enlargement of the spleen and increased the number of circulating ii) inflammatory monocytes, ii) other inflammatory immune cells from mice treated with cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) in red or with vehicle (PBS) in black. Data are shown as Mean ± SD of two independent experiments (N=8). **P <.01, ***P <.001, and ****P <.0001, Mann Whitney test.

Mice were monitored daily following the mini pump implant for any changes in body weight and appearance. Figure 4-2 shows that the body weights in mice treated with the cytokine cocktail, or vehicle (PBS), were nearly identical.

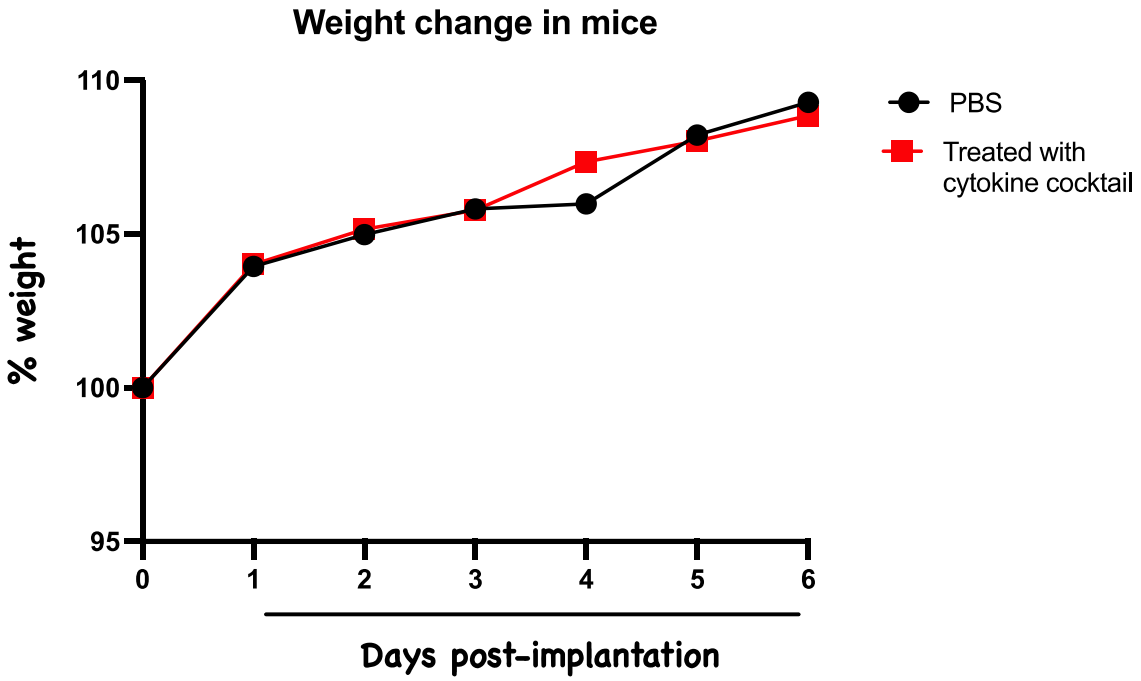

Figure 4-2 Mice weight post-implantation procedure. Each point on the line graph represents the mean of the body weight changes for all mice treated with the cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, and 2.083ng/hr IFN) in red or with vehicle (PBS) in black, as measured from day 0 prior to surgery to a day before sacrifice.

4.3 iCCR expression under sustained inflammation using subcutaneous osmotic mini pump implantation model

iREP mice and their non-fluorescent WT littermates were sacrificed on day 7 following implantation. After that, single-cell suspensions of BM and blood were prepared and stained with the antibody panel listed in Table 3-1 in the Materials and Methods section to identify leukocyte subsets. Then, flow cytometric analysis was performed to assess the expression of the iCCR reporters in classical inflammatory monocytes.

Cells were also collected from the membrane formed around the mini pump implant to determine iCCR reporter expression in recruited inflammatory monocytes and differentiated macrophages at the inflamed membrane. It is important to note that this membrane represents a new inflamed structure that allows us to study cell recruitment without being influenced by the presence of any resident cell populations.

4.3.1 Initial flow cytometry gating strategies

Four general gates were initially applied to all cells isolated from BM, blood, and the membrane surrounding the mini pump before analysing the expression of the iCCR reporters (Figure 4-3). First, cells were identified, and debris was removed by gating on SSC-A and FSC-A. Then, using viability dye, live cells were selected by gating on the unstained population to exclude all dead cells since they have higher levels of autofluorescence and non-specific antibody binding, which can result in false positive results. After that, cells were gated on FSC-A and FSC-H to select single cells and omit doublets. Leukocytes were finally identified by gating on the CD45-positive population.

4.3.1.1 Gating strategies applied to BM and peripheral blood

After gating on $CD45^+$ leukocytes, plotting Ly6c against CD11b allowed us to differentiate classical inflammatory monocytes ($CD11b^+ly6c^{hi}$) in BM and blood from intermediate and non-classical myocytes as well as from other $CD11b^+$ myeloid subsets (Figure 4-4). The expression of iCCR receptors in monocytes/macrophages is our primary focus because these cells have been found to express these receptors in combination[198].

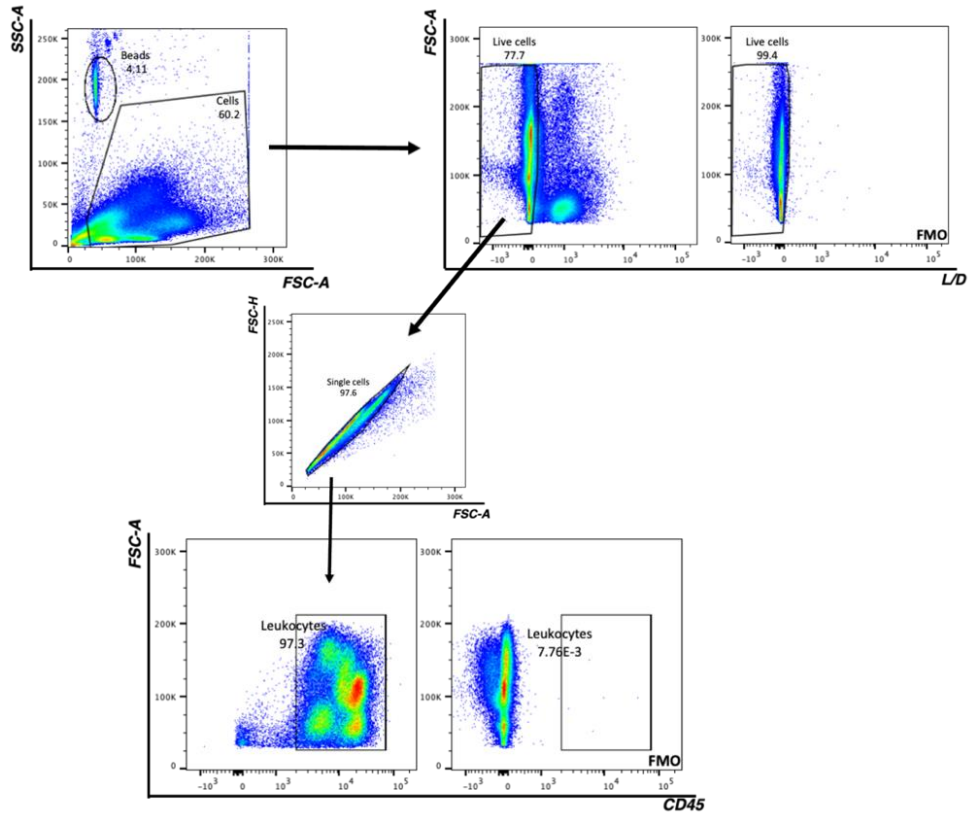

Figure 4-3 Initial gating strategy for cells of subcutaneous osmotic mini pump experiment. All the subsequent analyses of the flow cytometric data were analysed following this initial gating strategy, starting with selecting cells based on size and granularity using FSC-A versus SSC-A. Then, Dead cells were identified and removed by gating on non-stained cells. Doublets were excluded gating on single cells followed by CD45 for identifying Leukocytes. Cells were further gated before analysing the expression of iCCR.

Our group has found that neither neutrophils nor lymphocytes express these receptors and that CCR1 is only expressed by B cells in certain conditions, while CCR3 is exclusive to eosinophils. Therefore, $CD11b^{+}ly6c^{hi}$ classical inflammatory monocytes were then gated on SiglecF and CD19, markers for eosinophils and B cells, respectively, to remove any potentially contaminated eosinophils and B cells, and they were thus identified as $SiglecF^{-}CD19^{-}$. The remaining cells were next gated as $CD11c^{-}ly6G^{-}$ to further eliminate any potential presence of unwanted cell populations, such as neutrophils and DCs. After that, the BM and blood inflammatory monocytes were either gated on FSC-A and FSC-H to exclude doublets before analysing the reporter iCCR expression, or they were gated on CCR2 first, followed by doublet exclusion, and finally, iCCR analysis (Figure 4-4).

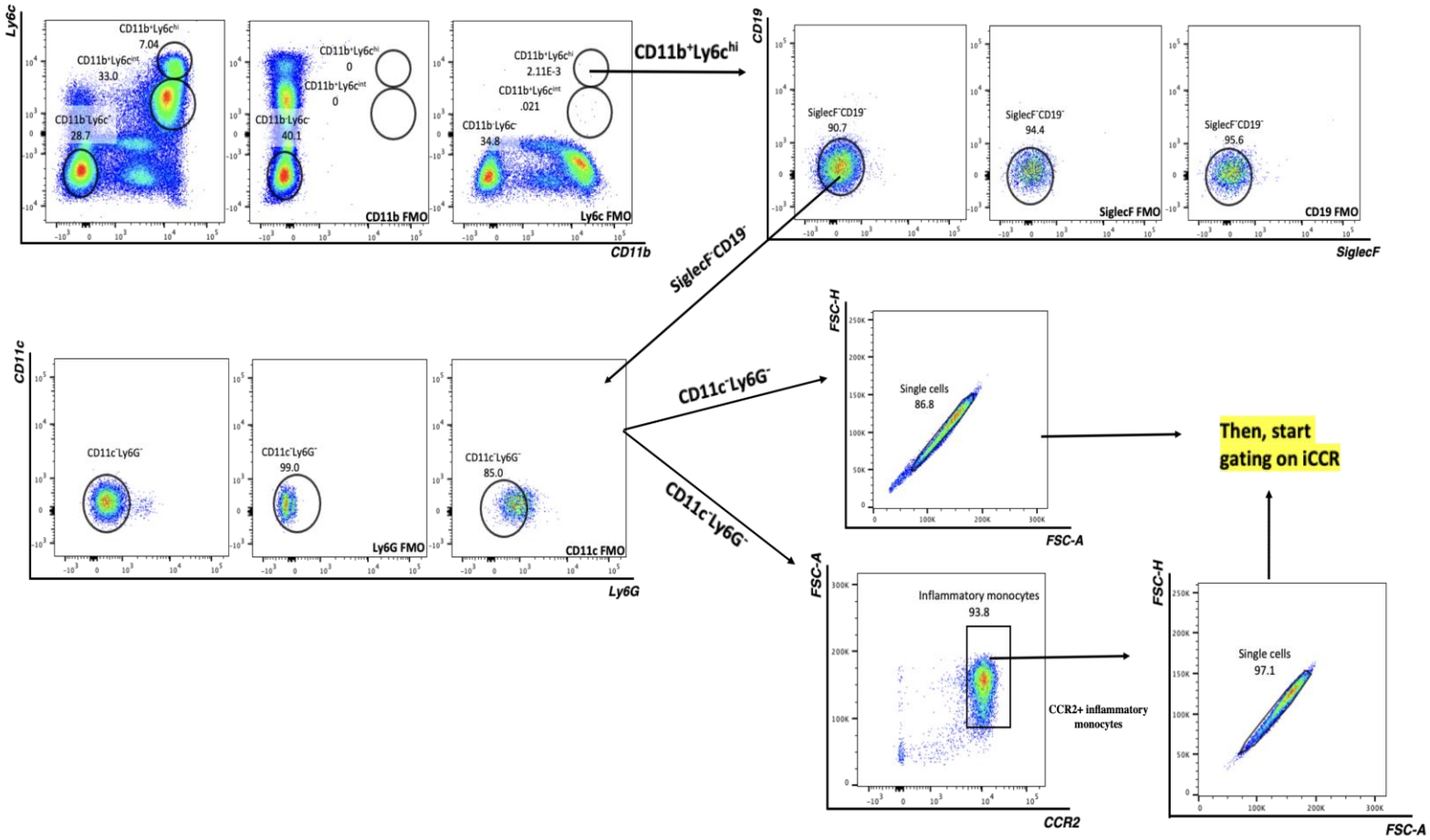

Figure 4-4 Gating strategy to analyse the expression of iCCR reporters in BM and peripheral blood inflammatory monocytes. Subcutaneous osmotic mini pumps infused with cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) or PBS were surgically implanted under the dorsal skin of mice. Seven days later, mice were culled, then BM and blood samples were analysed by FACS. Black arrows highlight the gating pathway. Leukocytes were identified as CD45+ as shown in Figure 4-2. Gating on CD11b and Ly6C, CD45^{+}cells were classified into three populations, and therefore inflammatory monocytes were defined as CD11b^{+}ly6c^{hi}. To improve purity of CD11b^{+}Ly6C^{hi} inflammatory monocytes were further analysed as SiglecF^{-}CD19^{-} then as CD11c^{-}Ly6G^{-}. Finally, Cells were either gated on single cells or CCR2 expression before analysing reporter iCCR expression.

4.3.1.2 Gating strategies applied to membrane

Different gating strategies were used to analyse iCCR reporter expression in inflammatory monocytes and differentiated macrophages in the inflamed membrane surrounding the mini pump. Ly6c and F480 were both used to distinguish between these two cell populations in the inflamed membrane.

Initially, $CD45^+$ cells were gated on CD11b and Ly6G to remove unwanted leukocyte subsets, and cells of interest were identified as $CD11b^+$ $Ly6G^-$. The remaining cells were subsequently gated as $CD11c^-$ and $SiglecF^-$ to further eliminate undesired cell types. Then, the target cells were determined by gating on Ly6c and F480. $Ly6c^{hi}$ $F480^{low}$ cells were identified as classically inflammatory monocytes, while $Ly6c^-F480^{hi}$ cells were identified as fully differentiated macrophages. Finally, the expression of each iCCR reporter protein was evaluated in each of them.

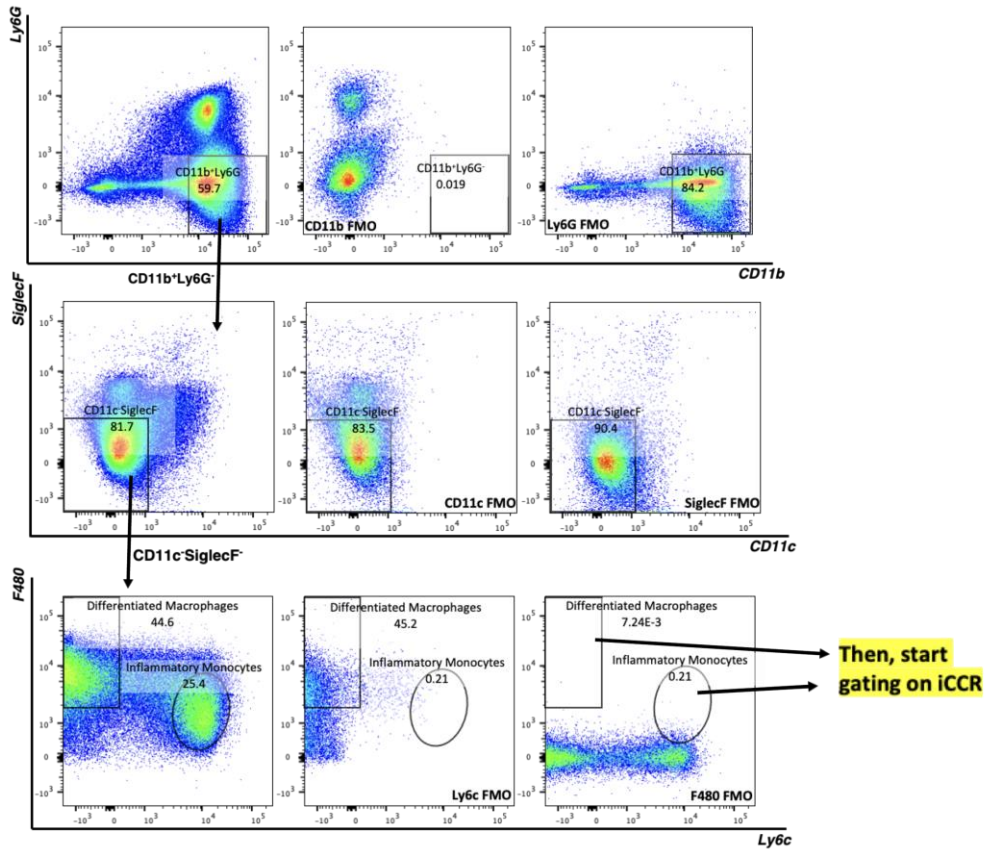

Figure 4-5 Gating strategy to analyse the expression of iCCR reporters in membrane inflammatory monocytes and macrophages. Seven days after surgically implanted

subcutaneous osmotic pumps with cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) or vehicle (PBS) under the dorsal skin of mice, the membrane around the minipumps was analysed by FACS. Black arrows highlight the gating pathway. Positive $CD45^{+}$cells were filtered from any unwanted cell populations. The population of interest was then determined as $CD11b^{+}Ly6G^{-}CD11c^{-}$ $SiglecF^{-}$. Finally, inflammatory monocytes and differentiated macrophages were gated on Ly6c and F480 and identified as $Ly6c^{hi}F480^{low}$ and $Ly6C^{-}F480^{hi}$, respectively. Cells were then analysed for the expression of iCCR reporters.

4.3.2 iCCR reporter expression in BM inflammatory monocytes

4.3.2.1 CCR2

Since it is well known that CCR2 is responsible for monocyte migration from the BM into circulation at rest and during inflammation[194, 309, 365], we first analysed the expression of the CCR2 reporter in BM classical inflammatory monocytes ($CD11b^{+}Ly6C^{hi}$) under sustained inflammation. As expected, and in line with previous reports[194, 198, 366], flow cytometric analysis in Figure 4-6, A and Ai show that the majority of $CD11b^{+}Ly6C^{hi}$ inflammatory monocytes were positive for mRuby2/CCR2, and about 95% $CD11b^{+}Ly6C^{hi}$ inflammatory monocytes expressed mRuby2/CCR2 in mice treated with the cytokine cocktail or vehicle (PBS). Also, there were no significant differences in the mRuby2/CCR2 MFI in $CD11b^{+}Ly6C^{hi}$ inflammatory monocytes from either group of mice (Figure 4-6 B, Bi). Thus, these data demonstrate that classical inflammatory monocytes predominantly express CCR2 in both resting and inflamed BM.

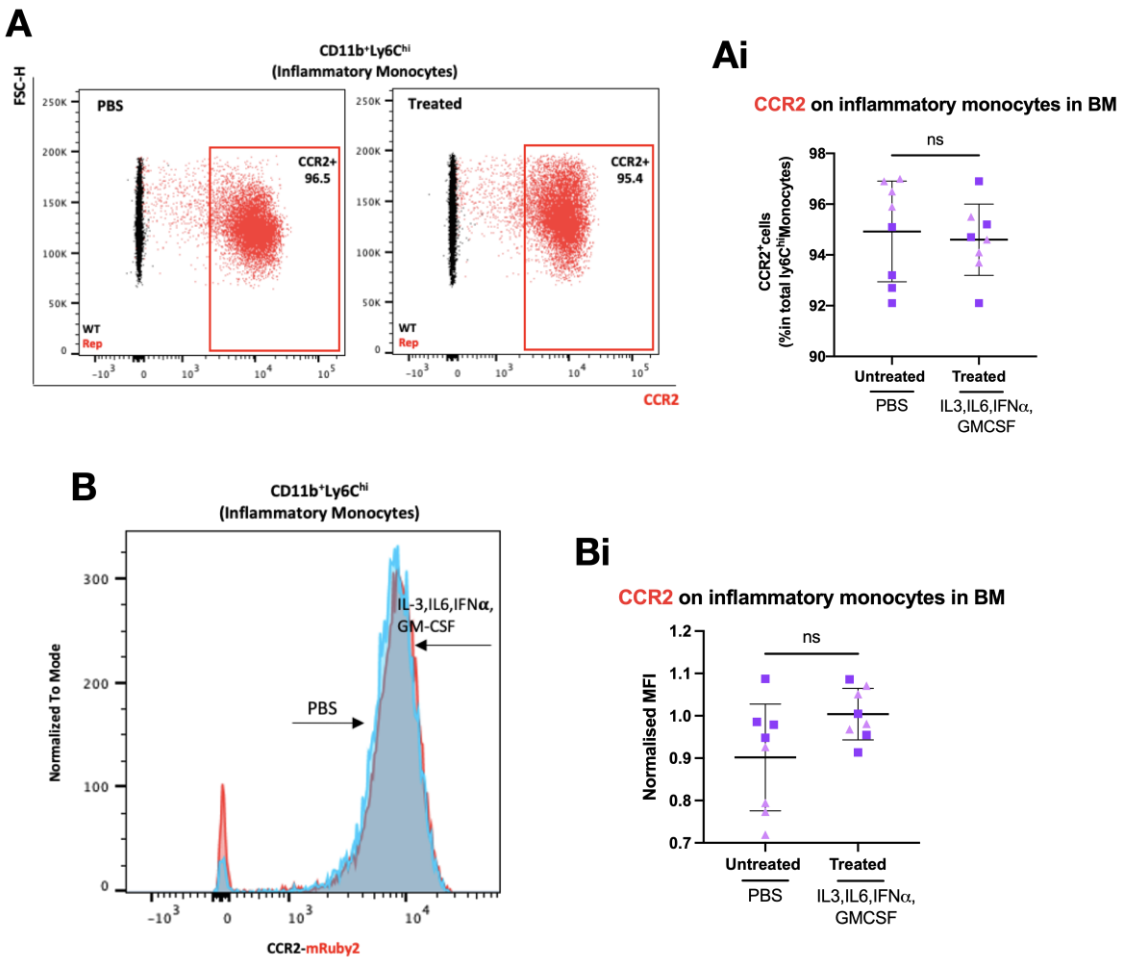

Figure 4-6 Effect of sustained inflammation on CCR2 reporter expression in BM inflammatory monocytes. Subcutaneous osmotic pumps loaded with a cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) or with vehicle (PBS) were surgically implanted under the dorsal skin of mice. Infusion of the inflammatory mediators was maintained for 7 days. After this time, mice were culled and BM inflammatory monocytes ($CD45^{+}CD11b^{+}Ly6C^{hi}$) were analysed. A) Flow cytometric analysis and (Ai) quantification of CCR2-mRuby2 expression. B) Histogram shows the fluorescent expression level of CCR2-mRuby2 in inflammatory monocytes from mice treated with cytokine cocktail (red) compared to vehicle (PBS) (blue). Bi) Graph shows the MFI of CCR2-mRuby2 expression. Data are shown as Mean ± SD of two independent experiments. n.s. not significant. Mann Whitney test.

4.3.2.2 Distribution of iCCR reporter expression in total BM inflammatory monocytes

Next, further analysis or characterisation of the combinatorial expression of the iCCR reporters in total $CD11b^{+}Ly6C^{hi}$ inflammatory monocytes was performed using flow cytometry. Figures 4-7 A and B show the FACS plots of mRuby2/CCR2 against Clover/CCR1 or iRFP682/CCR5, respectively. The results confirmed the previous observation that the bulk of $CD11b^{+}Ly6C^{hi}$ inflammatory

monocytes expresses only mRuby2/CCR2 in both mouse groups treated with either the cytokine cocktail or with vehicle (PBS). However, sustained inflammation led to an increase in the number of CD11b^{+}Ly6C^{hi} inflammatory monocytes that co-expressed mRuby2/CCR2 with Clover/CCR1 or iRFP682/CCR5, as shown in Figures 4-7 C and Ci. Therefore, to accurately quantify the expression of Clover/CCR1 and iRFP682/CCR5, only mRuby2/CCR2^{+} monocytes were gated on for the following analysis.

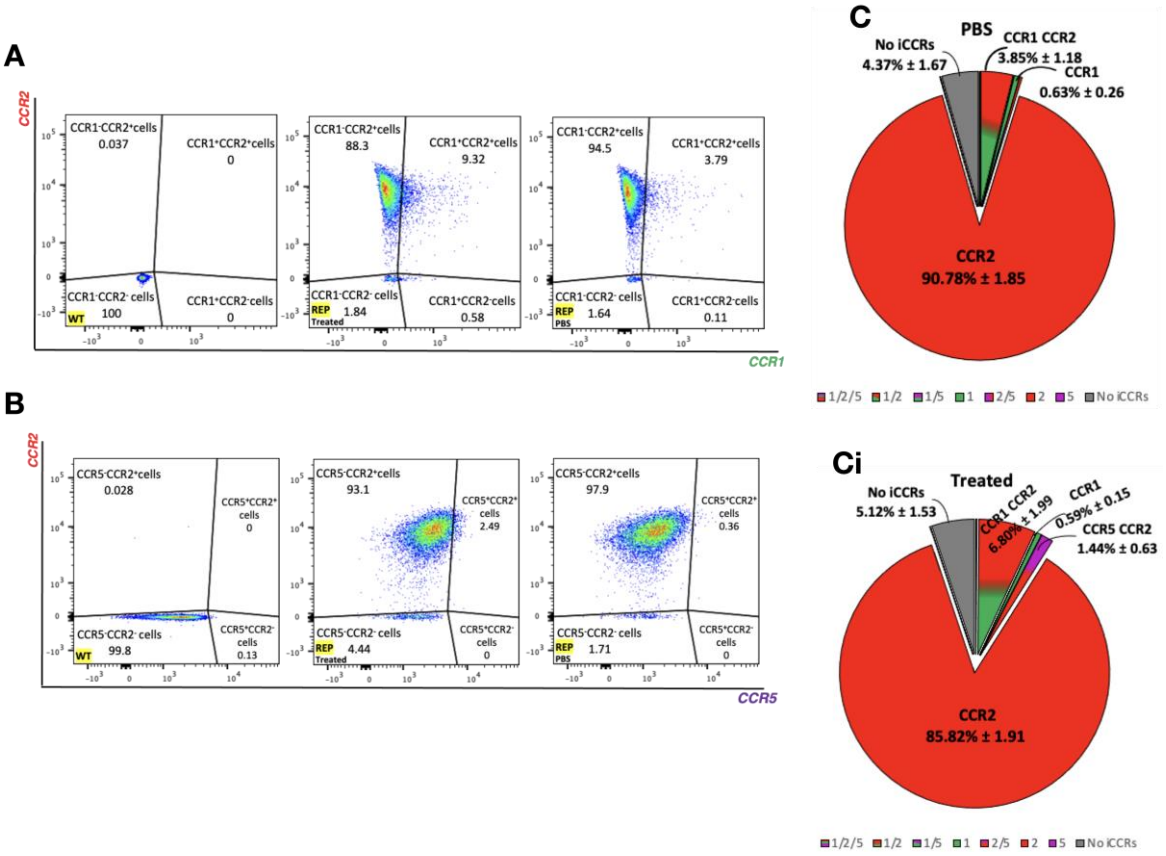

Figure 4-7 Combinatorial expression of reporter iCCRs in BM inflammatory monocytes. Flow cytometric analysis showing the co-expression of (A) CCR1-Clover and (B) CCR5-iRFP682 with CCR2-mRuby2 in CD45^{+}CD11b^{+}Ly6C^{hi} BM inflammatory monocytes. Positive expression was determined using non-fluorescent WT inflammatory monocytes. Pie charts summarising the distribution of the fluorescent receptors in BM inflammatory monocytes from mice treated with (C) vehicle (PBS) and (Ci) cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα). The expression of CCR2-mRuby2 is shown in red, the co-expression of CCR2-mRuby2 and CCR1-Clover is shown in red/green, red/purple for CCR2-mRuby2/CCR5-iRFP682 co-expression and no fluorescent reporter expression in grey. Data are shown from two independent experiments.

4.3.2.3 CCR1

The results in Figure 4-8 A and Ai show that Clover/CCR1 was significantly more highly expressed in CCR2^{+} inflammatory monocytes in the BM of mice treated with the cytokine cocktail than in CCR2^{+} inflammatory monocytes from mice treated with vehicle (PBS), with about 9% being Clover/CCR1 positive compared to 5%, respectively. This induction was also reflected in a 1.5-fold increase in Clover/CCR1 MFI in CCR2^{+} inflammatory monocytes in the BM of mice treated with the cytokine cocktail compared to vehicle (PBS) (Figure 4-8 B, Bi). Overall, sustained inflammation increased the number of CCR1-expressing inflammatory monocytes in the BM and the intensity of CCR1 expression in these cells.

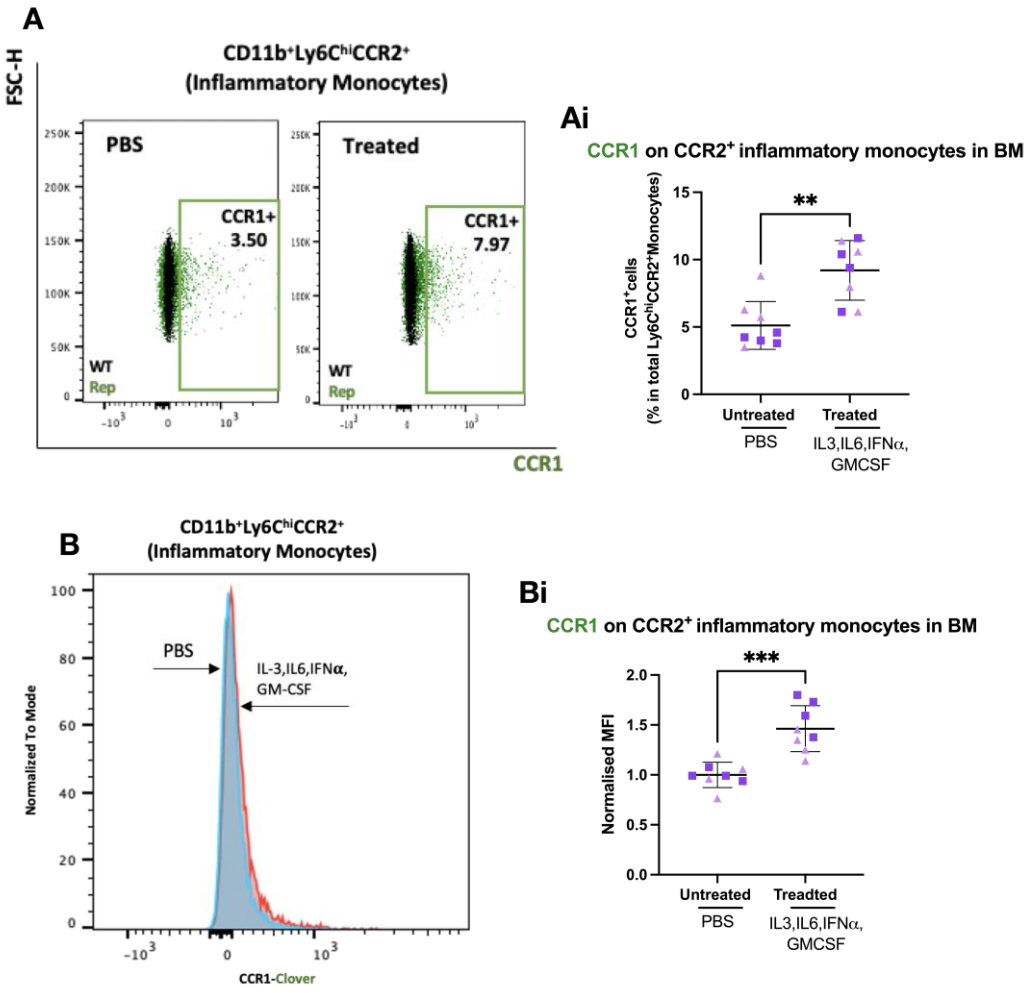

Figure 4-8 Effect of sustained inflammation on CCR1 reporter expression in BM CCR2+inflammatory monocytes. Subcutaneous osmotic pumps loaded with a cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) or with vehicle (PBS) were surgically implanted under the dorsal skin of mice. Infusion of the inflammatory mediators was

maintained for 7 days. After this time, mice were culled and BM inflammatory monocytes ($CD45^+CD11b^+Ly6C^{hi}CCR2^+$) were analysed. A) Flow cytometric analysis and (Ai) quantification of CCR1-Clover expression. B) Histogram shows the shift of the CCR1-Clover fluorescent expression in $CCR2^+$inflammatory monocytes from mice treated with cytokine cocktail (red) compared to vehicle (PBS) (blue). Bi) Graph shows the MFI of CCR1-Clover expression. Data are shown as Mean ± SD of two independent experiments. **P <.01 and ***P <.001. Mann Whitney test.

4.3.2.4 CCR5

Figure 4-9 A and Ai demonstrate that the number of iRFP682/CCR5-expressing $CCR2^+$ inflammatory monocytes in BM increased significantly after cytokine cocktail treatment (~2% CCR5 positive vs~0.3%), which was accompanied by higher MFI levels of iRFP682/CCR5 in this population (Figure 4-9 B, Bi). However, because of the small fraction involved, it was unclear whether this significant increase in iRFP682/CCR5 expressing $CCR2^+$ inflammatory monocytes would have biological importance. Overall, the data in Figure 4-9 shows that sustained inflammation causes a limited number of monocytes to express CCR5, which suggests that CCR5 plays a less significant role than CCR1 in monocyte function in inflammation.

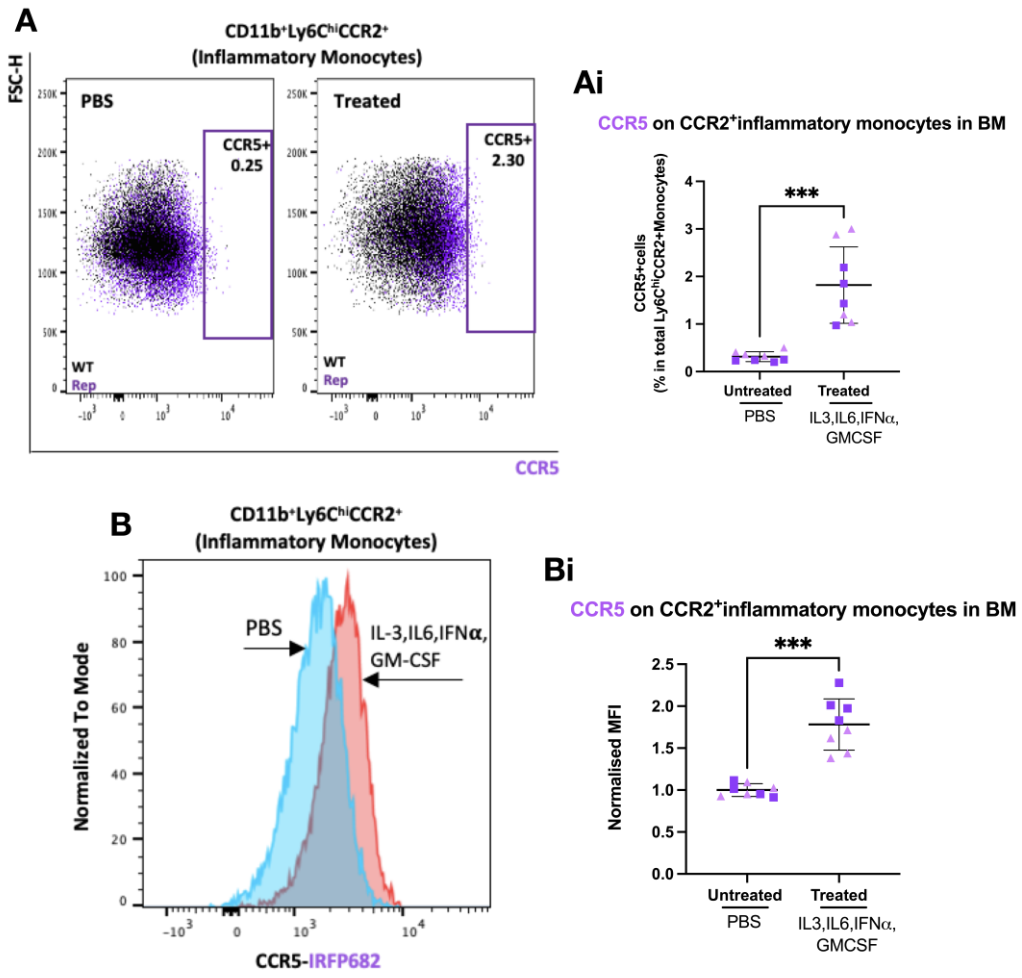

Figure 4-9 Effect of sustained inflammation on CCR5 reporter expression in BM CCR2^{+}inflammatory monocytes. Subcutaneous osmotic pumps loaded with a cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) or with vehicle (PBS) were surgically implanted under the dorsal skin of mice. Infusion of the inflammatory mediators was maintained for 7 days. After this time, mice were culled and BM inflammatory monocytes (CD45^{+}CD11b^{+}Ly6C^{hi}CCR2^{+}) were analysed. A) Flow cytometric analysis and (Ai) quantification of CCR5-iRFP682 expression. B) Histogram shows the shift of the CCR5-iRFP682 fluorescent expression in CCR2^{+}inflammatory monocytes from mice treated with cytokine cocktail (red) and vehicle (PBS) (blue). Bi) Graph shows the MFI of CCR5-iRFP682 expression. Data are shown as Mean ± SD of two independent experiments. ***P <.001. Mann Whitney test.

4.3.2.5 CCR3

Figure 4-10 shows no evidence of mTagBFP2/CCR3 expression in CCR2+ inflammatory monocytes in the BM of mice treated with either the cytokine cocktail or vehicle (PBS). Therefore, it is clear from these data that inflammatory monocytes do not express CCR3 and that CCR3 is not involved in monocyte migration in either resting or inflamed conditions. Therefore, CCR3 will not be included in our subsequent analysis.

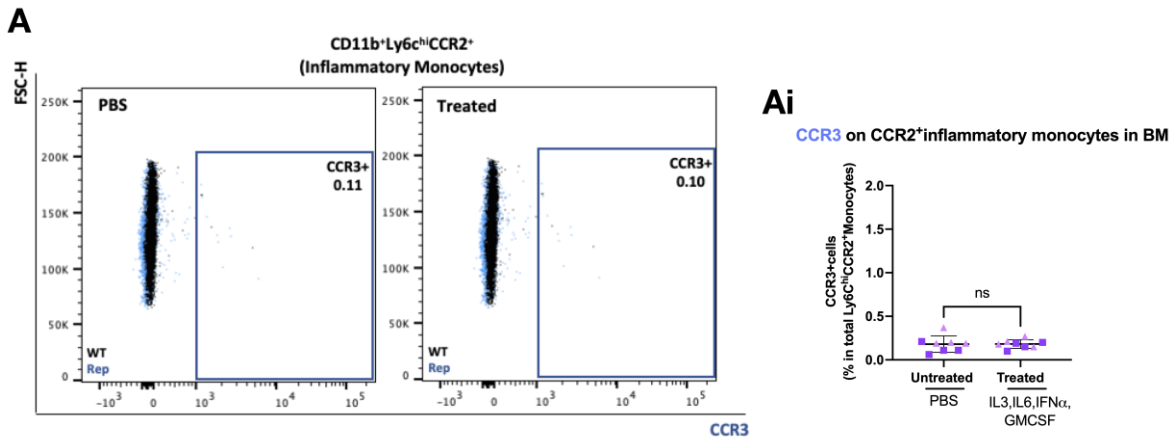

Figure 4-10 Effect of sustained inflammation on CCR3 reporter expression in BM CCR2^{+}inflammatory monocytes. A)Flow cytometric analysis and (Ai) quantification of CCR3-mTagBFP2 expression in BM inflammatory monocytes (CD45^{+}CD11b^{+}Ly6C^{hi}CCR2^{+}) from mice implanted with subcutaneous osmotic pumps loaded with a cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) or with vehicle (PBS). Data are shown as Mean ± SD of two independent experiments. n.s. not significant. Mann Whitney test.

4.3.3 Blood

4.3.3.1 CCR2

The expression of the CCR2 reporter in blood inflammatory monocytes was then analysed. Similar to the results observed in BM, most of the blood CD11b^{+}Ly6C^{hi} inflammatory monocytes from mice treated with the cytokine cocktail or vehicle (PBS) expressed mRuby2/CCR2 (Figure 4-11 A). However, the number of CCR2^{+} positive inflammatory monocytes was marginally and significantly lower in inflamed mice compared to vehicle (PBS)-treated mice (~97.8% vs ~99.6%, respectively) (Figure 4-11 Ai). This was also shown by a trend toward lower MFI levels of mRuby2/CCR2 in this population from mice treated with the cytokine cocktail compared to mice treated with the vehicle (PBS), but this was not statistically significant (Figure 4-11 B, Bi). Thus, inflammatory blood monocytes mainly express CCR2 during both rest and inflammation.

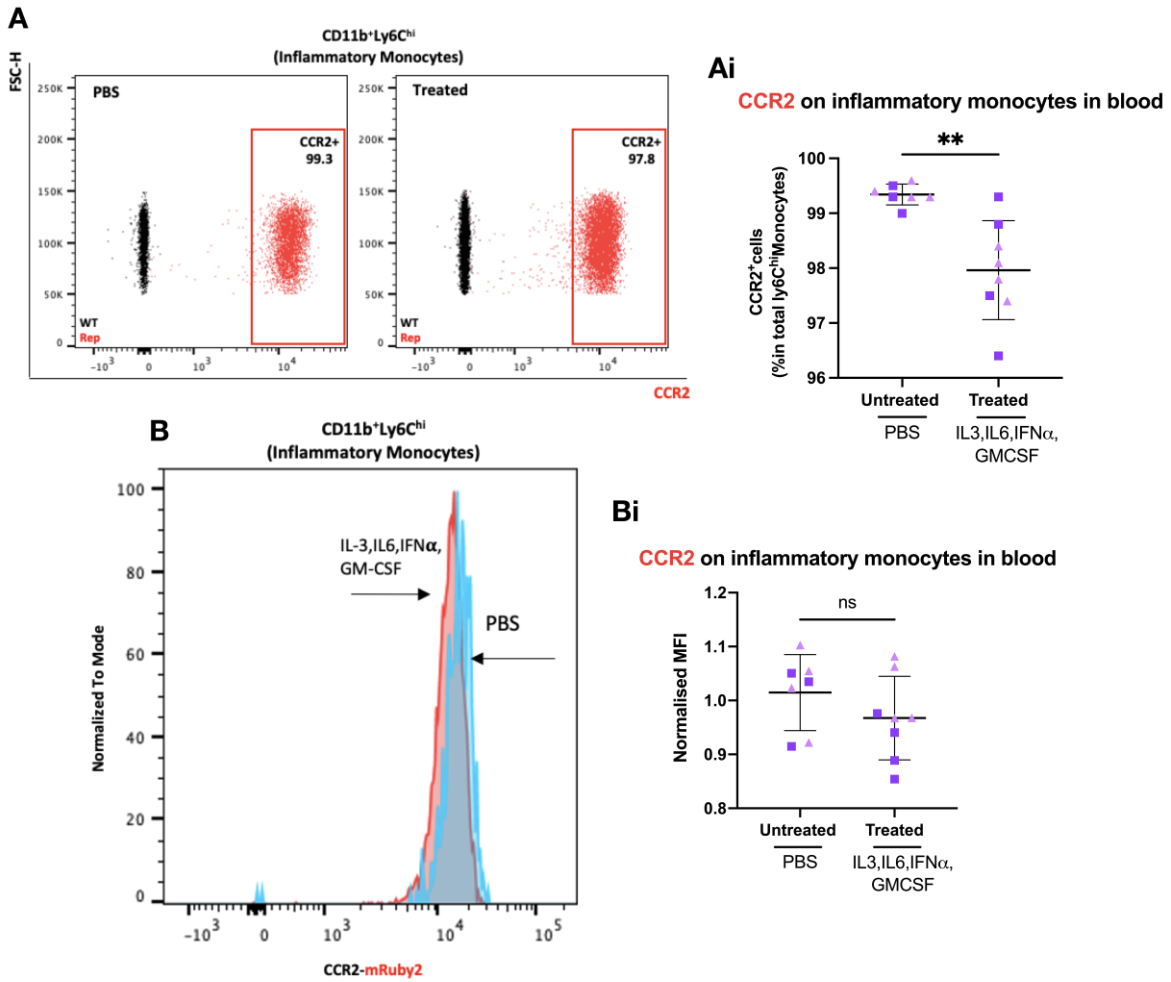

Figure 4-11 Effect of sustained inflammation on CCR2 reporter expression in blood inflammatory monocytes. Subcutaneous osmotic pumps loaded with a cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) or with vehicle (PBS) were surgically implanted under the dorsal skin of mice. Seven days later, mice were culled and circulating monocytes ($CD45^+CD11b^+Ly6C^{hi}$) were analysed. A) Flow cytometric analysis and (Ai) quantification of CCR2-mRuby2 expression. B) Histogram shows the level of CCR2-mRuby2 fluorescent expression in circulating monocytes from mice treated with cytokine cocktail (red) compared to vehicle (PBS) (blue). Bi) Graph shows MFI of CCR2-mRuby2 expression. Data are shown as Mean ± SD of two independent experiments. **P <.01 and ***P <.001. Mann Whitney test.

4.3.3.2 Distribution of iCCR reporter expression on total blood inflammatory monocytes

Then, mRuby2/CCR2 was plotted against either Clover/CCR1 or iRFP682/CCR5 to analyse combinatorial iCCR reporter expression in total inflammatory monocytes in the blood. As shown in the FACS plots in Figure 4-12 A and B, the majority of $CD11b^+Ly6C^{hi}$ monocytes in mice treated with the cytokine cocktail or vehicle (PBS) were only positive for mRuby2/CCR2, and the percentage of these cells was slightly lower in the cytokine cocktail-treated mice than in

vehicle (PBS)-treated mice (82.85% vs 93.23%, respectively) (Figure 4-12 C, Ci). As previously mentioned in the BM results, mice treated with the cytokine cocktail showed an increase in blood CD11b^{+}Ly6C^{hi} inflammatory monocytes co-expressing CCR2 with Clover/CCR1 or iRFP682/CCR5 in comparison to those treated with vehicle (PBS) (Figure 4-12 C, Ci). Therefore, for the subsequent analysis, only mRuby2/CCR2^{+} monocytes were chosen for gating to obtain an accurate measurement of their expression.

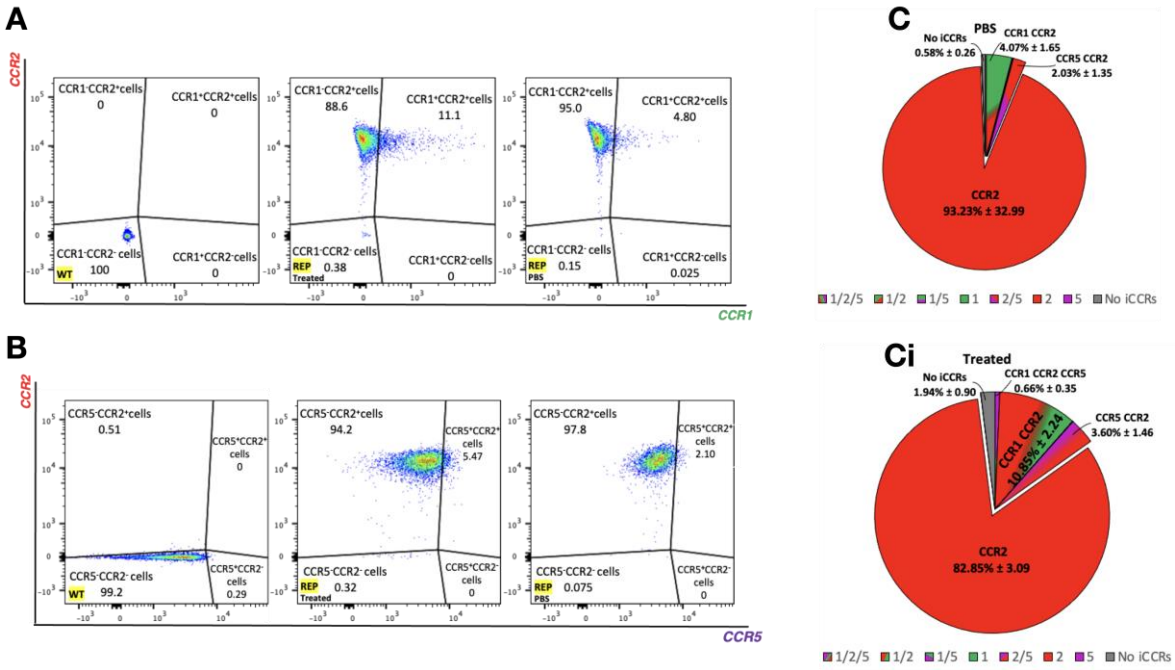

Figure 4-12 Combinatorial expression of reporter iCCR in blood inflammatory monocytes. Flow cytometric analysis showing the co-expression of (A) CCR1-Clover and (B) CCR5-iRFP682 with CCR2-mRuby2 in circulating inflammatory monocytes (CD45^{+}CD11b^{+}Ly6C^{hi}). Positive expression was determined using non-fluorescent WT inflammatory monocytes. Pie charts summarizing the distribution of CCR2-mRuby2 (red), CCR1-Clover (green), CCR5-iRFP682 (purple) and grey for no fluorescent reporter expression in circulating inflammatory monocytes from mice treated with (C) vehicle (PBS) and (Ci) cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα). Data are shown from two independent experiments.

4.3.3.3 CCR1

The number of CCR2^{+} inflammatory monocytes co-expressing Clover/CCR1 was higher than what we had previously shown in the BM, where approximately 12% of circulating CCR2^{+} monocytes from mice treated with the cytokine cocktail were Clover/CCR1 positive, compared to 5% from mice treated with vehicle (PBS)

(Figure 4-13 A, Ai). This was further confirmed by a similar 2.5-fold increase in Clover/CCR1 MFI in circulating CCR2^{+} monocytes from mice treated with the cytokine cocktail as compared to vehicle (PBS) (Figure 4-13 B, Bi). The results in Figure 4-13, taken together, indicate that acute inflammation not only results in an increase in CCR1^{+}CCR2^{+} monocytes in the blood but also increases the levels of CCR1 expression in these cells.

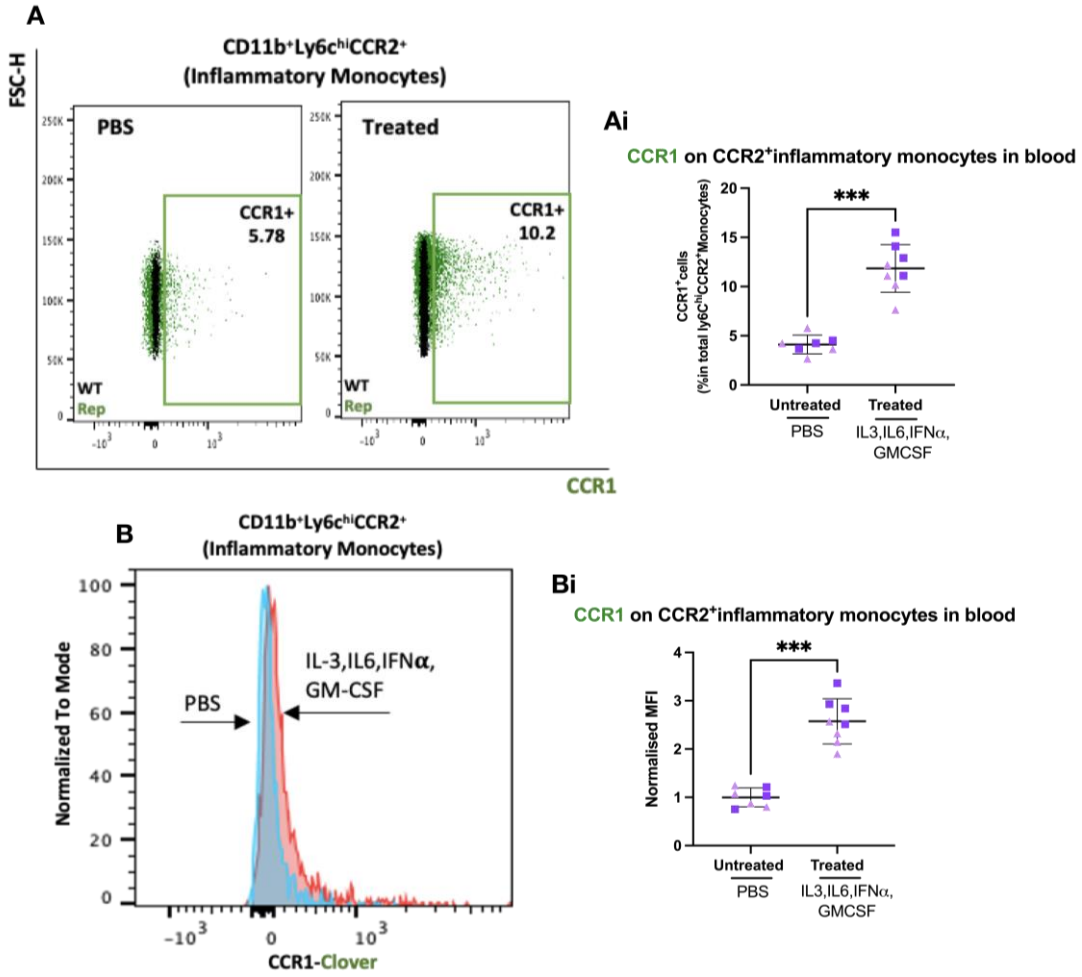

Figure 4-13 Effect of sustained inflammation on CCR1 reporter expression in blood inflammatory monocytes. Subcutaneous osmotic pumps loaded with a cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) or with vehicle (PBS) were surgically implanted under the dorsal skin of mice. Seven days later, mice were culled and circulating monocytes (CD45^{+}CD11b^{+}Ly6C^{hi}) were analysed. A) Flow cytometric analysis and (Ai) quantification of CCR1-Clover expression. B) Histogram shows the shift of the fluorescent expression of CCR1-Clover in circulating monocytes from mice treated with cytokine cocktail (red) compared to vehicle (PBS) (blue). Bi) Graph shows MFI of CCR1-Clover expression. Data are shown as Mean ± SD of two independent experiments. **P <.01 and ***P <.001. Mann Whitney test.

4.3.3.4 CCR5

The expression of iRFP682/CCR5 was then examined in CCR2^{+} inflammatory monocytes. As seen in Figure 4-14 A and Ai, mice treated with the cytokine cocktail showed an increase in the number of circulating CCR2^{+} inflammatory monocytes co-expressing iRFP682/CCR5, with roughly 5% of cells expressing CCR5 compared to 2% in mice treated with vehicle (PBS). Additionally, CCR2^{+} inflammatory monocytes displayed greater iRFP682/CCR5 MFI in the blood of mice treated with the cytokine cocktail compared to mice treated with vehicle (PBS)(Figure 4-14 B, Bi). Even though acute inflammation causes CCR2^{+} blood monocytes to express more CCR5, it appears that this still only accounts for a very small proportion of these cells.

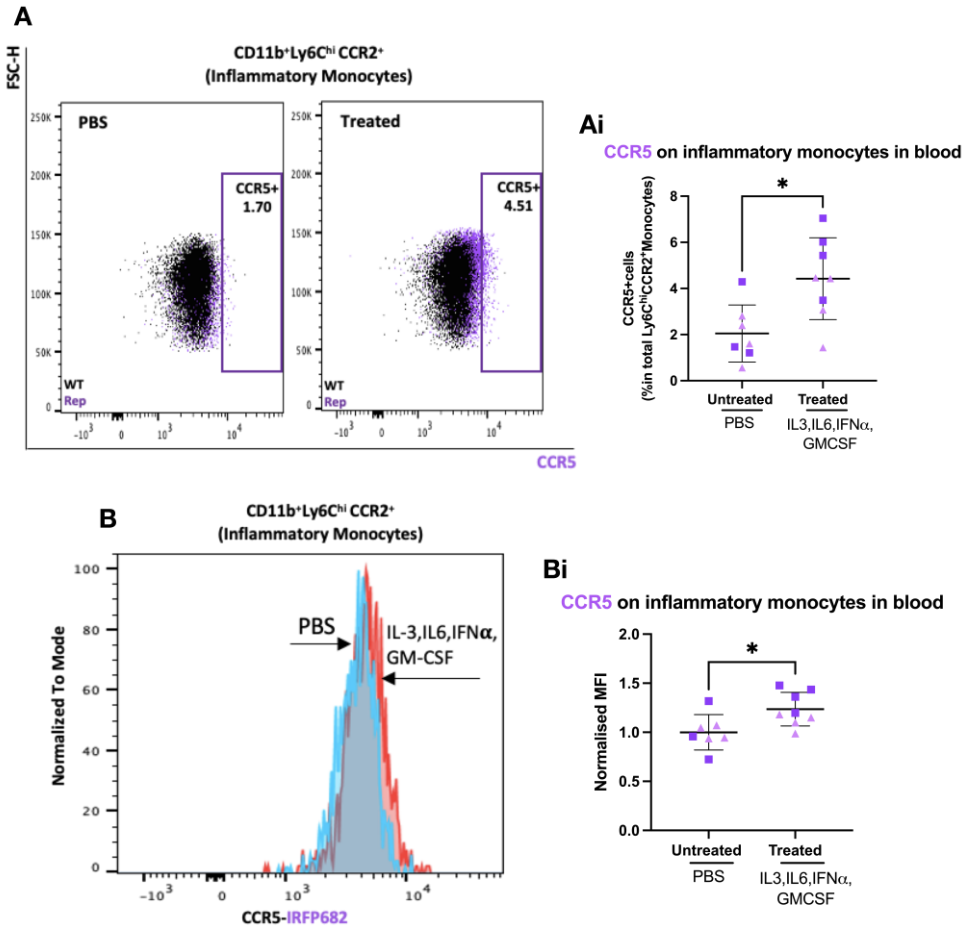

Figure 4-14 Effect of sustained inflammation on CCR5 reporter expression in blood inflammatory monocytes. Subcutaneous osmotic pumps loaded with a cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) or with vehicle (PBS) were surgically implanted under the dorsal skin of mice. Infusion of the inflammatory mediators was maintained for

7 days. After this time, mice were culled and circulating inflammatory monocytes ($CD45^+CD11b^+ly6c^{hi}$) were analysed. A) Flow cytometric analysis and (Ai) quantification of CCR5-iRFP682 expression. B) Histogram shows the shift of the fluorescent expression of CCR5-iRFP682 in inflammatory monocytes from mice treated with cytokine cocktail (red) and vehicle (PBS) (blue). Bi) Graph shows MFI of CCR5-iRFP682 expression. Data are shown as Mean ± SD of two independent experiments. *P <.05. Mann Whitney test.

4.3.4 Membrane

4.3.4.1 iCCR reporter expression in membrane inflammatory monocytes

4.3.4.1.1 CCR2

Next, inflammatory monocytes recruited into the inflamed membranes surrounding the mini pump were examined for reporter iCCR expression. As shown in Figure 4-15 A and Ai the number of inflammatory $Ly6C^{hi}F480^{low}$ monocytes expressing mRuby2/CCR2 was significantly decreased in the inflamed membrane of mice treated with the cytokines cocktail compared to membrane of mice treated with vehicle (PBS)(~78% vs ~88%, respectively). However, the majority of inflammatory monocytes still express mRuby2/CCR2, but to a lesser extent than what was found in the BM and blood. This reduction in mRuby2/CCR2 expressing inflammatory $Ly6C^{hi}F480^{low}$ monocytes was also reflected in a decrease in mRuby2/CCR2 MFI in these cells (Figure 4-15 B, Bi). Overall, these results show that inflammatory monocytes downregulate CCR2 expression when they first enter the inflamed tissue, suggesting that they are likely to be undergoing rapid differentiation at the inflamed site.

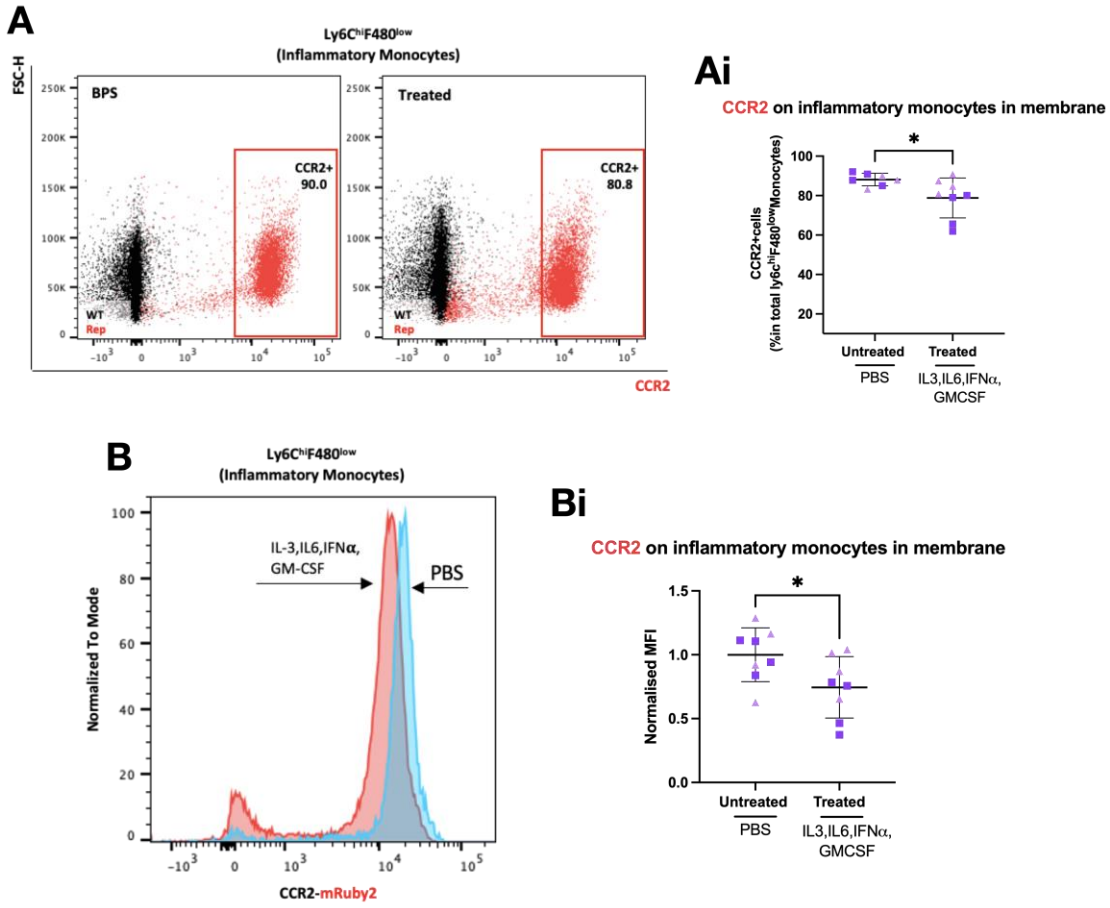

Figure 4-15 Effect of sustained inflammation on CCR2 reporter expression in membrane inflammatory monocytes. Mice were implanted with subcutaneous osmotic pumps. Seven days after the continuous release of inflammatory cytokines (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) or with vehicle (PBS), Ly6C^{hi}F480^{low} inflammatory monocytes from the minipump membrane were analysed for CCR2 expression. A) Flow cytometric analysis and (Ai) quantification of CCR2-mRuby2 expression. B) Histogram shows decrease in the level of CCR2-mRuby2 fluorescent expression by Ly6chiF480low monocytes from mice treated with cytokine cocktail (red) and vehicle (PBS) (blue). Bi) Graph shows the MFI rate of CCR2-mRuby2 expression. Data are shown as Mean ± SD of two independent experiments. *P <.05. Mann Whitney test.

4.3.4.1.2 Distribution of iCCR reporter expression on membrane inflammatory monocytes

The next step was to examine the combinatorial expression of the reporter iCCRs in the inflammatory monocytes. Inflammatory Ly6C^{hi}F480^{low} monocytes remained mainly expressing mRuby2/CCR2 inside the membrane with decreased expression in mice treated with the cytokine cocktail compared to vehicle (PBS). The percentage of inflammatory Ly6C^{hi}F480^{low} monocytes expressing mRuby2/CCR2 was lower than observed in BM and blood and about 66% vs 79.23%

in mice treated with the cytokine cocktail compared to vehicle (PBS), respectively (Figures 4-16 A and B). Therefore, to measure the expression of CCR1 and CCR5 in inflammatory monocytes in the membrane, gating was performed on total inflammatory Ly6ChiF480low monocytes instead of just CCR2-positive ones.

However, as observed in BM and blood, an increase in membrane Ly6C^{hi}F480^{low} inflammatory monocytes co-expressing mRuby2/CCR2 with Clover/CCR1 or iRFP682/CCR5 was observed in mice treated with the cytokine cocktail compared to vehicle (PBS). Overall, inflammatory monocytes in this model either exclusively expressed CCR2 or co-expressed CCR1 or CCR5 with CCR2, and there was no evidence that they expressed CCR1 or CCR5 on their own.

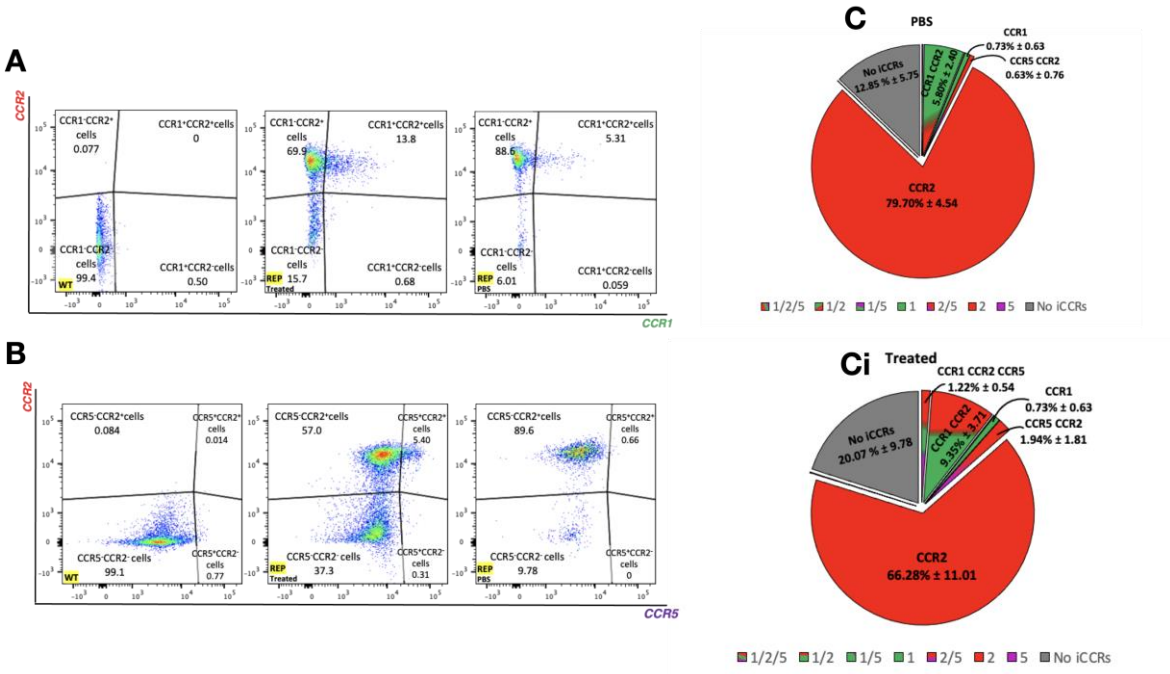

Figure 4-16 Distribution of the reporter iCCR expression in membrane inflammatory monocytes. Flow cytometric analysis showing the co-expression of CCR2-mRuby2 with (A) CCR1-Clover and (B) CCR5- iRFP682 in Ly6C^{hi}F480^{low} inflammatory monocytes isolated from minipump membrane. Pie charts summarizing the distribution of CCR2-mRuby2 (red), CCR1-Clover (green), CCR5-iRFP682 (purple) and grey for no fluorescent reporter expression in inflammatory monocytes from mice treated with (Ci) vehicle (PBS) and (Cii) cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα).

4.3.4.1.3 CCR1

As shown in Figure 4-17 A and Ai, inflammatory Ly6C^{hi}F480^{low} monocytes in the membrane of mice treated with the cytokine cocktail showed a similar increase in Clover/CCR1 expression to that seen in blood, with about 12% of inflammatory Ly6C^{hi}F480^{low} monocytes being positive for Clover/CCR1, compared to about 6% of mice treated with vehicle (PBS). Also, inflammatory Ly6C^{hi}F480^{low} monocytes significantly increased the Clover/CCR1 MFI by about 2.7-fold in the membrane of mice treated with the cytokine cocktail compared to vehicle (PBS) (Figure 4-17 B and Bi). In general, inflammatory monocytes in the inflamed membrane maintain the same levels of CCR1 expression as those detected in circulating monocytes, indicating that they were recently recruited into the inflamed area.

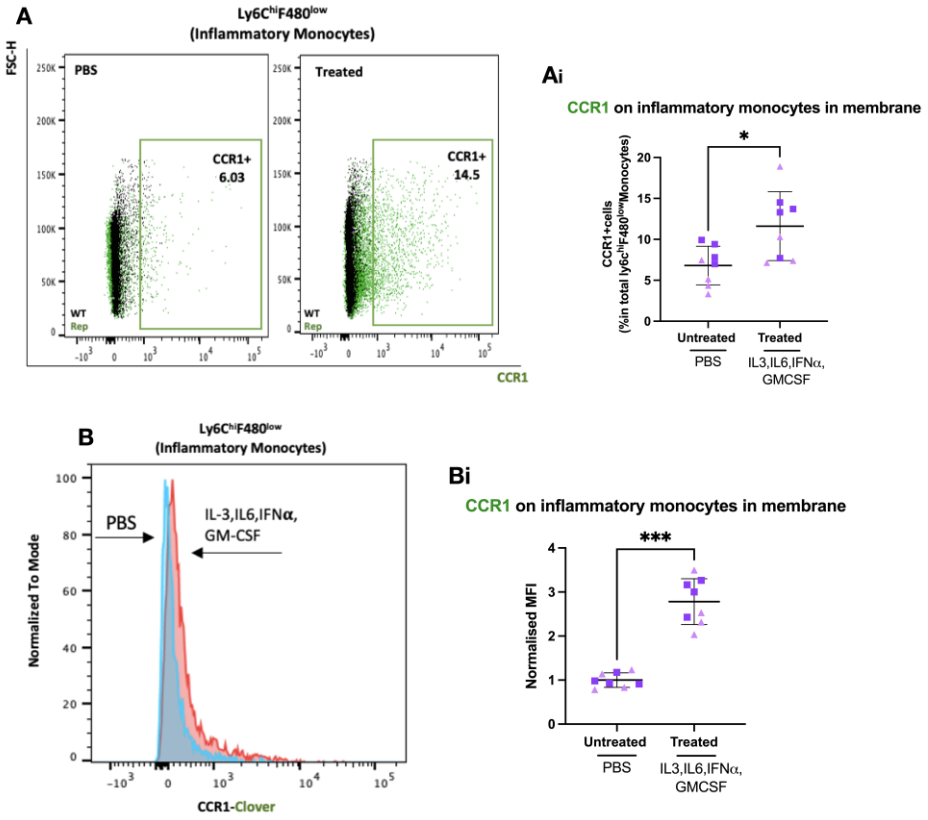

Figure 4-17 Effect of sustained inflammation on CCR1 reporter expression in membrane inflammatory monocytes. Implanted Subcutaneous osmotic pumps loaded with a cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) or with vehicle (PBS) for seven days were analysed for CCR1 expression in membrane Ly6C^{hi}F480^{low} inflammatory

monocytes. A) Flow cytometric analysis and (Ai) quantification of CCR1-Clover expression. B) Histogram shows the shift of CCR1-Clover fluorescent expression in Ly6C^{hi}F480^{low} monocytes from mice treated with cytokine cocktail (red) compared to vehicle (PBS) (blue). Bi) Graph shows the MFI of CCR1-Clover expression. Data are shown as Mean ± SD of two independent experiments. *P <.05 and ***P <.001. Mann Whitney test.

4.3.4.1.4 CCR5

Again, consistent with the observation in BM and blood, a small fraction of inflammatory Ly6C^{hi}F480^{low} monocytes were positive for iRFP682/CCR5 in the membrane of mice treated with the cytokine cocktail compared to vehicle PBS (~3% CCR5 positive vs~1%, respectively) (Figure 4-18 A, Ai). MFI for iRFP682/CCR5 was also increased by about 1.5-fold in CCR5 positive inflammatory Ly6C^{hi}F480^{low} monocytes in the membrane of mice treated with the cytokine cocktail compared to vehicle (PBS) (Figure 4-18 B, Bi). The results in Figure 4-18 show that sustained inflammation results in a small accumulation of inflammatory monocytes that express CCR5, which generally remains low throughout the BM and blood until they are recruited into the inflamed membrane.

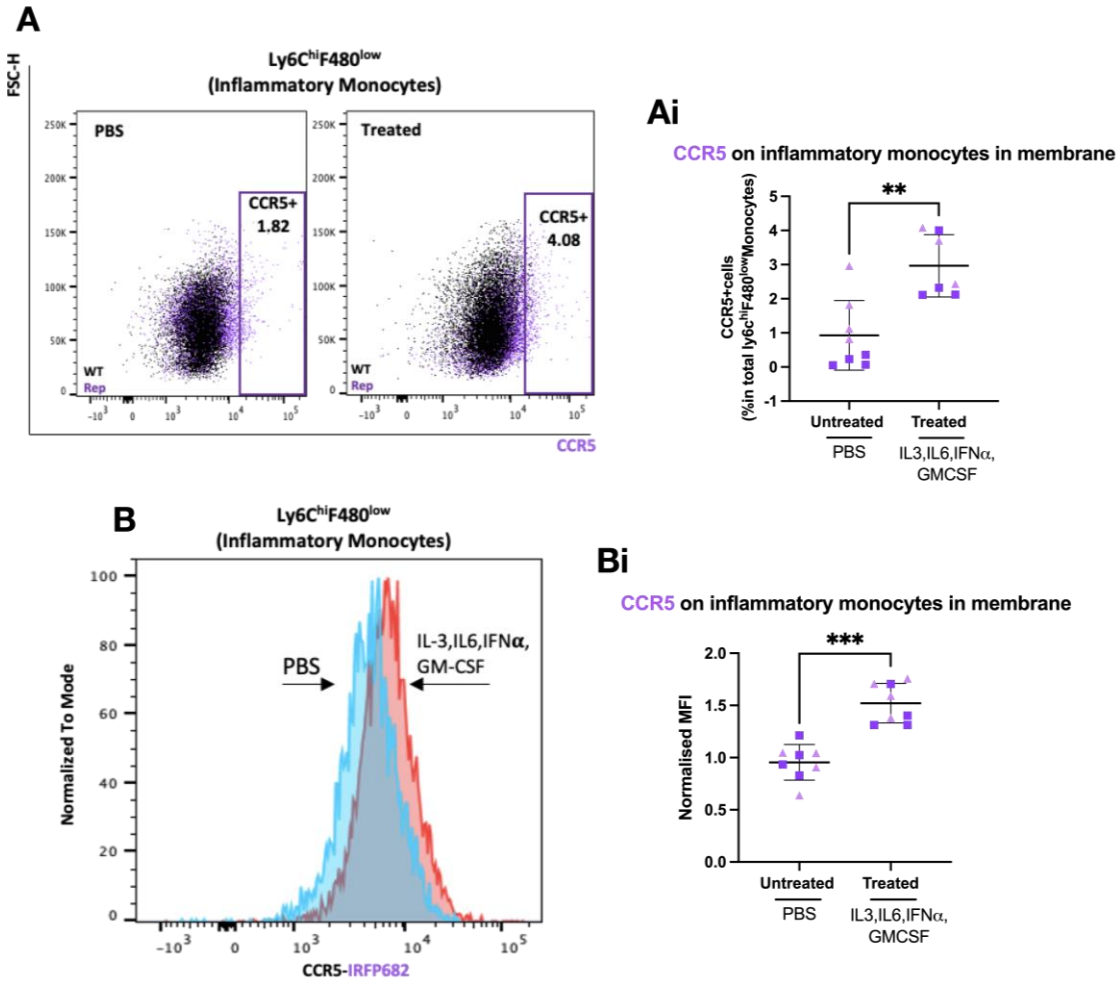

Figure 4-18 Effect of sustained inflammation on CCR5 reporter expression in membrane inflammatory monocytes. A) Flow cytometric analysis and (Ai) quantification of CCR5-iRFP682 expression in Ly6C^{hi}F480^{low} inflammatory monocytes isolated from the membrane of osmatic minipump mice. B) Histogram shows the shift of the fluorescent expression CCR5-iRFP682 in Ly6C^{hi}F480^{low} monocytes from mice treated with cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) in red compared to vehicle (PBS) in blue. Bi) Graph shows the MFI rate of CCR5-iRFP682 expression. Data are shown as Mean ± SD of two independent experiments. ***P <.01 and **P <.001. Mann Whitney test.

4.3.4.2 iCCR reporter expression in membrane differentiated macrophages

4.3.4.2.1 CCR2

Next, the expression of the iCCR reporters on monocyte-derived macrophages was examined using flow cytometry. As shown in Figure 4-19 A and Ai, there were no significant differences in the number of Ly6C^{-}F480^{hi} differentiated macrophages expressing mRuby2/CCR2 in the membrane of mice treated with the cytokine cocktail or vehicle (PBS) since mRuby2/CCR2 was

expressed by nearly 40% of this population in the membrane of both groups. Also, MFI for mRuby2/CCR2 remained unchanged in Ly6C^{-}F480^{hi} differentiated macrophages in the membrane of either group (Figure 4-19 B and Bi). Overall, these results support the finding from the previous chapter that differentiated macrophages express high levels of CCR2 but that this expression is unaffected by inflammatory conditions and remains constant.

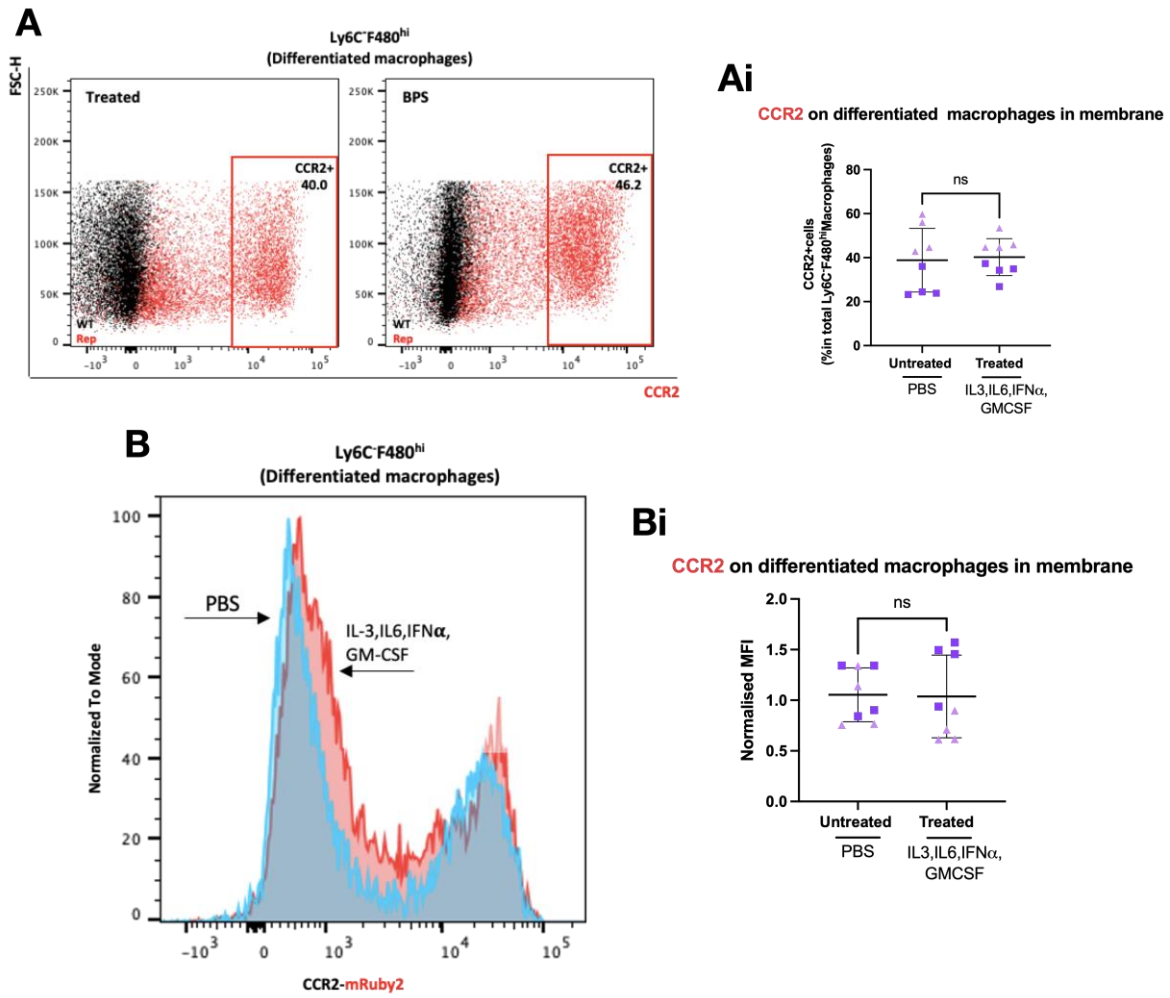

Figure 4-19 Effect of sustained inflammation on CCR2 reporter expression in membrane differentiated macrophages. A) Flow cytometric analysis and (Ai) quantification of CCR2-mRuby2 expression in Ly6C^{-} F480^{hi} differentiated macrophages from minipump membrane. B) Histogram shows the fluorescent expression level of CCR2-mRuby2 in Ly6C^{-} F480^{hi} differentiated macrophages from mice treated with cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) in red and vehicle (PBS) in blue. Bi) Graph shows the MFI of CCR2-mRuby2 expression. Data are shown as Mean ± SD of two independent experiments. n.s. not significant. Mann Whitney test.

4.3.4.2.2 Distribution of iCCR reporter expression in membrane differentiated macrophages

The combinatorial expression of the iCCRs reporter in Ly6C$^-$F480^{hi} differentiated macrophages was then further characterised by flow cytometry. As shown in Figure 4-20, differentiated Ly6C$^-$F480^{hi} macrophages did not exclusively express mRuby2/CCR2, in contrast to inflammatory monocytes. iRFP682/CCR5 expression was found to be also expressed independently of mRuby2/CCR2. However, it was only seen in a small proportion of differentiated Ly6C$^-$F480^{hi} macrophages in the membrane with about 5% in mice treated with vehicle PBS and increased to almost 8% of mice treated with cytokine cocktail (Figure 4-20 C, Ci). iRFP682/CCR5 and mRuby2/CCR2 were co-expressed by a higher percentage of Ly6C$^-$F480^{hi} differentiated macrophages (17% in the membrane of mice treated with the cytokine cocktail and 11% in the membrane of mice treated with vehicle (PBS)) (Figure 4-20 C, Ci). The number of triple-positive macrophages for CCR1, CCR2, and CCR5 in the membranes of mice treated with the cytokine cocktail was 2.5 times higher (from 3% to 8%) than that of mice treated with vehicle (PBS) (Figure 4-20 C, Ci).

Overall, as expected in the first chapter, the expression of CCR1 and CCR5 by macrophages fluctuates in response to inflammation. In this model of sustained inflammation, CCR5 expression dominates CCR1 expression and is expressed by a large fraction of macrophages.

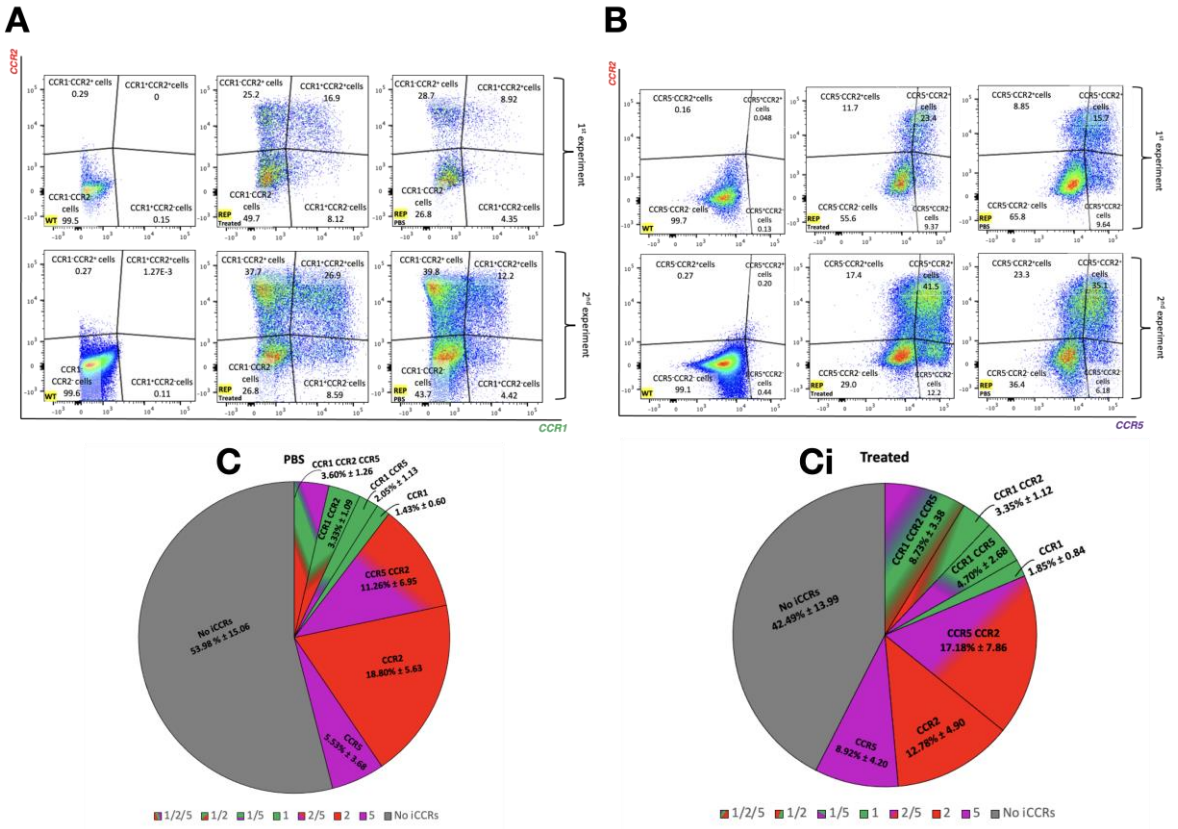

Figure 4-20 Distribution of the reporter iCCR expression in membrane differentiated macrophages. Representative FACS plots showing the expression of (A) CCR1-Clover and (B) CCR5-iRFP682 and their co-expression with CCR2-mRuby2 in membrane Ly6C^{-} F480^{hi} differentiated macrophages for each experimental repeat of subcutaneous osmotic mini pumps modal. Pie charts summarising the distribution of CCR1-Clover (green), CCR2-mRuby2 (red) and CCR5-iRFP682 (purple) in Ly6C^{-}F480^{hi} differentiated macrophages from membrane of (C) vehicle (PBS) and (Cii) cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) treated mice. Data are shown from two independent experiments.

4.3.4.2.3 CCR1

Figure 4-21 shows that mice treated with the cytokine cocktail had slightly more Ly6C^{-}F480^{hi} differentiated macrophages expressing Clover/CCR1 than mice treated with vehicle (PBS) (around 20% vs 10%, respectively). This was also reflected by a 1-fold increase in Clover/CCR1 MFI in Ly6C^{-}F480^{hi} differentiated macrophages in the inflamed membrane. Overall, these findings suggest that in response to sustained inflammation, macrophages increase their expression of CCR1, which implies that CCR1 participates in their localization within tissues and potentially enhances their ability to respond to inflammation more effectively.

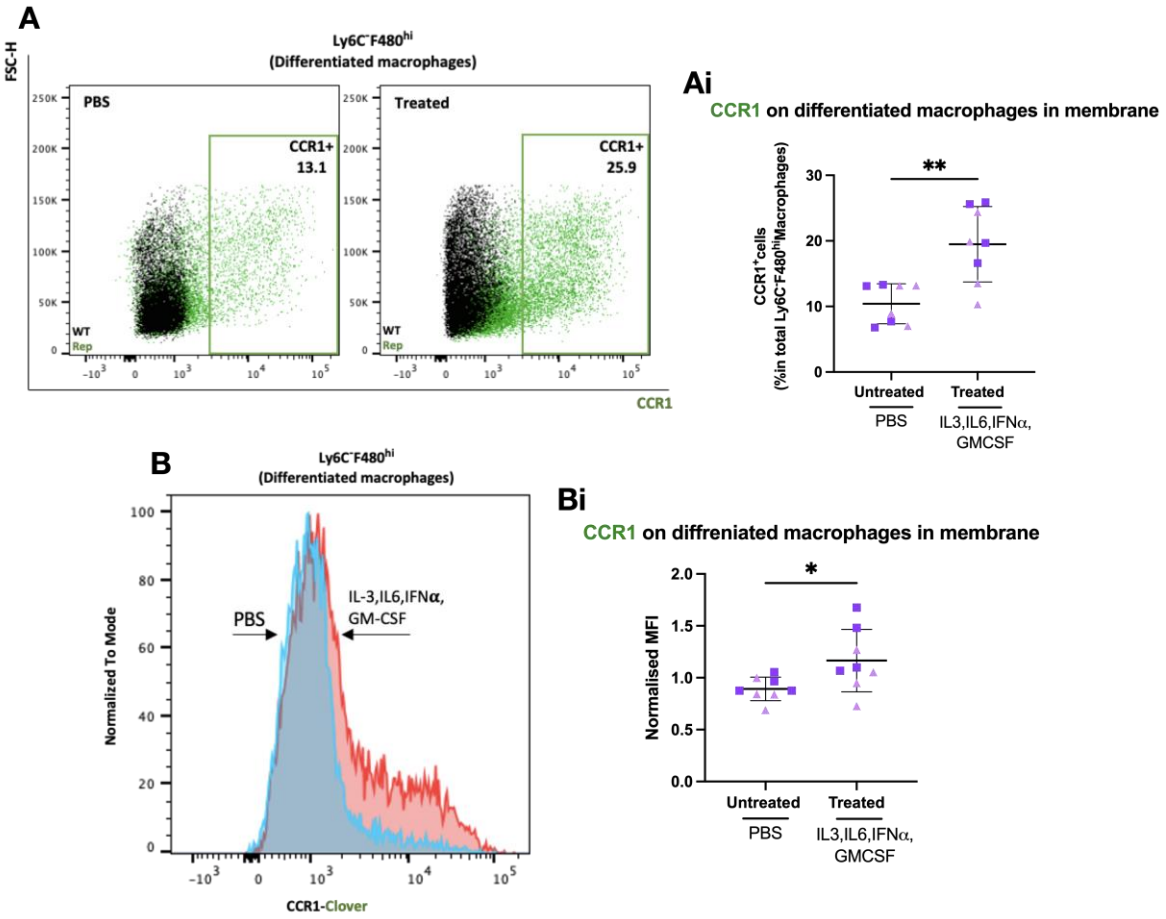

Figure 4-21 Effect of sustained inflammation on CCR1 reporter expression in membrane differentiated macrophages. A) Flow cytometric analysis and (Ai) quantification of CCR1-Clover expression in Ly6C$^-$ F480^{hi} differentiated macrophages from minipump membrane. B) Histogram shows the shift of CCR1-Clover fluorescent expression in Ly6C$^-$F480^{hi} differentiated macrophages from mice treated with cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) in red and vehicle (PBS) in blue. Bi) Graph shows the MFI rate of CCR1-Clover. Data are shown as Mean ± SD of two independent experiments. *P <.05 and **P <.001. Mann Whitney test.

4.3.4.2.4 CCR5

iRFP682/CCR5 was expressed by 20% of Ly6CnegF480hi differentiated macrophages in the resting membrane of mice treated with vehicle PBS; however, this expression was upregulated, similar to what was observed for CCR1, by nearly 2-fold to reach 40% of Ly6CnegF480hi differentiated macrophages in the inflamed membrane of mice treated with cytokine cocktail (Figure 4-22 A, Ai). There were no detectable differences in MFI for iRFP682/CCR5 in Ly6CnegF480hi differentiated macrophages in either resting or inflamed membranes (Figure 4-22

B, Bi). Overall, CCR5 is highly expressed in macrophages in response to sustained inflammation, suggesting CCR5 plays a role in macrophage recruitment.

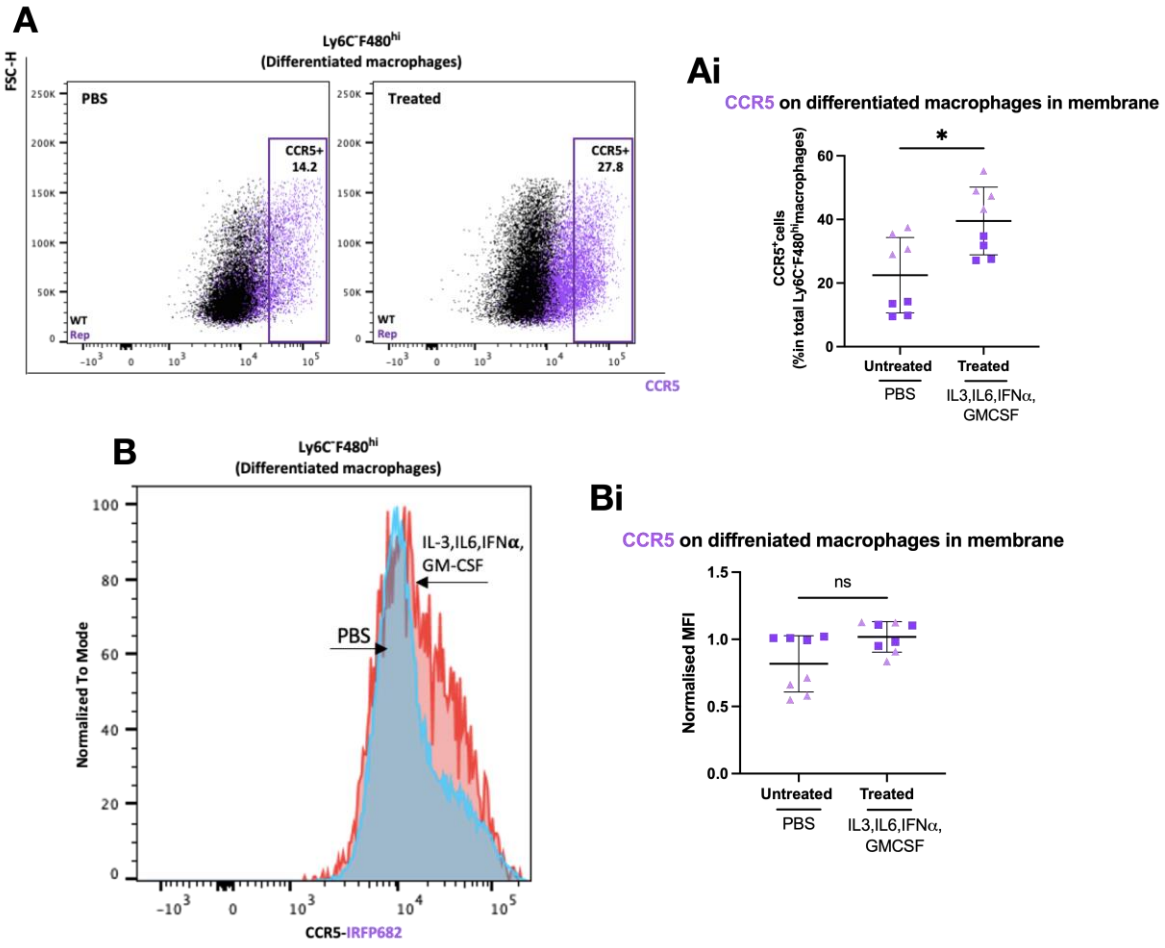

Figure 4-22 Effect of sustained inflammation on CCR5 reporter expression in membrane differentiated macrophages. A) Flow cytometric analysis and (Ai) quantification of CCR5-iRFP682 expression in Ly6C$^-$ F480^{hi} differentiated macrophages from minipump membrane. B) Histogram shows the level of the CCR5-iRFP682 fluorescent expression in Ly6C$^-$ F480^{hi} differentiated macrophages from mice treated with cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) in red and vehicle (PBS) in blue. Bi) Graph shows the MFI rate of CCR5-iRFP682. Data are shown as Mean ± SD of two independent experiments. *P <.05 and **P <.001. Mann Whitney test.

4.4 Discussion and conclusion

The inflammatory iCCRs are well-documented for their expression in leukocytes during various inflammatory stimuli, such as monocytes in atherosclerotic plaques[275], macrophages in CNS lesions[367], and during pulmonary inflammation[368] as well as T-cells and NK-cells in viral infection[369, 370], highlighting the significance of these receptors in regulating leukocyte migration during the inflammatory and immune response. However, the apparent redundancy and promiscuity of receptor-ligand interaction, along with the need for a proper in-vivo mouse model, have hampered our ability to precisely understand the combinatorial or individual roles of iCCRs in orchestrating the inflammatory response and thus effectively target them in inflammatory diseases.

These receptors are known for their tight chromosomal clustering, and they share a high degree of structural homology, in which their genetic proximity effectively prevents chromosomal recombination and results in difficulties in generating multi-receptor knock-out mice. The high level of similarity between different iCCRs also makes it challenging to develop specific antibodies for them. This was confirmed by Medina-Ruiz et al.[198] who used cells from mice with a complete deletion of the entire iCCR locus to show non-specific binding of iCCR antibodies. Also, it was stated in their study that there are no high-quality commercially available CCR1 antibodies, and those that are available do not convincingly detect the receptor. These challenges are addressed by the novel iCCR reporter mouse strain (iREP) used in this study, which has the potential to significantly improve our understanding of the role of these receptors in the inflammatory immune response, especially in terms of whether all iCCRs are expressed simultaneously by leukocytes or at different times.

The data presented in this chapter show that the REP mice can effectively allow us to track the fluctuations in iCCR expression in monocytes as they respond to acute inflammation and differentiate into macrophages at the site of inflammation in real time. To mimic inflamed condition, iREP mice were implanted with a subcutaneous osmotic pump loaded with IL-3, IL-6, GM-CSF and IFNα or PBS (control) to provide continuous release of cytokines into the circulation. The analysis of iCCR-REP mice showed no substantial differences in the expression of CCR2 in $CD11b^{+}Ly6C^{hi}$ inflammatory monocytes from BM of mice treated with cytokine cocktails or vehicle (PBS). However, there was a slight decrease in CCR2 expression in $CD11b^{+}$Ly6Chi inflammatory monocytes in inflamed blood. Despite this, most $CD11b^{+}Ly6C^{hi}$ inflammatory monocytes primarily and exclusively expressed CCR2 in both BM and blood at rest and during sustained inflammation, which is consistent with earlier findings from other studies[198, 366]. Our data also revealed that sustained inflammation causes an increase in the small proportion of $CD11b^{+}$ $Ly6C^{hi}$ inflammatory myocytes that co-express CCR1 and CCR2 in BM (~9%) and blood (~12%). The expression of both CCR1 and CCR2 on CD11b+ Ly6Chi inflammatory monocytes might give these cells an advantage over those expressing CCR2 alone. CCR2 is crucial for the egress of monocytes from the BM into circulation and their recruitment to sites of inflammation. Therefore, the co-expression of both CCR1 and CCR2 on these cells may enhance their migration towards inflammation through two potential mechanisms.

One mechanism is that the co-expression of CCR1 and CCR2 may speed the migration of these cells towards inflammation by strengthening the adhesion of monocytes to blood vessel walls through CCR1. This is supported by the findings of Weber et al., [371] who found that blocking CCR1 in laminar flow experiments

(simulating shear flow) significantly inhibited CCL5-induced arrest of monocytes but blocking CCR5 had no effect.

Another mechanism is that co-expression of CCR1 and CCR2 might facilitate monocyte recruitment to inflamed sites since these cells benefit from expressing CCR1 and can still access the inflamed site even if CCR2 is not present. This was supported by the study of Dyer et al. [194], who found that a minority of Ly6Chi monocytes can still enter inflamed tissues even in the absence of CCR2.

This fraction co-expressing CCR1 remained at the same levels when CD11b+ Ly6Chi inflammatory monocytes infiltrated the inflamed site (or membrane surrounding the minipump), suggesting that they had just been recruited. However, the number of CD11b+ Ly6Chi inflammatory monocytes expressing CCR2 and the level of CCR2 expression in these cells were both significantly downregulated in the membrane of mice treated with cytokine cocktails compared to vehicle (PBS). This finding suggests that monocytes rapidly downregulate CCR2 to start differentiation, given that this process has been well-documented in the previous studies[198, 355] and is thought to occur to prevent them from returning to circulation[354]

Our analyses of Ly6C-F480hi differentiated macrophages revealed that these cells prominently expressed CCR5 in the membrane over CCR1, which was expressed at lower levels, and that the number of these cells expressing CCR5 was significantly higher in inflamed membrane from mice treated with cytokine cocktail than from mice treated vehicles (PBS) (40% vs 20%, respectively). Given that our analysis was performed on fully differentiated macrophages, it supports our previous chapter's conclusion that macrophages fluctuate CCR1 and CCR5 expression in response to inflammation.

In conclusion, our findings highlight the non-redundant role of CCR2 in attracting monocytes toward the site of inflammation, with a majority of the inflammatory monocytes being solely and exclusively positive for CCR2. Our results also suggest that CCR1 contributes to monocyte recruitment, at least in this inflammatory context, as there is a slight increase in the proportion of inflammatory monocytes expressing both CCR1 and CCR2. On the other hand, CCR5 is expressed by only a small proportion of inflammatory monocytes and is mainly found in macrophages, indicating its potential role in macrophage localisation rather than monocyte recruitment.

Chapter 5 Transcriptomic Analysis of iCCR Reporter Expressing Ly6C^{hi} Monocytes

5.1 Overview

As shown in the previous chapter, sustained inflammation triggers changes in iCCR reporter expression on Ly6C^{hi} monocytes, suggesting the importance of these receptors for inflammatory monocyte recruitment. While the vast majority of Ly6C^{hi} inflammatory monocytes (>92%) express CCR2 regardless of inflammation, a small proportion of monocytes also co-express CCR1. While there have been extensive studies on the role and importance of CCR2 in inflammatory monocyte recruitment[57, 193, 194, 309, 365, 366, 372], the purpose of CCR1 expression by a small percentage of monocytes is unclear. While the expression of CCR1 in inflammatory monocytes has been described before[355, 373-376], and seen as an example of redundancy in the chemokine system, other data suggest that the role of CCR1 in inflammatory monocytes could instead be an example of specificity. If redundancy were at play, one might expect CCR1 expression to be more widespread in monocytes as it is supposed to function as a "back-up" for CCR2. Instead, CCR1 expression appears to be restricted to a small percentage of monocytes, possibly suggesting a specific role in the recruitment of a specific inflammatory monocyte subset to inflamed sites. In this model, CCR5 expression was slightly increased on a small fraction of BM and circulating Ly6C^{hi} monocytes; however, the significance remains unclear.

In this chapter, we examined Ly6C^{hi} inflammatory monocytes with different iCCR expression patterns to determine whether they represent phenotypically discrete cell types or simple variations on the overall populations of Ly6C^{hi} monocytes. Therefore, Ly6C^{hi} monocytes expressing reporters for CCR1, CCR2, or both CCR1 and CCR2 were sorted from BM of mice at rest and under sustained inflammation using the subcutaneous osmotic mini-pump model. This chapter presents an extensive analysis of the Ly6Chi monocyte transcriptomic data.

5.2 Transcriptional analysis of iCCR reporter expressing Ly6C^{hi} monocytes from mice at rest and under sustained inflammation using the subcutaneous osmotic mini pump implantation model

To address the aim of this chapter, iREP mice and non-fluorescent WT mice were either left untreated (resting) or implanted with the subcutaneous osmotic mini pumps loaded with IL-3, IL-6, GM-CSF, and IFNα for 7 days to induce sustained inflammation, as described in Figure 5-1. Then, based on reporter expression, inflammatory CD11b^{+} Ly6C^{hi} monocytes expressing only mRuby2/CCR2 or both Clover/CCR1 and mRuby2/CCR2 from both resting and inflamed BM were sorted by Dr. Laura Medina-Ruiz in RLT buffer using BD Biosciences FACS Aria III sorter and then RNA was isolated using RNeasy Micro Kit (Qiagen), along with a small population of these cells in inflamed BM expressing Clover/CCR1 independently of mRuby2/CCR2 (Figure 5-1).

The isolated RNA underwent further analysis at the Glasgow Polyomics Facility to construct libraries for sequencing, using NEBNext® Single Cell/Low Input RNA Library Prep Kit for Illumina (New England Biolabs). Subsequently, these libraries were sequenced on an Illumina NextSeq 2000 with paired-end sequencing, followed by quality control assessments performed by the Glasgow Polyomics Facility to ensure the integrity of the reads. Finally, all the transcriptomics analyses were done using R Studio to identify differential gene expression between inflammatory Ly6C^{hi} monocytes expressing different iCCR reporters.

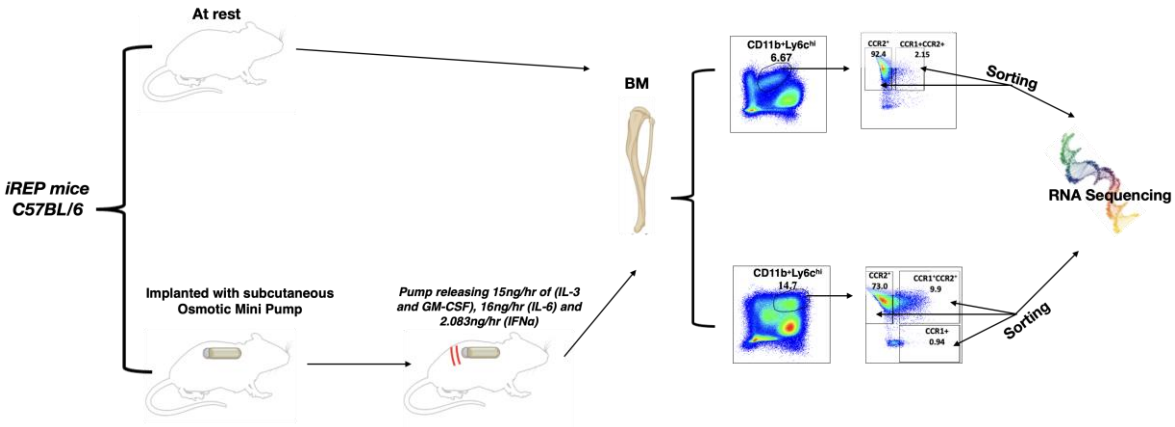

Figure 5-1 Sorting Ly6C^{hi} inflammatory monocytes expressing iCCR reporters in resting and inflamed BM. The schematic diagram represents the outline of the experiment. First, BM was isolated from iREP mice, both at rest and implanted subcutaneously with osmotic pumps. The osmotic pumps deliver the loaded cytokine cocktail indicated above into the circulation for 7 days to induce systemic inflammation. Then, CD11b^{+}Ly6C^{hi} monocytes were analysed for the expression of CCR1-Clover and CCR2-mRuby2. Finally, inflammatory monocytes expressing reporter CCR2 or CCR1/CCR2 were sorted from resting and inflamed BM, and inflammatory monocytes expressing reporter CCR1 were sorted from inflamed BM for transcriptomic analysis.

5.2.1 Gating strategy for sorting iCCR reporter expressing Ly6C^{hi} inflammatory monocytes

Before sorting Ly6Chi inflammatory monocytes with different iCCR reporter expressions, cells were first gated based on FSC-A and SSC-A to exclude debris, as shown in Figure 5-2. After this, leukocytes were identified as CD45-positive on live cells. Inflammatory monocytes were then identified as CD11b^{+} LY6C^{hi} cells. These cells were further purified by gating as being negative for Ly6G, SiglecF, and CD19 to eliminate any potential contamination from other leukocyte subsets. Single cells were gated, and doublets were excluded using FSC-A and FSC-H before sorting cells based on iCCR reporter expression. Finally, for RNA sequencing, inflammatory monocytes expressing mRuby2/CCR2 and co-expressing CCR1/Clover and CCR2/mRuby2 were sorted from the BM of mice at rest or under sustained inflammation, while inflammatory monocytes only expressing CCR1/Clover were sorted from the BM of mice under sustained inflammation (Figure 5-2).

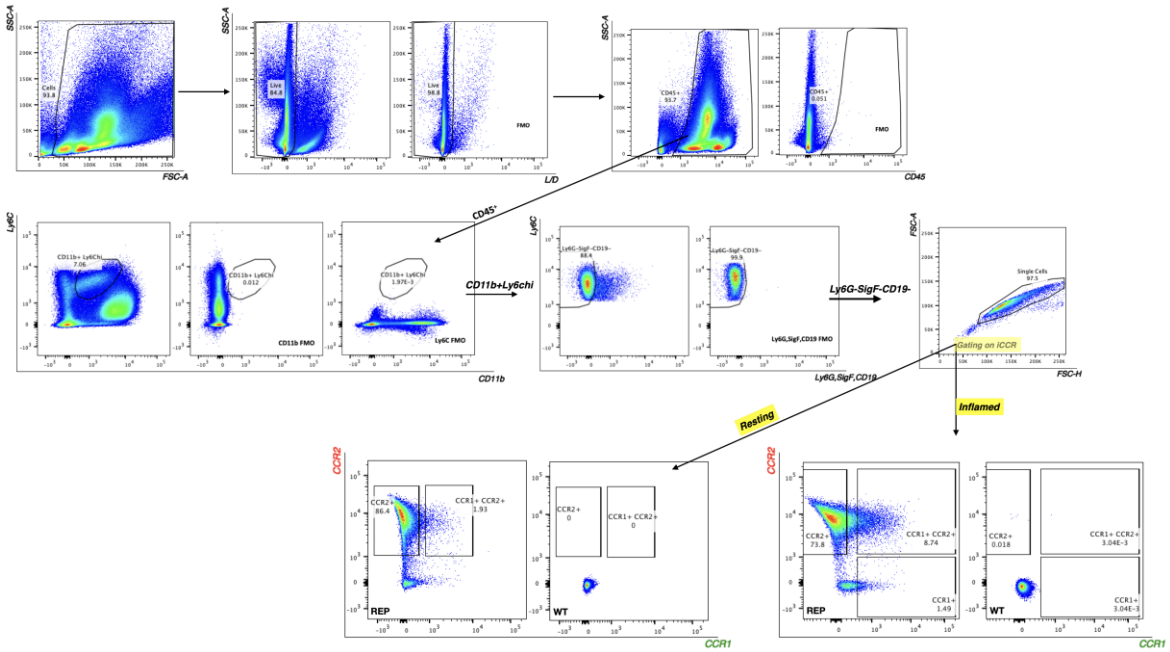

Figure 5-2 Gating strategy for sorting Ly6C^{hi} inflammatory monocytes expressing reporter iCCR in resting and inflamed BM. Black arrows highlight the gating pathway. FSC-A and SSC-A were first used to select cells, followed by selecting live cells by gating on non-stained cells for the live-dead marker. Next, leukocytes were gated as CD45-positive then inflammatory monocytes were defined as CD11b^{+}Ly6C^{hi}Ly6G^{-}SiglecF^{-}CD19-. Finally, gating on single cells to remove cell clumps before sorting cells based on CCR1-Clover and CCR2-mRuby2 expression.

5.2.2 Comparison of the gene expression profile between rest and inflamed ly6C^{hi} monocytes expressing either CCR2 or both CCR1 and CCR2 reporters

Given that the differentiation of Ly6C^{hi} monocytes into iNOS+ macrophages or monocyte-derived dendritic cells (Mo-DCs) can be influenced by environmental signals present during inflammation and can be predicted by their different transcriptional profiles, as demonstrated by Menezes et al.,[377], we were interested to understand how inflammation, in general, can alter the gene expression profile of monocytes, which can have significant impact on their characteristics and behaviour. To achieve this, we compared the transcriptomic profiles of inflammatory Ly6C^{hi} monocytes in the BM of mice under sustained inflammation, expressing either only CCR2 or both CCR1 and CCR2, to those inflammatory Ly6C^{hi} monocytes in the BM of mice at rest, expressing the same reporters.

5.2.2.1 Resting vs. inflamed

We first used a principal component analysis (PCA) to determine the transcriptional similarities among the gene expression profiles of Ly6C^{hi} monocytes expressing different iCCR reporters under both inflamed and resting conditions, as shown in Figure 5-3. PCA analysis involved comparing the entire transcriptomic profiles of Ly6C^{hi} monocytes that express CCR1/Clover or CCR2/mRuby2, as well as those co-expressing CCR1/Clover and CCR2/mRuby2, under both resting and inflamed conditions. The results showed that inflamed Ly6C^{hi} monocytes expressing CCR2/mRuby2 and those co-expressing CCR1/Clover and CCR2/mRuby2 were located closely together but separated from their respective resting groups. This indicates that inflammation has a clear impact on the transcription profiles of these monocytes. However, regardless of their inflamed or resting state, Ly6C^{hi} monocytes that independently expressed CCR1/Clover were clearly separated from CCR2/mRuby2 expressing and CCR1/Clover and CCR2/mRuby2 co-expressing Ly6C^{hi} monocytes, indicating that the transcriptional profiles of these monocytes are markedly different from the other iCCR-expressing monocytes.

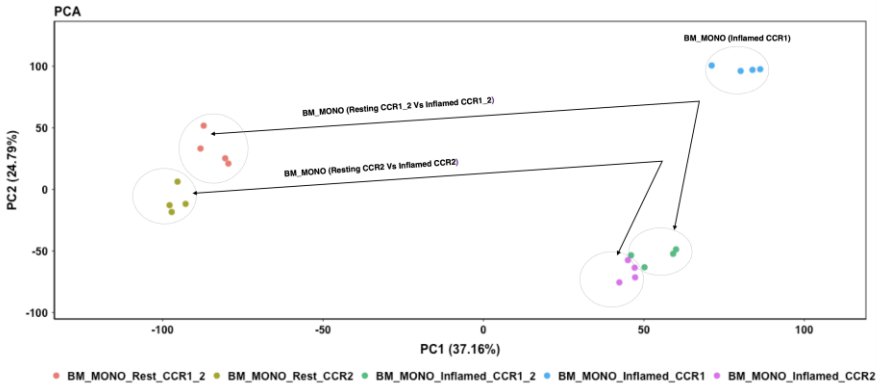

Figure 5-3 PCA scatterplot of the transcription profile of Ly6C^{hi} monocytes expressing different iCCR reporters in resting and inflamed BM. BM was isolated from iREP mice at rest and inflamed for 7 days with subcutaneous osmotic pumps loaded with a cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα). Ly6C^{hi} monocytes positive for CCR1-Clover, CCR2-mRuby2, or both CCR1-Clover/CCR2-mRuby2 were then sorted for RNA sequencing. PCA comparing the transcribed genes of BM Ly6C^{hi} monocytes from resting and inflamed mice, showing the distribution of samples in accounted variance PC1 (37.16%) on the x-axis and PC2 (24.72%) on the y-axis. Each colour represents a sample group.

The correlation heat map in Figures 5-4 A and B, in line with the PCA plot, further supports the observed differences in gene expression profiles between resting and inflamed Ly6C^{hi} monocytes expressing only CCR2/mRuby2 or those co-expressing CCR1/Clover and CCR2/mRuby2, respectively. It also shows a strong correlation between the gene expression values among sample replicates.

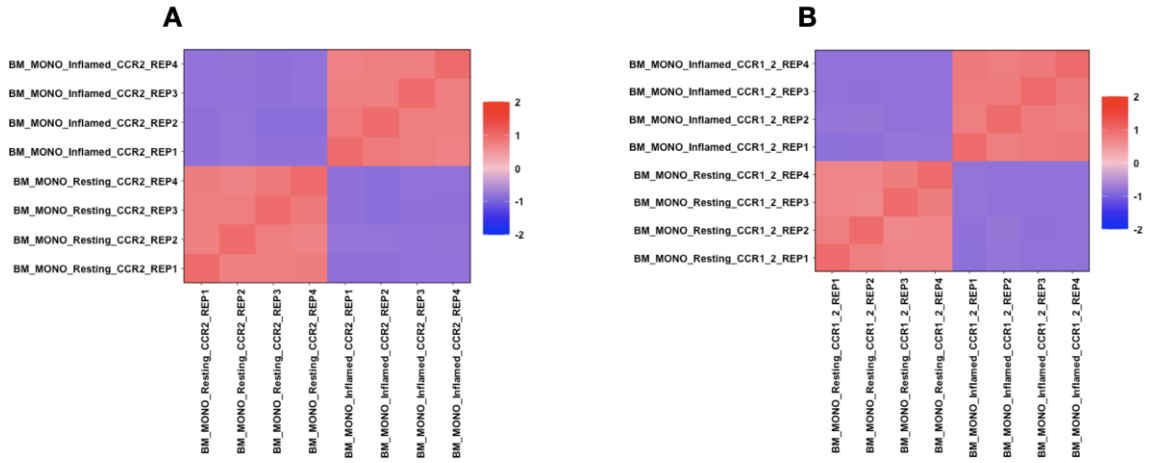

Figure 5-4 Correlation heatmap confirming the transcriptional differences between resting and inflamed Ly6C^{hi} monocytes expressing different iCCR reporters. The heatmap shows the correlation analysis of the transcriptional profiles of Ly6C^{hi} monocytes expressing A) CCR2-mRuby2 and B) CCR1-Clover/CCR2-mRuby2 from both resting and inflamed BM samples. The heatmap indicates the level of correlation or similarity between sample replicates and within sample groups. Colour intensity represents the level of correlation, with red indicating a positive correlation and blue indicating a negative correlation. Both axes are hierarchically clustered, using Spearman distance to group transcriptionally similar samples.

Both Ly6C^{hi} monocyte groups showed similar changes in their gene expression profiles under sustained inflammation, with a significant overlap in the top differentially expressed genes compared to their respective resting groups (not shown). Thus, the subsequent analysis in this section will focus on identifying highly upregulated and downregulated genes in inflamed monocytes expressing only CCR2/mRuby2 compared to their resting counterparts. Transcriptional changes in inflamed and resting CCR2/mRuby2-expressing Ly6C^{hi} monocytes are shown in Figure 5-5, A, which also validates the consistency of differentially expressed genes within and between replicates. A total of 5212 genes were

significantly differentially expressed, with 3297 downregulated and 1915 upregulated in inflamed Ly6C^{hi} monocytes expressing CCR2/mRuby2 compared to their resting counterparts (Figure 5-4, B).

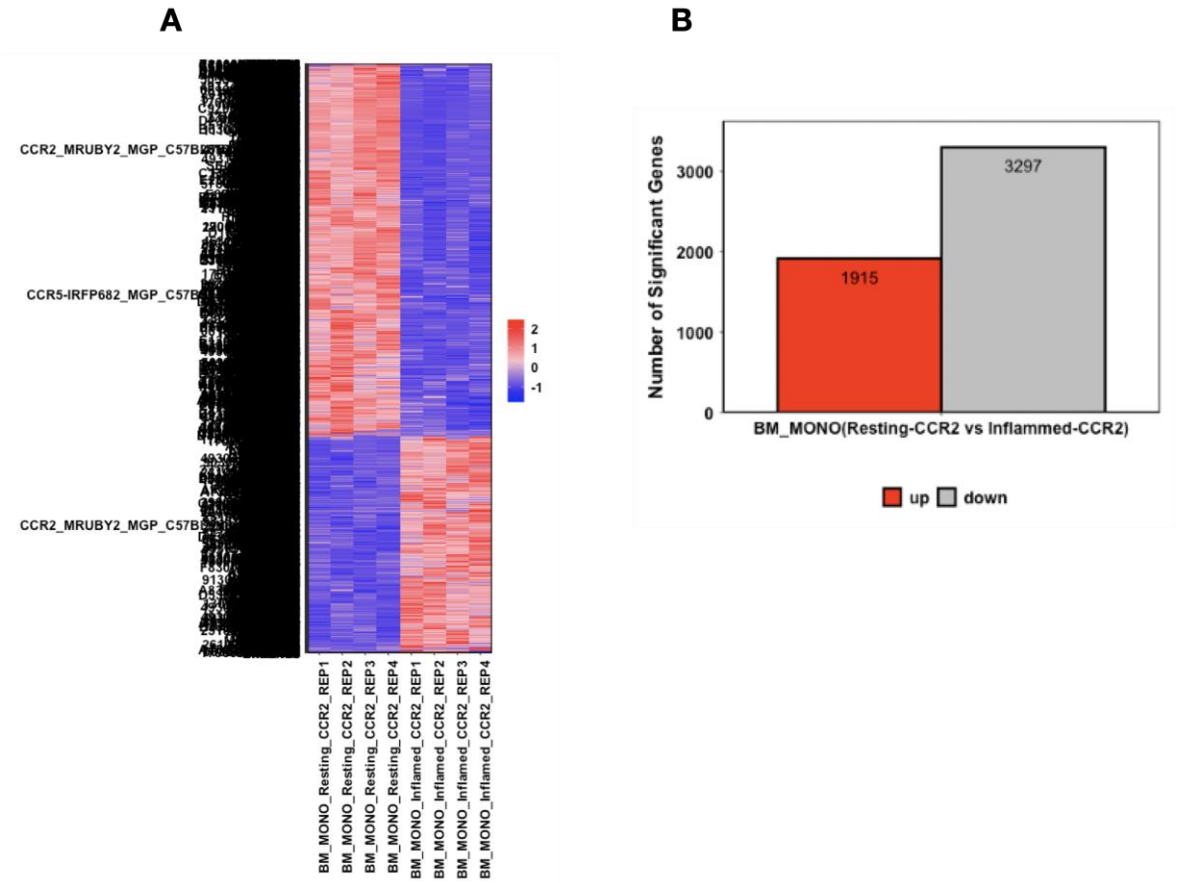

Figure 5-5 Transcriptional analysis of reporter CCR2^{+} monocytes in resting and inflamed BM. A) heatmap shows the patterns of differentially expressed genes (p.adj 0.05, absolute log2 fold > 1.0) in CCR2-mRuby2-expressing Ly6C^{hi} monocytes from resting and inflamed BM. B) bar graph shows the number of these genes with upregulated genes in red and downregulated genes in grey. Colour intensity of the heatmap represents the expression level, blue for downregulated genes and red for upregulated genes. The y-axis is hierarchically clustered, using Spearman distance. All the expression levels were scaled using a Z−score.

5.2.2.1.1 Upregulated differentially expressed genes

As previously mentioned, 1915 genes were found to be upregulated in inflamed CCR2/mRuby2-positive Ly6C^{hi} monocytes compared to resting ones. To gain insights into the potential roles of these genes in inflammation, a box plot of the top 40 of the upregulated genes was generated in Figure 5-6 A.

At the top of the list is HTRA3, a gene that encodes a protein belonging to the family of serine proteases[378]. This protein is responsible for cleaving and deactivating TGFß[378]. TGFß plays a crucial role in suppressing inflammation by inhibiting the activation and effector function of immune cells such as NK cells and by promoting the differentiation of Tregs[379, 380]. As a result, this could imply that inflammatory monocytes upregulated HTRA3 in response to acute inflammation to promote a pro-inflammatory response.

Another upregulated gene on the list is DAB2, which encodes an adaptor protein that has been found to facilitate the binding of Smad2/3 to the TGFß-receptor, resulting in downstream activation of the TGF-beta signalling pathway[381]. In contrast to HTRA3, inflammatory monocytes upregulated DAB2, which acts as a positive regulator of the TGFß pathway by promoting downstream signalling to ensure a proper immune response. Together, these two genes ensure immune regulation and balance, with HTRA3 acting as a negative regulator to prevent excessive immune suppression and DAB2 acting as a positive regulator to promote appropriate immune responses.

CDH1, also known as epithelial cadherin, is one of the top upregulated genes, and it encodes a transmembrane glycoprotein called cadherins. These cadherins, which are mainly expressed by epithelial cells, function in cell-cell adhesion as well as maintaining the integrity of epithelial tissue[382]. Similarly, F11R, or junctional adhesion molecule-A (JAM-A), is a gene that encodes a transmembrane protein expressed in both endothelial and epithelial cells, which is also involved in adhesion, maintaining tight junctions and the transendothelial migration of leukocytes[383, 384].

Both CDH1 and F11R can have homophilic interactions, meaning they bind to the same molecule on the surface of immune cells[382, 385]. This suggests that

inflammatory monocytes upregulate both CDH1 and F11R to pass through epithelial and endothelial barriers by making homophilic interactions with these molecules.

The MUC1 and MUC20 genes encode a type of mucin that is located in the cell membrane and on the surface of cells, respectively. These mucins are highly glycosylated and are found in various epithelial tissues. Some mucins, such as those discovered on the surface of colon carcinoma cells, can bind to E-selectin and P-selectin on the surface of endothelial cells[386]. Therefore, by binding to selectins on the endothelial surface, mucins on the surface of inflammatory monocytes could assist in anchoring them to pass through endothelial barriers and reach the site of inflammation, where they can perform their immune functions.

The CYP11A1 gene encodes a protein that converts cholesterol into pregnenolone, a vital precursor required to produce all steroid hormones[387]. Interestingly, this gene was also found to be upregulated in inflammatory monocytes during acute inflammation.

This finding suggests that the upregulation of the CYP11A1 gene in inflammatory monocytes could lead to increased production of steroid hormones, which can have both anti-inflammatory and pro-inflammatory effects. Cortisol, for example, can suppress the immune response and limit tissue damage during inflammation[388, 389]. At the same time, aldosterone can have pro-inflammatory effects and contribute to the activation of immune cells and the promotion of local inflammation[390].

The LIPG, NOV, and ITK genes are among the most upregulated genes in response to inflammatory stimuli. LIPG, also known as Endothelial lipase (EL), encodes a protein that plays a role in lipoprotein metabolism and monocyte adhesion to the endothelium[391]. Its expression is upregulated in response to

inflammatory cytokines, which can lead to the progression of inflammation, as observed in human atherosclerosis. This upregulation can contribute to the accumulation of lipids in the vascular wall and the induction of monocyte adhesion, both key features in the development of atherosclerosis[392-394]. However, EL can also facilitate the uptake of cholesterol-containing lipoproteins, supplying the energy required for tissue repair [391]. Therefore, although EL upregulation in response to inflammatory cytokines by monocytes can promote their pro-inflammatory response, it can also contribute to tissue repair.

IL-2 inducible T-cell kinase (ITK) encodes a non-receptor tyrosine kinase that participates in several downstream signaling pathways, including PI3K and PKC, which are two crucial downstream signalling pathways for chemokine receptor-mediated signalling[395, 396]. Therefore, ITK may indirectly affect the recruitment and activation of immune cells, such as inflammatory monocytes, during inflammation by controlling these pathways. Nephroblastoma Overexpressed gene (NOV), also called CCN3, has been shown to enhance renal fibrosis and promote the pro-inflammatory immune response by increasing CCL2 and IL-6 expression and cell infiltration after obstructive neuropathy[397]. This suggests that, following acute inflammation, inflammatory monocytes may increase NOV expression to attract additional monocytes to the inflamed site and enhance the pro-inflammatory response.

Figure 4-6 B shows that the upregulated genes in CCR2/mRuby2-positive Ly6C^{hi} inflammatory monocytes are predominantly enriched for Gene Ontology (GO) terms that mediate biological processes or functions in the context of inflammation. For instance, the GO term "regulation of inflammatory response" implies that these genes may play a role in controlling the inflammatory response. In general, the upregulated genes in inflammatory monocytes either activate or

regulate the inflammatory response, indicating that these cells aim to maintain a fine balance of pro- and anti-inflammatory signals, which is essential for achieving appropriate resolution of inflammation.

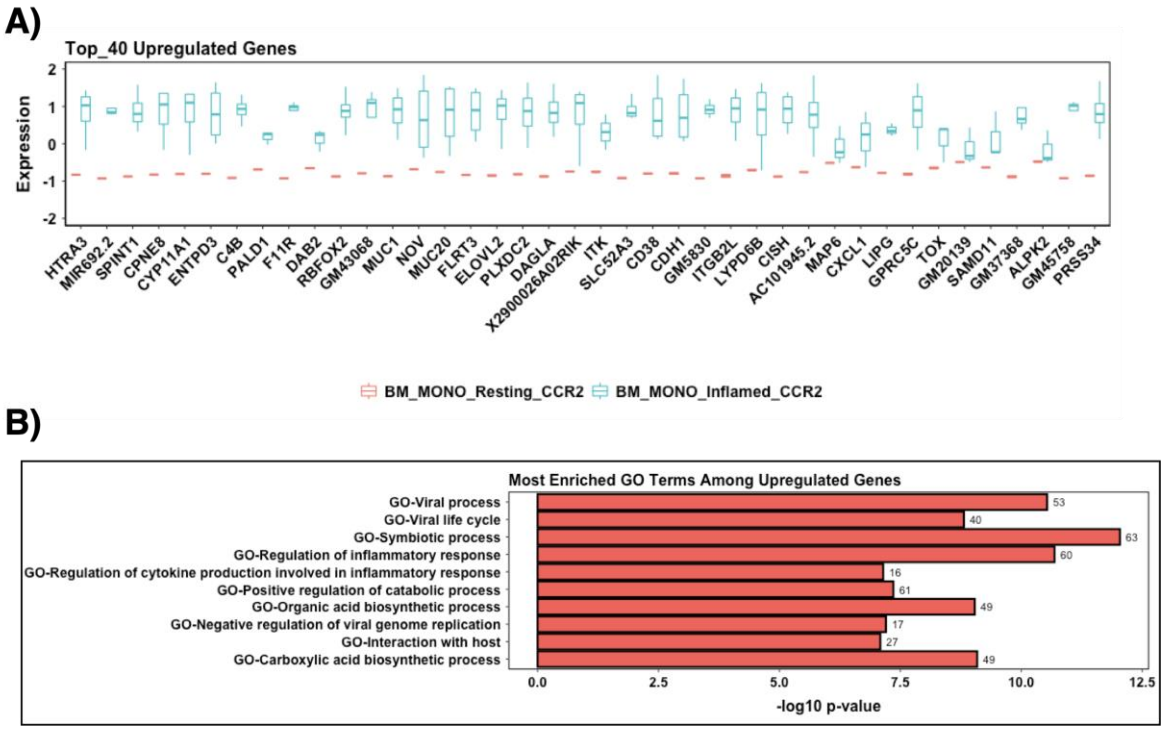

Figure 5-6 Upregulated differentially expressed genes in CCR2⁺ monocytes from resting and inflamed BM. RNA sequencing was performed on CCR2⁺Ly6C^{hi} monocytes sorted from BM of both resting and inflamed mice with a cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) for 7 days. A) Box plot showing the top 40 upregulated genes in inflamed CCR2⁺Ly6C^{hi} monocytes (blue) relative to resting CCR2⁺Ly6C^{hi} monocytes (red). The x-axis displays the differentially expressed genes (p.adj < 0.05, log2 fold > 0), and the y-axis displays the Z-score of the expression level. B) Bar graph presenting the top 10 enriched terms of gene ontology (GO) for the differentially expressed genes between inflamed and resting BM CCR2⁺Ly6C^{hi} monocytes. The y-axis shows the term of the gene set, and the x-axis shows the -log10 p-value. GO terms were selected using GO databases, biological process (BP) and p-values <.05.

5.2.2.1.2 Downregulated differentially expressed genes

Out of the 3297 downregulated genes, we focused on the top 40 genes that showed the most significant differential expression, as shown in Figure 5-7 A. The APOE gene, for example, encodes a protein called apolipoprotein E, which is involved in transporting cholesterol and other lipids in the bloodstream, and is therefore essential for lipid metabolism[398]. In addition to its role in lipid

metabolism, studies have shown that APOE has anti-inflammatory properties. For example, the expression of APOE in monocytes and macrophages can suppress NF-κB signalling when exposed to LPS[399]. Moreover, exogenous APOE administration to a monocyte-macrophage cell line can suppress the production of pro-inflammatory cytokines (IL-6, IL-1ß, and TNFα) following TLR3 and TLR4 activation using poly I:C and LPS, respectively[400]. Therefore, it seems that inflamed monocytes downregulate the APOE gene to enhance activation of the NF-κB pathway, which is responsible for inducing inflammatory mediators required in response to acute inflammation.

On the other hand, one of the top downregulated genes is PPP1R2-PS1, which encodes a protein that inhibits the activity of Protein Phosphatase 1 (PP1). PP1 is a type of serine/threonine phosphatase involved in regulating many cellular processes and signal transduction[401]. For example, one study has found that PP1 can suppress the production of pro-inflammatory cytokines in macrophages by inactivating NF-κB and MAPK signalling pathways[402]. This finding suggests that inflammatory monocytes might be able to balance and prevent excessive immune responses by downregulating both PPP1R2-PS1 and APOE, where PPP1R2-PS1 downregulation negatively regulates NF-κB signalling by increasing the activity of PP1, which inhibits NF-κB, and APOE downregulation, which positively regulates NF-B signalling.

According to the GO analysis in Figure 5-7 B, the downregulated genes in inflamed monocytes were found to be mainly involved in normal biological processes, cell cycle, and transcription regulation. The finding suggests that inflamed monocytes direct most of their energy towards expressing genes that facilitate the clearance of infections since cell division is a highly energy-demanding process.

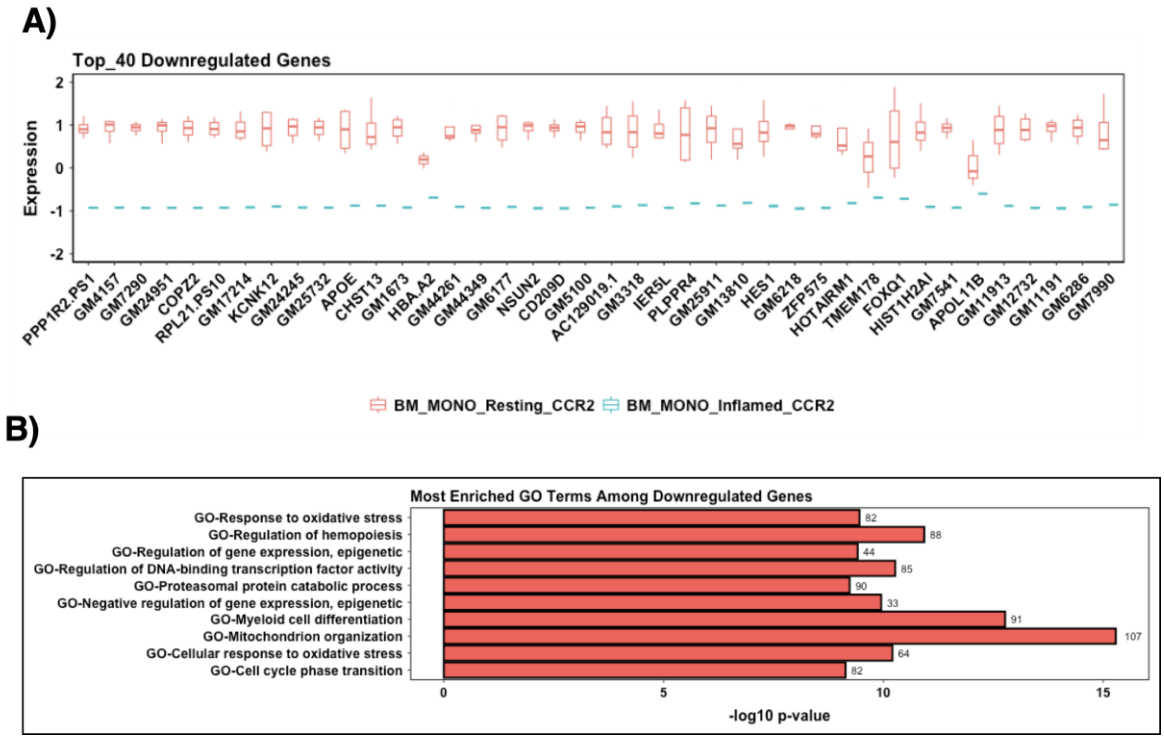

Figure 5-7 Downregulated differentially expressed genes in CCR2⁺ monocytes from resting and inflamed BM. RNA sequencing was performed on CCR2⁺Ly6C^{hi} monocytes sorted from BM of both resting and inflamed mice with a cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) for 7 days. A) Box plot showing the top 40 downregulated genes in inflamed CCR2⁺Ly6C^{hi} monocytes (blue) relative to resting CCR2⁺Ly6C^{hi} monocytes (red). The x-axis displays the differentially expressed genes (p.adj < 0.05, log2 fold < 0), and the y-axis displays the Z-score of the expression level. B) Bar graph presenting the top 10 enriched terms of gene ontology (GO) for the differentially expressed genes between inflamed and resting BM CCR2⁺Ly6C^{hi} monocytes. The y-axis shows the term of the gene set, and the x-axis shows the -log10 p-value. GO terms were selected using GO databases, biological process (BP) and p-values <.05.

5.2.3 Comparison of gene expression profile between each group of ly6c^{hi} monocytes in sustained inflammation

The transcriptomic profiles of Ly6C^{hi} monocytes expressing CCR2/mRuby2 alone, co-expressing CCR1/Clover and CCR2/mRuby2 or expressing only CCR1/Clover under sustained inflammation were next compared with each other.

5.2.3.1 Inflamed_CCR2 vs inflamed_CCR1_2

The PCA plot, which considers the entire transcriptome of these monocytes, demonstrates that Ly6C^{hi} monocytes expressing CCR2/mRuby2 alone were located

close to Ly6C^{hi} monocytes co-expressing both CCR1/Clover and CCR2/mRuby2. However, they were not clustered together, indicating a slight variation in the gene expression profiles between the two populations (Figure 5-8 A). In line with the PCA plot, the volcano plot in Figure 5-8 B also shows significant differences in the gene expression profiles between Ly6C^{hi} monocytes expressing only CCR2/mRuby2 and those expressing both CCR1/Clover and CCR2/mRuby2, with approximately 66 genes upregulated, and 103 genes downregulated, as shown in the bar graph (Figure 5-8 C).

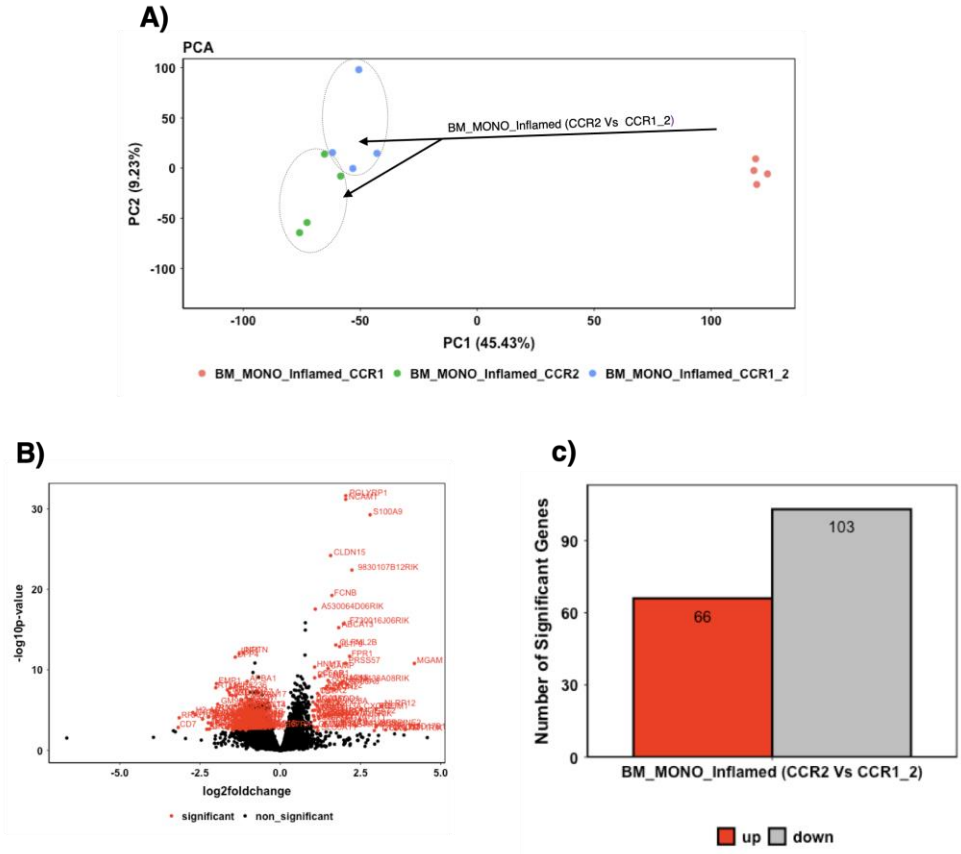

Figure 5-8 CCR2^{+} monocytes are transcriptionally distinct from CCR1_2^{+} Ly6Chi monocytes in inflamed BM. Mice were implanted with subcutaneous osmotic pumps. Seven days after the continuous release of inflammatory cytokines (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα), CCR2+, CCR1_2^{+} and CCR1^{+} Ly6C^{hi} monocytes were sorted from BM cells for RNA sequencing. A) Principal component analysis (PCA) comparing the transcribed genes of Ly6C^{hi} monocyte groups, showing the percentage of variance in the PC1 (45.43%) on the x-axis and PC2 (24.72%) on the y-axis. Each colour represents a sample group. The expression level was scaled using a Z−score. B) Volcano plot showing in red the differentially expressed genes in CCR2^{+} relative to CCR1_2^{+} Ly6C^{hi} monocytes. Genes toward the right (upregulated), the left (downregulated) and the top (most significant). C) Bar graph represents the number of upregulated genes (red) and downregulated (grey) in CCR2^{+} relative to CCR1_2^{+} Ly6C^{hi} monocytes. Significant genes (p.adj < 0.05, absolute log2 fold > 1.0) were selected.

5.2.3.1.1 Upregulated differentially expressed genes

66 genes were highly expressed in Ly6C^{hi} monocytes expressing both CCR1/Clover and CCR2/mRuby2, compared with Ly6C^{hi} monocytes expressing only CCR2/mRuby2. The top 40 significantly differentially expressed genes among these upregulated ones were therefore examined, and the list is shown in the box plot in Figure 5-9 A. To find out the lineage affiliation of these top 40 upregulated genes, we next examined expression in the immGen data sets. Figure 5-9 B shows that these differential transcripts were most prevalent in neutrophil. Then, an overrepresentation analysis (ORA) was performed to identify the biological pathways and functions involved in the 66 differentially upregulated genes (Figure 5-9 C). The results of the analysis, which are in line with immGen findings, show that the top 10 enriched gene sets were related to the function and activity of neutrophils, as they were found to be associated with processes such as neutrophil chemotaxis, granulocyte chemotaxis, and neutrophil migration (Figure 5-9 C).

One of the highly differentially upregulated genes in Ly6C^{hi} monocytes expressing both CCR1/Clover and CCR2/mRuby2, compared to those expressing CCR2/mRuby2 exclusively, is PRSS57, which encodes a serine protease stored in the primary granules or "azurophilic granules" of neutrophils. These proteases regulate pro-inflammatory cytokine activity and contribute to the immune defence mechanism by aiding neutrophil-mediated host defence against pathogens[403, 404]. In addition, the list of top upregulated genes included MPO (Myeloperoxidase) and ELANE (Elastase, Neutrophil Expressed), which are genes expressed in neutrophils and associated with primary granules. Furthermore, the list also shows the presence of secondary granule-associated genes, including NGP (Neutrophilic Granule Protein) and LCN2 (Lipocalin-2), also known as neutrophil gelatinase-associated lipocalin-2[405, 406]. Both IL1F9 or also called IL-36γ, and

CXCR2 genes are upregulated. IL1F9 has been shown to facilitate neutrophil migration by stimulating the production of CXCL8, a ligand that binds to CXCR2, the primary chemokine receptor expressed on neutrophils[407]. Overall, comparing CCR1/Clover and CCR2/mRuby2 co-expressing Ly6C^{hi} monocytes to those that only express CCR2/mRuby2 revealed some differentially expressed genes and upregulated genes in the CCR1/Clover and CCR2/mRuby2 co-expressing Ly6C^{hi} monocytes are characteristically involved in neutrophil function and activities. This suggests that Ly6C^{hi} monocytes expressing both CCR1/Clover and CCR2/mRuby2 represent a distinct population of Ly6C^{hi} monocytes with a component of neutrophilic gene expression.

5.2.3.1.2 Downregulated differentially expressed genes

Ly6C^{hi} monocytes co-expressing CCR1/Clover and CCR2/mRuby2 showed a decrease in the expression of 103 genes compared to Ly6C^{hi} monocytes expressing only CCR2/mRuby2. Among these downregulated genes, the top 40 were selected for further analysis, and they are presented in the box plot in Figure 5-10 A. These top 40 downregulated genes were typically expressed in monocytes, DCs, and macrophages, as indicated by the immGen data sets in Figure 5-10 B.

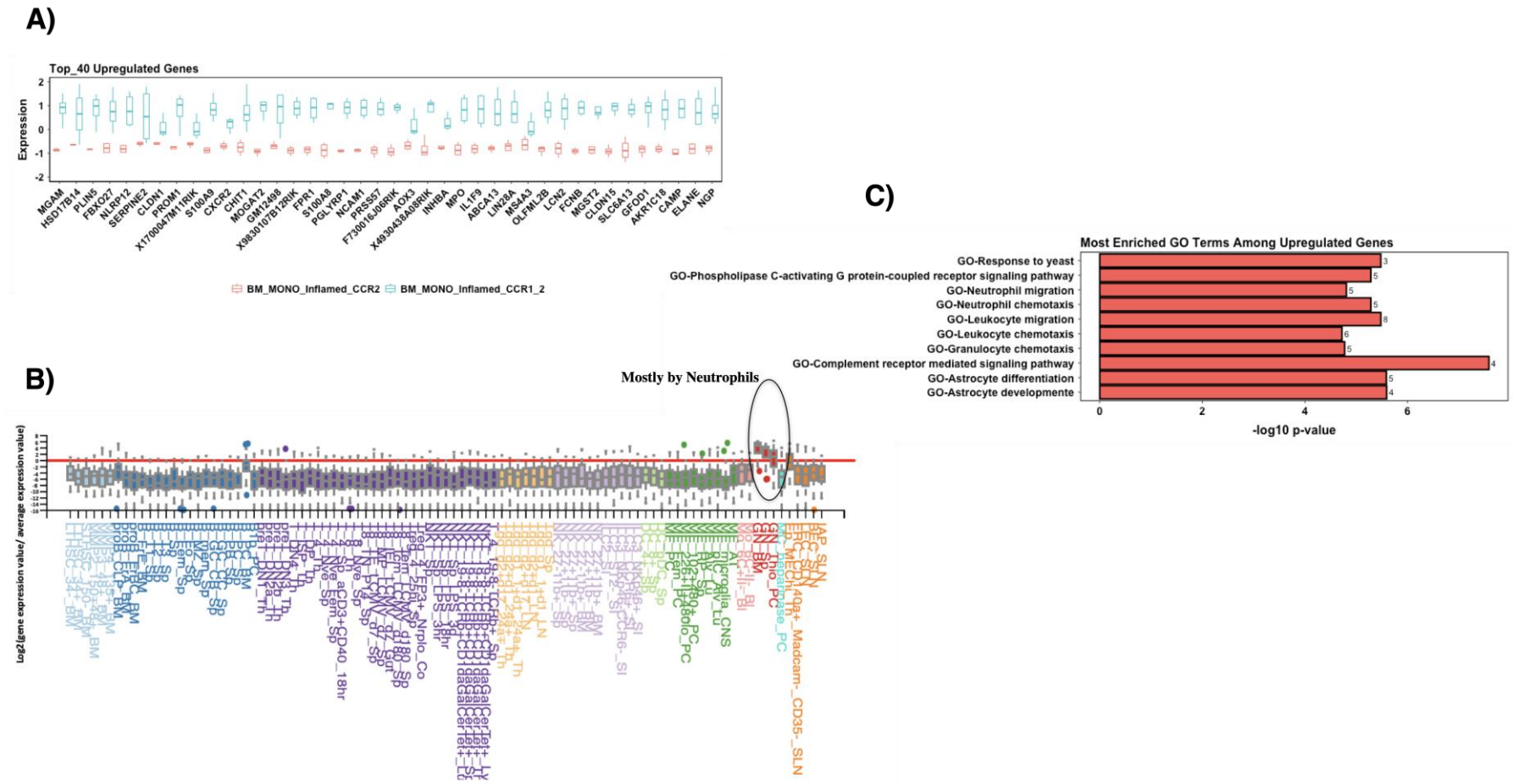

Figure 5-9 Analysis of upregulated differentially expressed genes in CCR2^{+} and CCR1_2^{+} monocytes from inflamed BM. Top 40 upregulated genes in CCR1_2^{+} relative to CCR2^{+} Ly6Chi monocytes from mice treated with a cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) showing: A) Box plot with the list of differentially expressed genes (p.adj < 0.05, log2 fold > 0) on the x-axis and Z-score of the expression level on the y-axis, and B) The upregulated gene expression profile compared to ImmGen data sets. C) Bar graph summarising the top 10 enriched gene ontology (GO) terms for the differentially expressed genes in CCR1_2^{+} relative to CCR2^{+} Ly6C^{hi} monocytes. The y-axis shows the term of the gene set, and the x-axis shows the -log10 p-value. GO terms were selected using GO databases, biological process (BP) and p-values <.05.

The ORA analysis of 103 downregulated genes shows that the top 10 enriched gene sets are associated with biological pathways and functions related to various aspects of the immune response, including leukocyte differentiation and hemopoiesis, with most GO terms specifically related to antigen processing and presentation, a typical feature of antigen-presenting cells (APCs) (Figure 5-10 C).

Among the top 40 downregulated genes, CIITA, H2-EB1, H2-AA, and CD74 are all involved in antigen presentation through the MHC class II pathway. CIITA (class II transactivator), for example, regulates the assembly of the MHC class II complex[408], while H2-AA (histocompatibility 2, class II antigen A, alpha) and H2-EB1 (histocompatibility 2, class II antigen E, beta) encode the alpha and beta chains of MHC-II molecules, respectively. In addition, CD74 assists in the folding and assembly of MHC-II molecules and helps to stabilise the peptide-MHC-I complex[409, 410]. Overall, inflammatory monocytes that only express reporter CCR2 upregulate APC-associated genes, allowing them to initiate the adaptive immune response during acute inflammation. Conversely, Ly6C^{hi} monocytes co-expressing both CCR1/Clover and CCR2/mRuby2 upregulate neutrophil markers while downregulating these genes that might typically be associated with inflammatory monocytes.

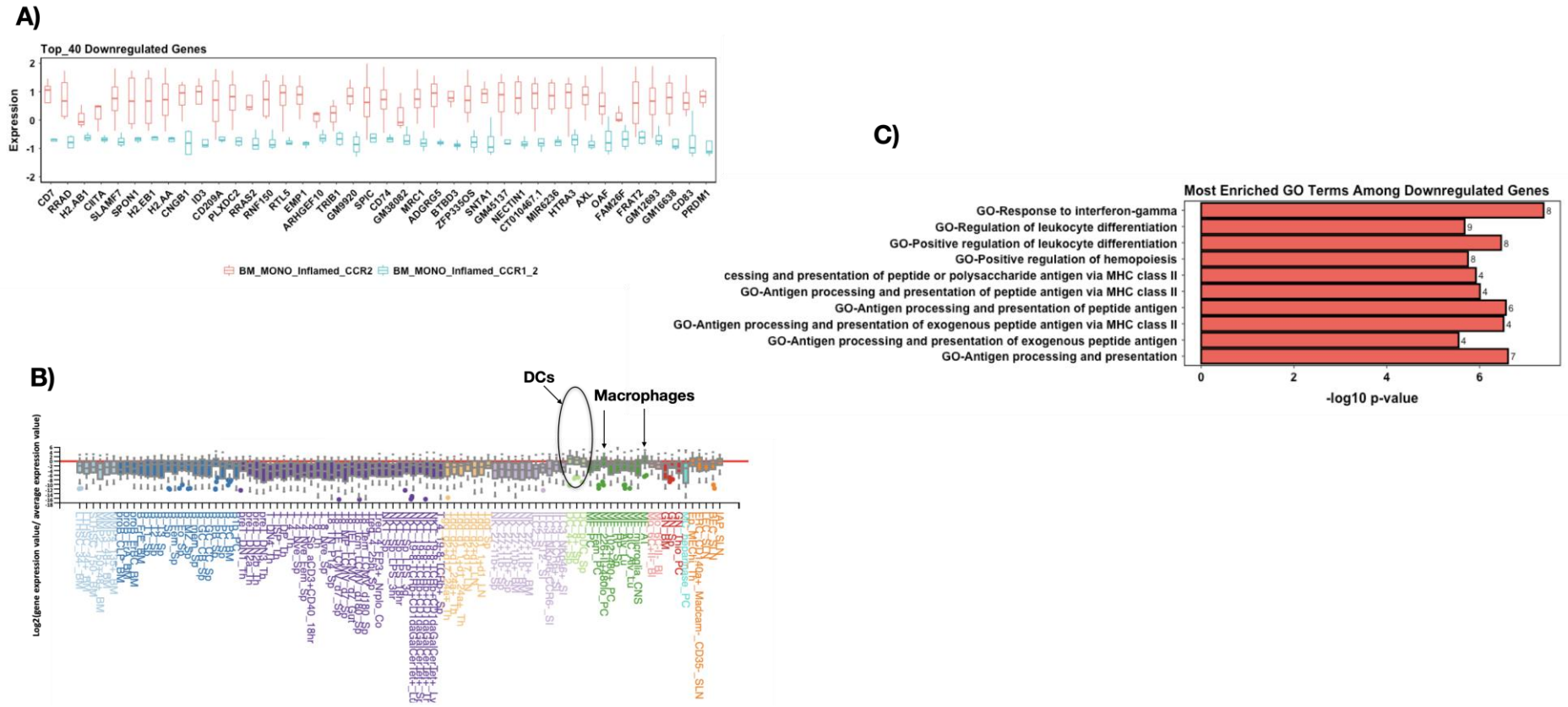

Figure 5-10 Analysis of downregulated differentially expressed genes in CCR2⁺ and CCR1_2⁺ monocytes from inflamed BM. Top 40 downregulated genes in CCR1_2⁺ relative to CCR2⁺ Ly6C^{hi} monocytes from mice treated with a cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) showing: A) Box plot with the list of differentially expressed genes (p.adj < 0.05, log2 fold < 0) on the x-axis and Z-score of the expression level on the y-axis, and B) The downregulated gene expression profile compared to ImmGen data sets. C) Bar graph summarising the top 10 enriched gene ontology (GO) terms for the differentially expressed genes in CCR1_2⁺ relative to CCR2⁺ Ly6c^{hi} monocytes. The y-axis shows the term of the gene set, and the x-axis shows the -log10 p-value. GO terms were selected using GO databases, biological process (BP) and p-values <.05.

5.2.3.2 Inflamed_CCR2 vs inflamed_CCR1

Figure 5-11 A displays the PCA plot that considers the gene expression profiles of 18,000 transcripts in BM Ly6C^{hi} monocytes with different iCCR reporter expression during acute inflammation. The plot illustrates a clear separation between Ly6C^{hi} monocytes that express CCR2/mRuby2 alone and those expressing CCR1/Clover, indicating significant variation in their gene expression profiles. This observation is consistent with the volcano plot, which indicates significant changes between the gene expression profiles of Ly6C^{hi} monocytes expressing exclusively CCR2/mRuby2 and those expressing CCR1/Clover (Figure 5-11 B). The bar graph in Figure 5-11 C shows that in monocytes that expressed CCR2/mRuby2 as compared to those that expressed CCR1/Clover, 2958 genes were upregulated, and 2657 genes were downregulated.

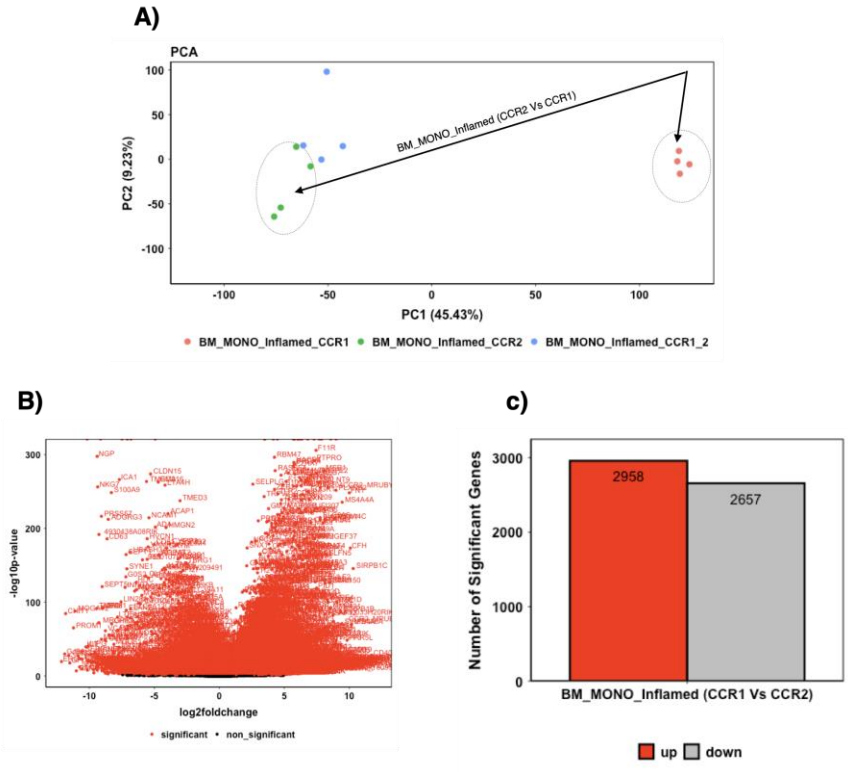

Figure 5-11 Transcriptional differences between CCR1^{+} and CCR2^{+} Ly6C^{hi} monocytes in inflamed mice. RNA sequencing was performed on CCR1+, CCR2+, and CCR1_2+ Ly6Chi monocytes sorted from BM cells of mice implanted with subcutaneous osmotic pumps loaded with a

cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) for 7 days. A) Principal component analysis (PCA) comparing the transcribed genes of Ly6Chi monocyte groups, showing the percentage of variance in the PC1 (45.43%) on the x-axis and PC2 (24.72%) on the y-axis. Each colour represents a sample group. The expression level was scaled using a Z−score. B) Volcano plot showing the differentially expressed genes (red) in CCR1+ relative to CCR2+ Ly6Chi monocytes. Genes toward the right (upregulated), the left (downregulated) and the top (most significant). C) Bar graph represents the number of upregulated genes (red) and downregulated (grey) in CCR1+ relative to CCR2+ Ly6Chi monocytes. Significant genes (p.adj < 0.05, absolute log2 fold > 1.0) were selected.

5.2.3.2.1 Upregulated differentially expressed genes

To further analyze these genes, the top 40 upregulated genes were selected and presented in the box plot (Figure 5-12 A). According to the immGen data, the top 40 upregulated transcripts were found to be associated with monocytes, DCs, and macrophages across all cell types (Figure 5-12 B).

ORA analysis was conducted on all 2958 upregulated genes, and the GO terms of the top 10 enriched gene sets were found to be associated with biological processes related to the immune response to inflammatory stimuli, such as interferon signalling, cytokine production, leukocyte activation, myeloid differentiation, and cell-cell adhesion (Figure 5-12 C). For example, SPON1 (Spondin 1), at the top of the list, encodes a protein involved in adhesion and may encourage monocyte recruitment to the inflamed site; CD40 encodes a co-stimulatory molecule that interacts with CD40L expressed on activated T cells; and MS4A4A (Membrane Spanning 4-Domains A4A), whose expression is restricted to monocytes and monocyte-derived macrophages but not granulocytes or lymphocytes[411]. These findings suggest that upregulated genes in Ly6C^{hi} monocytes expressing only CCR2 are associated with their response to inflammation. Specifically, these genes appear to be involved in preparing the monocytes to migrate, differentiate, and trigger a pro-inflammatory response to combat inflammation at the inflamed site.

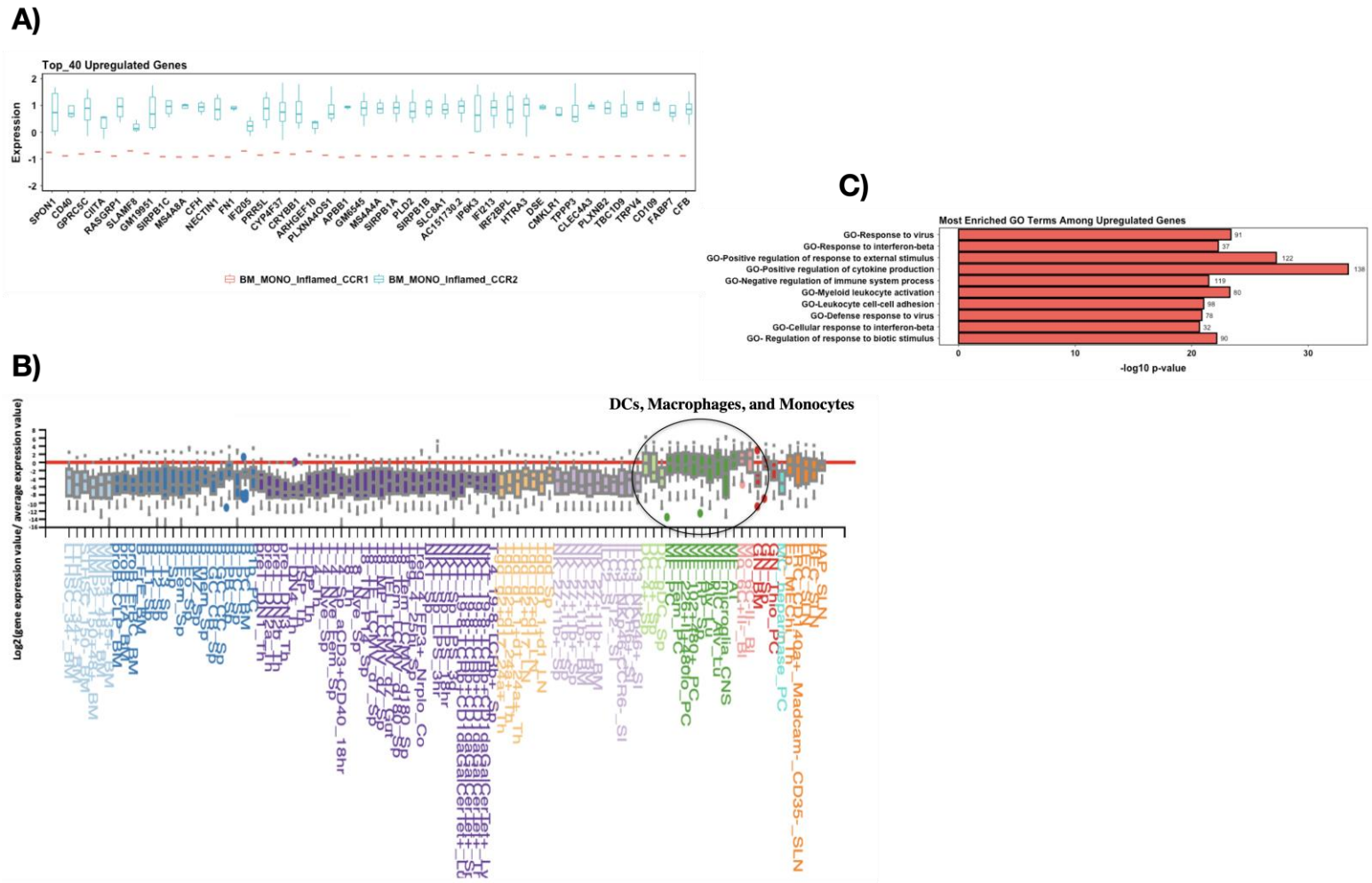

Figure 5-12 Analysis of upregulated differentially expressed genes in CCR1⁺ and CCR2⁺ monocytes from inflamed BM. Top 40 upregulated genes in CCR2⁺ relative to CCR1⁺ Ly6c^{hi} monocytes from mice treated with a cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) showing: A) Box plot with the list of highly upregulated genes (p.adj < 0.05, log2 fold > 0) on the x-axis and Z-score of the expression level on the y-axis, and B) The upregulated gene expression profile compared to ImmGen data sets. C) Bar graph summarising the top 10 enriched gene ontology (GO) terms for the differentially expressed genes in CCR1+ relative to CCR2+ Ly6chi monocytes. The y-axis shows the term of the gene set, and the x-axis shows the -log10 p-value. GO terms were selected using GO databases, biological process (BP) and p-values <.05.

5.2.3.2.2 Downregulated differentially expressed genes

A total of 2657 genes were significantly lower in Ly6C^{hi} monocytes expressing CCR2/mRuby2 compared to those expressing only CCR1/Clover. To further investigate these genes, the top 40 downregulated genes were identified and displayed in the box plot (Figure 5-13 A). The immGen data showed that the top 40 downregulated genes were predominantly expressed in neutrophils across all cell types (Figure 5-13 B). This finding indicates that the genes with higher expression in Ly6Chi monocytes expressing CCR1/Clover are primarily associated with neutrophils (Figure 5-13 B). Furthermore, ORA analysis of the 2657 downregulated genes showed several GO terms related to cellular processes, such as organelle fission, DNA repair, and recombination, suggesting that in response to an inflammatory stimulus, Ly6C^{hi} monocytes expressing only CCR1/Clover are not dead but rather are involved in various cellular processes and post-mitotic cells (Figure 5-13 C).

Interestingly, the top-downregulated gene, ERG (ETS Transcription Factor ERG), is highly expressed in neutrophils in the bone marrow of healthy donors[412] and plays a role in the terminal maturation of neutrophils[413]. Also, at the top of the list is GFI1 (Growth Factor Independent 1 Transcriptional) which encodes a zinc-finger transcription factor important for myeloid cell differentiation into neutrophils. Therefore, Gfi1-deficient mice have been found to exhibit severe neutropenia, and their myeloid cells are unable to differentiate into granulocyte[414, 415]. CAMP (Cathelicidin Antimicrobial Peptide) encodes an antimicrobial peptide that is produced by neutrophils during host defence against pathogens and stored in their granules.

Thus, the data in Figure 5-13 demonstrate a significant difference in the transcriptional profile between Ly6C^{hi} monocytes expressing only CCR2/mRuby2

compared to those expressing CCR1/Clover, indicating that Ly6C^{hi} monocytes expressing only CCR1/Clover are phenotypically distinct cell types. Furthermore, most genes with higher expression in Ly6C^{hi} monocytes expressing CCR1/Clover are associated with neutrophil defence and maturation. This observation suggests that Ly6C^{hi} "monocytes" expressing CCR1/Clover may represent a subpopulation of neutrophils that express Ly6C.

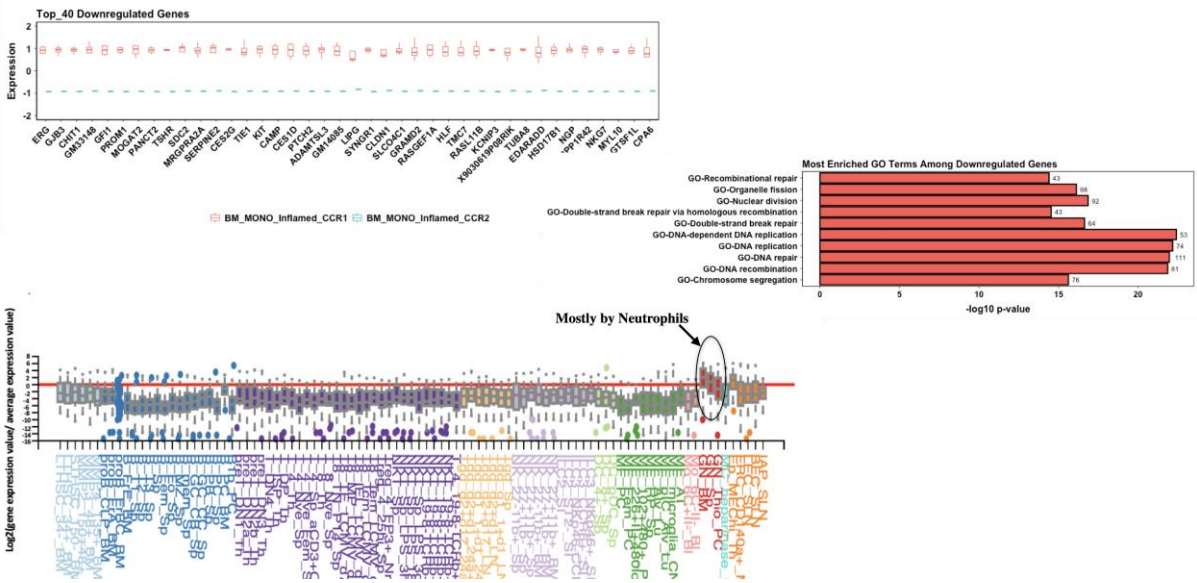

Figure 5-13 Analysis of downregulated differentially expressed genes in CCR1^{+} and CCR2^{+} monocytes from inflamed BM. Top 40 upregulated genes in CCR1^{+} relative to CCR2^{+} Ly6C^{hi} monocytes from mice treated with a cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) showing: A) Box plot with the list of highly downregulated genes (p.adj < 0.05, log2 fold < 0) on the x-axis and Z-score of the expression level on the y-axis, and B) The downregulated gene expression profile compared to ImmGen data sets. C) Bar graph summarising the top 10 enriched gene ontology (GO) terms for the differentially expressed genes in CCR1^{+} relative to CCR2^{+} Ly6C^{hi} monocytes. The y-axis shows the term of the gene set, and the x-axis shows the -log10 p-value. GO terms were selected using GO databases, biological process (BP) and p-values <.05.

5.2.3.3 Inflamed_CCR1 vs inflamed_CCR1_2

As observed in the previous comparison, there is also a clear separation between the sample group of Ly6C^{hi} monocytes expressing both CCR2/mRuby2 and CCR1/Clover compared to those solely expressing CCR1/Clover, indicating significant differences in gene expression profiles (Figure 5-14 A). Figures 5-14 B and

C demonstrate these differences with the volcano plot and bar graph, respectively, showing that Ly6C^{hi} monocytes positive for both CCR2/mRuby2 and CCR1/Clover had 2566 genes upregulated and 2589 genes downregulated in comparison with those solely expressing CCR1/Clover.

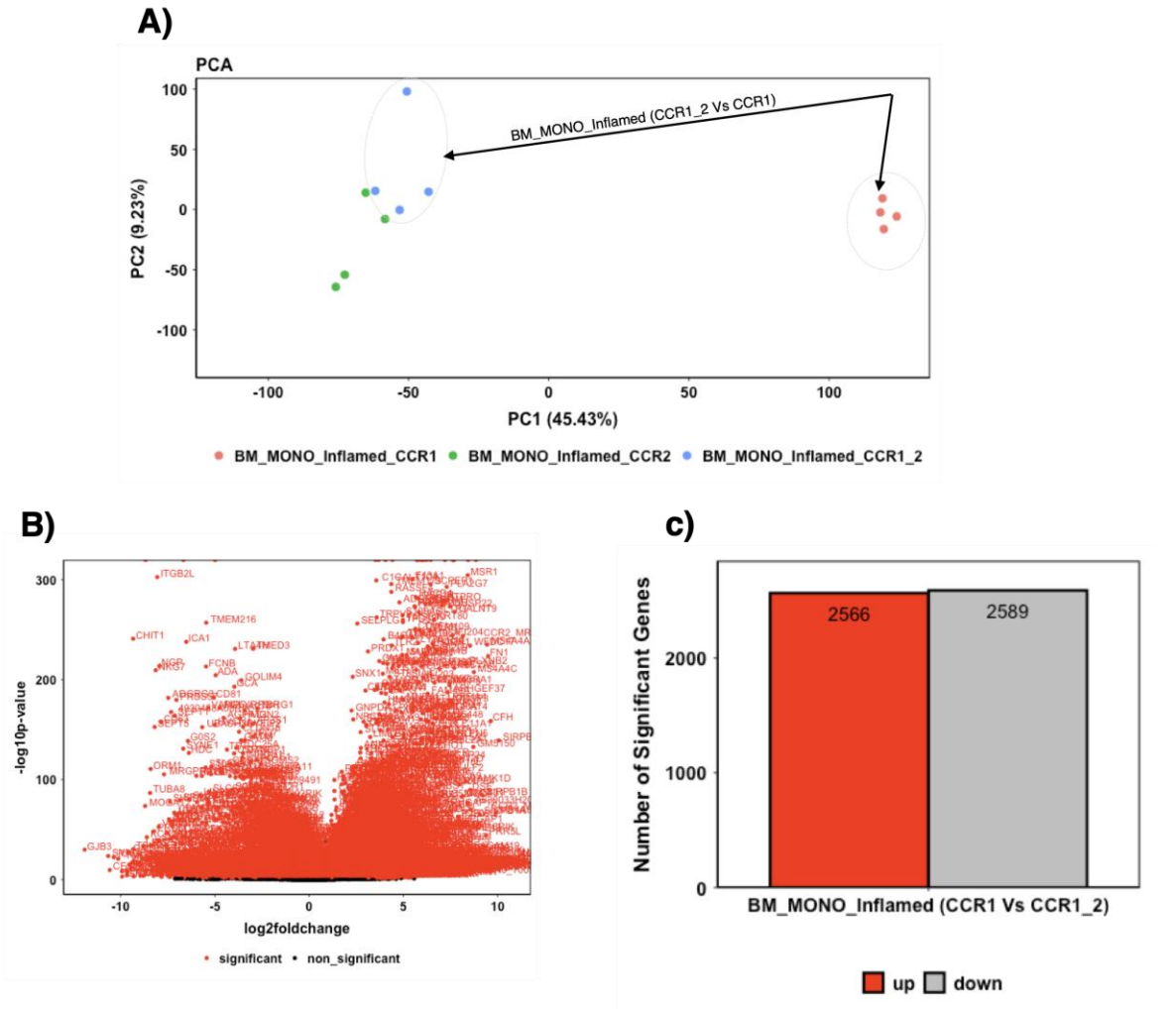

Figure 5-14 Transcriptional differences between CCR1^{+} and CCR1_2^{+} Ly6C^{hi} monocytes in inflamed mice. RNA sequencing was performed on CCR1^{+}, CCR2^{+}, and CCR1_2^{+} Ly6C^{hi} monocytes sorted from BM cells of mice implanted with subcutaneous osmotic pumps loaded with a cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) for 7 days. A) Principal component analysis (PCA) showing the three groups of Ly6C^{hi} monocytes are transcriptionally distinct, where PC1 (45.43% variance) on the x-axis and PC2 (24.72% variance) on the y-axis. Each colour represents a sample group. The expression level was scaled using a Z−score. B) Volcano plot presenting the differentially expressed genes (red) in CCR1_2^{+} relative to CCR1^{+} Ly6C^{hi} monocytes. Genes toward the right (upregulated), the left (downregulated) and the top (most significant). C) Graph showing the number of upregulated genes (red) and downregulated (grey) in CCR1_2^{+} relative to CCR1^{+} Ly6C^{hi} monocytes. Significant genes (p.adj < 0.05, absolute log2 fold > 1.0) were selected.

5.2.3.3.1 Upregulated differentially expressed genes

Looking at the top 40 differentially upregulated genes, a similar list was obtained as in the previous comparison (Figure 5-15 A). This confirms the earlier findings that Ly6C^{hi} monocytes co-expressing both CCR2/mRuby2 and CCR1/Clover and those solely expressing CCR2/mRuby2 are quite similar, with some significant differences but clearly distinct from Ly6C^{hi} monocytes solely expressing CCR1/Clover. According to immGen data, monocytes, macrophages, and DCs express the top 40 upregulated genes in Ly6C^{hi} monocytes that express both CCR2/mRuby2 and CCR1/Clover compared to those that only express CCR1/Clover (Figure 5-15 B). Additionally, the ORA analysis of all upregulated genes and the GO terms of the top 10 enriched gene sets were identical to those found in the previous comparison and associated with biological processes related to the immune response to inflammatory stimuli (Figure 5-15 C).

In general, these data suggest that Ly6C^{hi} monocytes solely expressing CCR1/Clover are a clearly distinct population, while Ly6C^{hi} monocytes expressing both CCR2/mRuby2 and CCR1/Clover appear to be a subpopulation of monocytes.

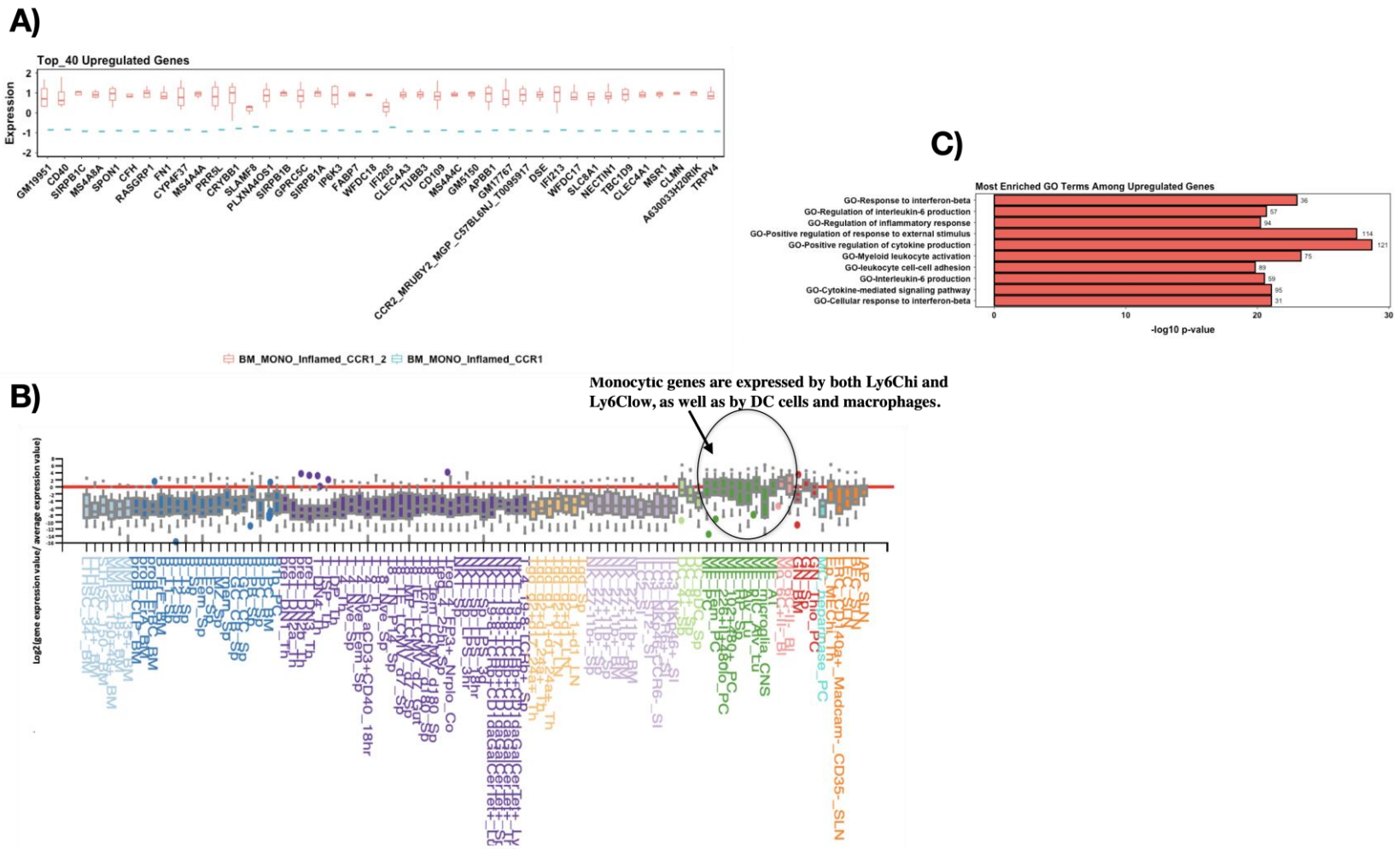

Figure 5-15 Analysis of upregulated differentially expressed genes in CCR1_2$^+$ and CCR1$^+$ monocytes from inflamed BM. Top 40 upregulated genes in CCR1_2$^+$ relative to CCR1$^+$ Ly6C^{hi} monocytes from mice treated with a cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) showing: A) Box plot with the list of highly upregulated genes (p.adj < 0.05, log2 fold > 0) on the x-axis and Z-score of the expression level on the y-axis, and B) The upregulated gene expression profile compared to ImmGen data sets. C) Bar graph summarising the top 10 enriched gene ontology (GO) terms among upregulated differentially expressed genes in CCR1_2$^+$ relative to CCR1$^+$ Ly6C^{hi} monocytes. The y-axis shows the term of the gene set, and the x-axis shows the -log10 p-value. GO terms were selected using GO databases, biological process (BP) and p-values <.05.

5.2.3.3.2 Downregulated differentially expressed genes

Similar to the previous comparison, the top 40 downregulated genes in Ly6C^{hi} monocytes expressing both CCR2/mRuby2 and CCR1/Clover were identical to those found to be downregulated in Ly6C^{hi} monocytes expressing only CCR2/mRuby2 in comparison to those expressing only CCR1/Clover (Figure 5-16 A). Notably, these top 40 downregulated genes, which were found to be more highly expressed in Ly6C^{hi} monocytes expressing only CCR1/Clover, were primarily expressed by neutrophils across all cell types, as evidenced by the immGen data sets presented in Figure 5-16 B. Furthermore, consistent with the previous comparison, most of the top 10 enriched gene sets were associated with cellular processes (Figure 5-16 C).

Overall, these data demonstrate that the transcriptional profiles of Ly6C^{hi} monocytes expressing only CCR1/Clover are distinct from other Ly6C^{hi} monocyte groups, and that the cells positive for CCR1/Clover express genes typically associated with neutrophils, suggesting that these cells may not belong to the monocyte population, but rather represent a population of neutrophils that express Ly6C.

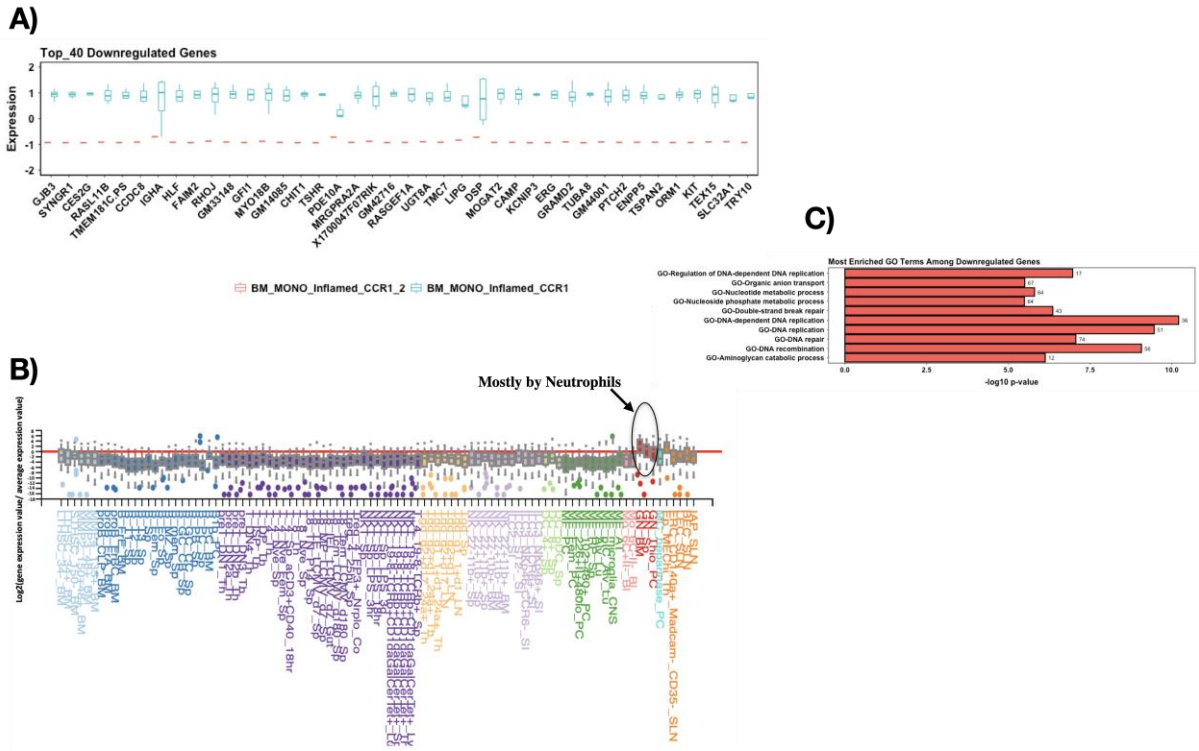

Figure 5-16 Analysis of downregulated differentially expressed genes in CCR1_2$^+$ and CCR1$^+$ monocytes from inflamed BM. Top 40 downregulated genes in CCR1_2$^+$ relative to CCR1$^+$ Ly6C^{hi} monocytes from mice treated with a cytokine cocktail (15ng/hr IL-3, 15ng/hr GM-CSF, 16ng/hr IL-6, 2.083ng/hr IFNα) showing: A) Box plot with the list of highly downregulated genes (p.adj < 0.05, log2 fold < 0) on the x-axis and Z-score of the expression level on the y-axis, and B) The downregulated gene expression profile compared to ImmGen data sets. C) Bar graph summarising the top 10 enriched gene ontology (GO) terms for the differentially expressed genes in CCR1_2$^+$ relative to CCR1$^+$ Ly6C^{hi} monocytes. The y-axis shows the term of the gene set, and the x-axis shows the -log10 p-value. GO terms were selected using GO databases, biological process (BP) and p-values <.05.

5.2.4 Comparison of gene expression profile between each group of iCCR expressing ly6C^{hi} monocytes under resting condition

Based on our previous findings, which showed that there were significant differences in gene expression profiles between Ly6C^{hi} monocytes co-expressing CCR2/mRuby2 and CCR1/Clover and those expressing only CCR2/mRuby2 in BM of mice under sustained inflammation and that some genes associated with neutrophil characteristics were expressed in Ly6C^{hi} monocytes expressing both CCR2/mRuby2 and CCR1/Clover. In the next section, we compared the transcriptional profiles of these two Ly6C^{hi} monocytes from BM of mice at rest to determine whether these

differences still exist or are related to the inflammatory effects on cellular characteristics.

5.2.4.1 Resting_CCR2 vs Resting_CCR1_2

The transcriptome contained more than 14,000 transcripts, and the PCA plot demonstrates a clear separation between Ly6C^{hi} monocytes that co-express CCR2/mRuby2 and CCR1/Clover and those that express only CCR2/mRuby2 (Figure 5-17 A). However, there was some heterogeneity among sample replicates within the same group, as not all replicates clustered together. In line with that, the volcano plot confirms significant changes in gene expression profiles between Ly6C^{hi} monocytes that co-express CCR2/mRuby2 and CCR1/Clover and those that express only CCR2/mRuby2 (Figure 5-17 B). 436 of the 843 differentially expressed genes were upregulated in Ly6C^{hi} monocytes that co-express CCR2/mRuby2 and CCR1/Clover, while 407 were downregulated in comparison to Ly6C^{hi} monocytes that only expressed CCR2/mRuby2 (Figure 5-17 C).

5.2.4.1.1 Upregulated differentially expressed genes

436 genes were found to be differentially upregulated in Ly6C^{hi} monocytes that co-expressed CCR2/mRuby2 and CCR1/Clover, compared to monocytes that only expressed CCR2/mRuby2. The top 40 upregulated genes were selected and analysed using the immGen datasets to learn more about their lineage of affiliation patterns (Figure 5-18 A). These genes were found to be predominantly expressed by neutrophils and red pulp macrophages (Figure 5-18 B). This finding is consistent with our previous comparison between these two monocyte types under sustained inflammation, which showed that the top 40 upregulated genes in Ly6C^{hi} monocytes

co-expressing CCR2/mRuby2 and CCR1/Clover were primarily expressed in neutrophils.

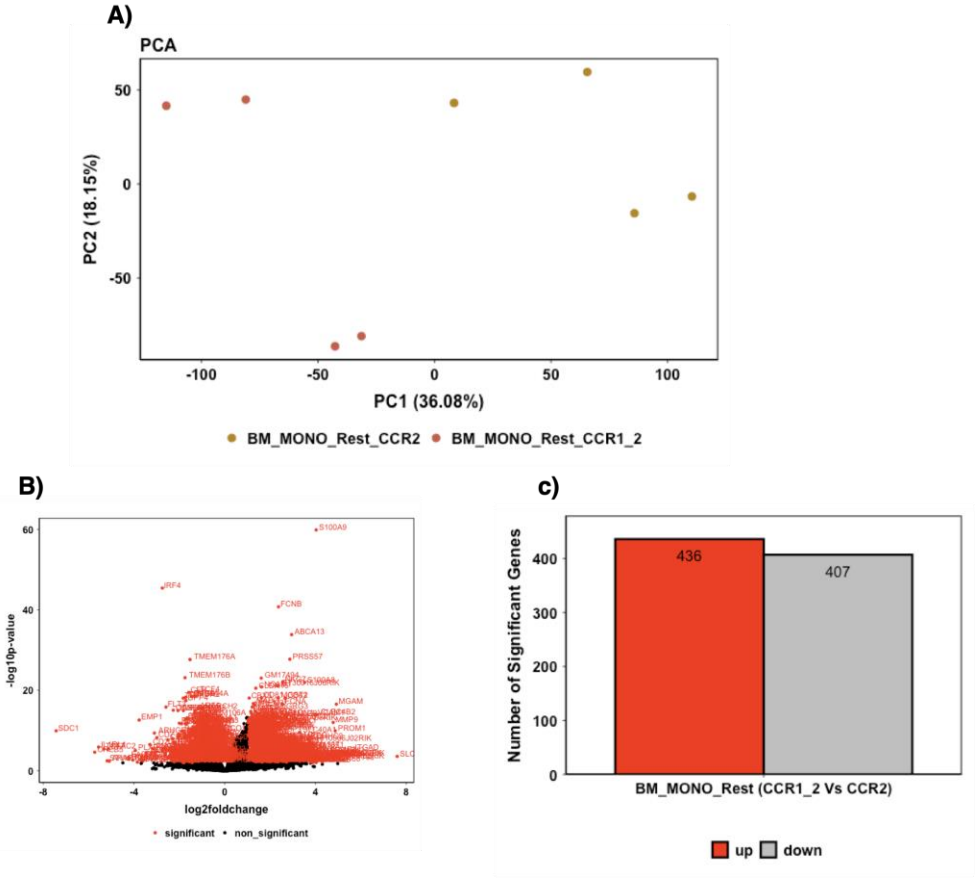

Figure 5-17 CCR2^{+} monocytes are transcriptionally distinct from CCR1_2^{+} Ly6C^{hi} monocytes in resting mice. RNA sequencing was performed on CCR2^{+} and CCR1_2^{+} Ly6C^{hi} monocytes sorted from BM cells of iREP mice at rest. A) Principal component analysis (PCA) showing the distribution of Ly6C^{hi} monocytes across the transcribed genes, where PC1 (45.43% variance) on the x-axis and PC2 (24.72% variance) on the y-axis. Each colour represents a sample group. The expression level was scaled using a Z−score. B) Volcano plot presenting the differentially expressed genes (red) in CCR1_2^{+} relative to CCR2^{+} Ly6C^{hi} monocytes. Genes toward the right (upregulated), the left (downregulated) and the top (most significant). C) Graph showing the number of upregulated genes (red) and downregulated (grey) in CCR1_2^{+} relative to CCR2^{+} Ly6C^{hi} monocytes. Significant genes (p.adj < 0.05, absolute log2 fold > 1.0) were selected.

The ORA analysis of all 436 upregulated genes identified that the top 10 enriched gene sets were associated with various cellular and molecular functions (Figure 5-18 C). Upon examining the top 40 gene list, it was noteworthy that S100A9 and S100A8 encode proteins that belong to the S100 family, which are involved in diverse cellular processes, such as the cell cycle, proliferation, differentiation, and

calcium homeostasis[416]. These cytosolic proteins exist as heterodimers and account for about 45% of the cytosolic protein content of neutrophils, whereas their expression is 40 times less prevalent in monocytes[417]. S100A8/A9 expression increases in neutrophils during inflammation, especially during NETosis, a process in which neutrophils release their DNA to trap and kill invading pathogens. It can serve as a marker for NETosis[418]. MMP9, a gene associated with neutrophil granules, was also detected in the list.

In general, there were significant differences in the gene expression profiles between Ly6C^{hi} monocytes co-expressing CCR2/mRuby2 and CCR1/Clover, compared to monocytes only expressing CCR2/mRuby2, with several genes characteristic of neutrophil function upregulated in Ly6C^{hi} monocytes co-expressing CCR2/mRuby2 and CCR1/Clover. In contrast, Ly6C^{hi} monocytes expressing CCR1/Clover independently upregulated transcription factors that promote neutrophil maturation and development. Therefore, based on these findings, Ly6C^{hi} monocytes co-expressing reporter CCR2 and CCR1 may represent a subpopulation of monocytes with neutrophil characteristics, whereas Ly6C^{hi} monocytes solely expressing reporter CCR1 are more similar to neutrophils than monocytes.

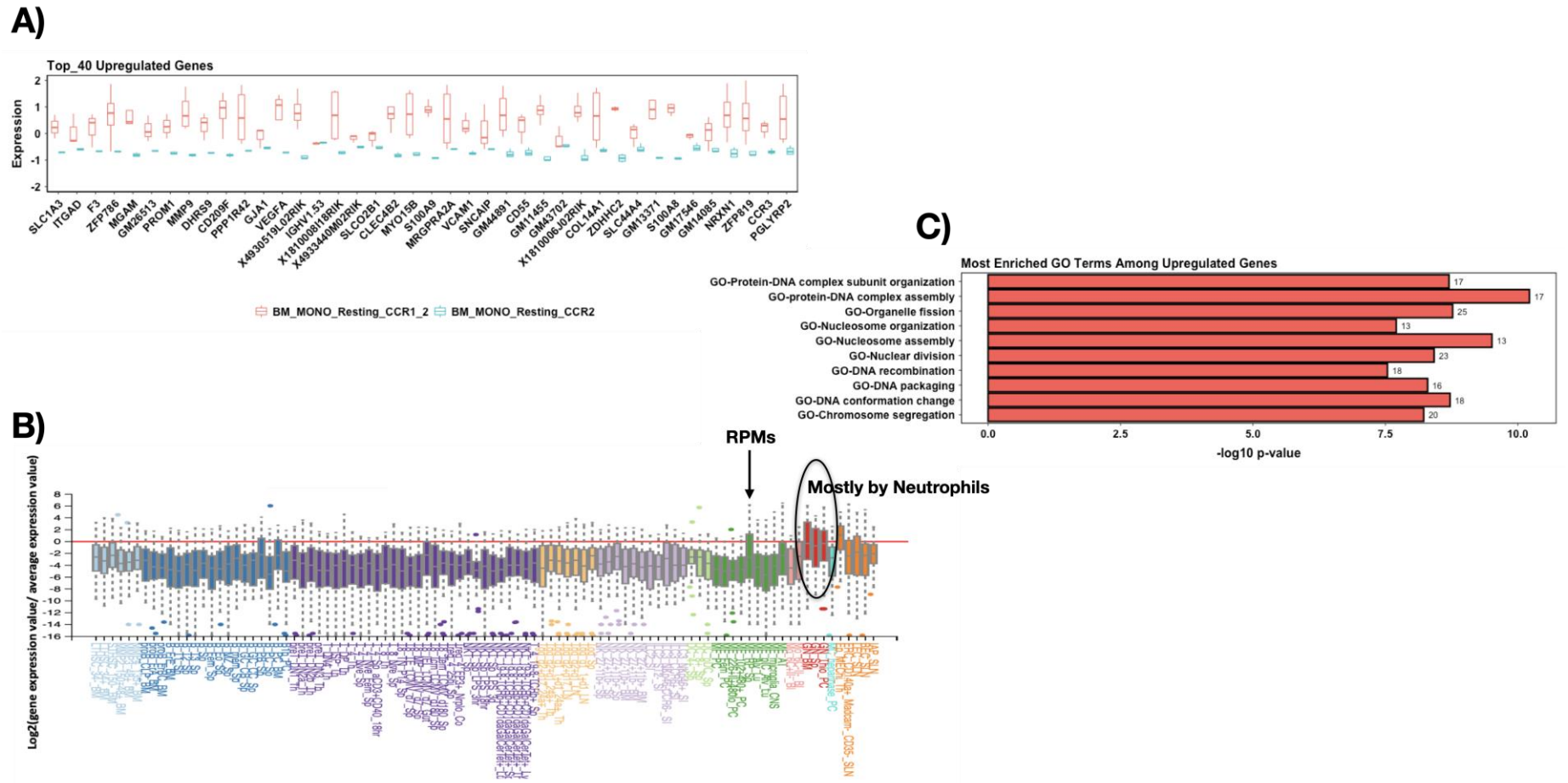

Figure 5-18 Analysis of upregulated differentially expressed genes in CCR1_2^{+} and CCR2^{+} monocytes from resting BM. Top 40 upregulated genes in CCR1_2^{+} relative to CCR2^{+} Ly6C^{hi} monocytes from BM cells of iREP mice at rest showing: A) Box plot with the list of highly upregulated genes (p.adj < 0.05, log2 fold > 0) on the x-axis and Z-score of the expression level on the y-axis, and B) The upregulated gene expression profile compared to ImmGen data sets. C) Bar graph presenting the top 10 enriched terms of gene ontology (GO) for the differentially expressed genes in CCR1_2+ relative to CCR2+ Ly6C^{hi} monocytes. The y-axis shows the term of the gene set, and the x-axis shows the -log10 p-value. GO terms were selected using GO databases, biological process (BP) and p-values <.05.

5.2.4.1.2 Downregulated differentially expressed genes

Compared to Ly6C^{hi} monocytes expressing only CCR2/mRuby2, Ly6C^{hi} monocytes expressing both CCR2/mRuby2 and CCR1/Clover were found to downregulate 407 genes. By analysing the top 40 downregulated genes shown in Figure 5-19 A, immGen datasets confirmed that these genes were primarily expressed in DCs, and macrophages (Figure 5-19 B). Furthermore, the ORA analysis of all downregulated genes revealed that the top 10 enriched GO terms were associated with immune process, such as the Toll-like receptor signalling pathway, positive regulation of cytokine production, regulation of leukocyte differentiation, migration, and T cell activation, indicating that Ly6Chi monocytes expressing CCR2/mRuby2 are ready to respond rapidly to external stimuli or pathogens (Figure 5-19 C).

Overall, Ly6C^{hi} monocytes that co-express CCR2/mRuby2 and CCR1/Clover show downregulated expression of genes associated with typical monocyte functions, suggesting a reduction in those functions. At the same time, these cells acquire genes contributing to the functional properties of neutrophils, indicating the development of some neutrophil-like characteristics. Therefore, the observed differences in the gene expression profiles of BM Ly6C^{hi} monocytes co-expressing both CCR2/mRuby2 and CCR1/Clover compared to those expressing only CCR2/mRuby2 under sustained inflammation and at rest are linked to genes contributing to functional properties of neutrophils. These results suggest they are a subset of Ly6C^{hi} monocytes with neutrophilic characteristics.

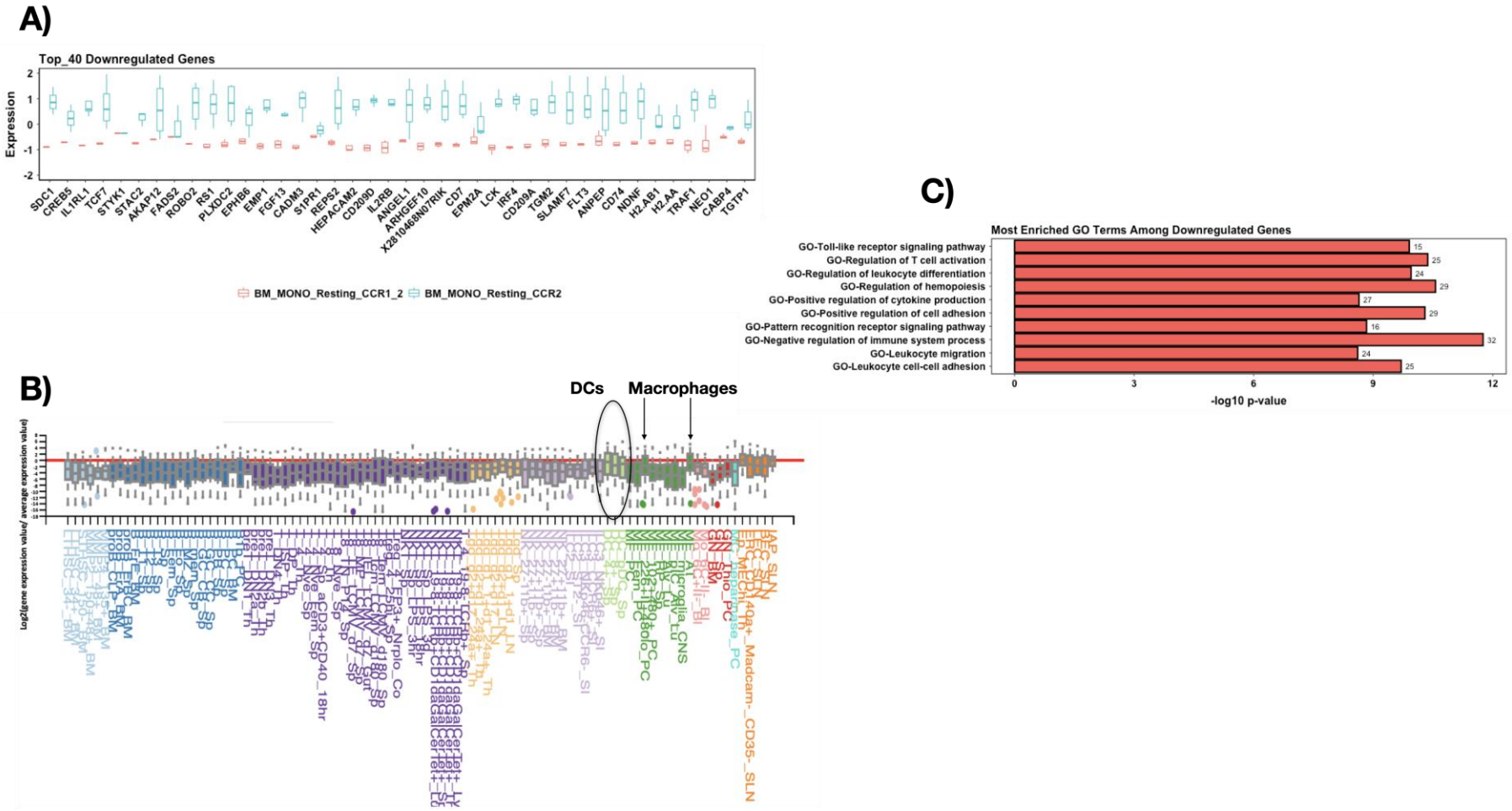

Figure 5-19 Analysis of downregulated differentially expressed genes in CCR1_2^{+} and CCR2^{+} monocytes from resting BM. Top 40 downregulated genes in CCR1_2^{+} relative to CCR2^{+} Ly6C^{hi} monocytes from BM cells of iREP mice at rest showing: A) Box plot with the list of highly downregulated genes (p.adj < 0.05, log2 fold < 0) on x-axis and Z-score of the expression level on y-axis, and B) The downregulated gene expression profile compared to ImmGen data sets. C) Bar graph presenting the top 10 enriched terms of gene ontology (GO) for the differentially expressed genes in CCR1_2+ relative to CCR2^{+} Ly6C^{hi} monocytes. The y-axis shows the term of the gene set, and the x-axis shows the -log10 p-value. GO terms were selected using GO databases, biological process (BP) and p-values <.05.

5.3 Discussion and conclusion

Our current understanding of the precise roles of chemokines in leukocyte mobilization and recruitment, as well as their dynamics within and between tissues, is limited. Furthermore, the fact that individual leukocyte subsets can express multiple receptors simultaneously suggests functional redundancy within the system, which adds to its complexity. For example, Ly6C^{hi} monocytes have been shown to use not only CCR2 and CCR5 but also CX3CR1 to accumulate within atherosclerotic plaques[275]. In our previous chapter, we found that while most Ly6C^{hi} inflammatory monocytes express reporter CCR2 regardless of inflammation, a minority of these cells also express reporter CCR1. The reason for this upregulation remains unknown, and it could indicate a redundant role for iCCR in monocyte migration. However, if redundancy were the case, we would expect CCR1 expression to be more widespread in monocytes.

To learn more about this, Ly6C^{hi} monocytes expressing only mRuby2/CCR2 and those expressing both mRuby2/CCR2 and Clover/CCR1 were isolated from the BM of iREP and WT mice at rest and under sustained inflammation along with a population of Ly6C^{hi} monocytes that independently express Clover/CCR1 under sustained inflammation. Then, RNA sequencing was conducted to compare the transcriptomic profiles and determine whether these cells are phenotypically discrete cell types or a homogeneous population of Ly6C^{hi} monocytes with different iCCR expressions.

The results shed light on the gene expression profiles of Ly6C^{hi} monocytes co-expressing mRuby2/CCR2 and Clover/CCR1. Significant differences were observed when compared to Ly6Chi monocytes expressing only mRuby2/CCR2. In the inflamed BM, 169 genes were significantly differentially expressed, while 843 genes showed significant differential expression in resting BM. The top 40

upregulated genes in both conditions were mainly associated with neutrophils, as evidenced by immGen data sets. Notably, among these top 40 upregulated genes in Ly6C^{hi} monocytes co-expressing mRuby2/CCR2 and Clover/CCR1 under sustained inflammation were those encoding proteins associated with neutrophil granules, such as ELANE, MPO, NGP, and LCN2, as well as those involved in neutrophil mobilisation and migration, such as CXCR2 and IL-36γ. In contrast, the top 40 downregulated genes were involved in antigen presentation, a characteristic feature of monocytes and their derived DCs and macrophages. Therefore, these findings indicate that Ly6C^{hi} monocytes expressing both CCR1/Clover and CCR2/mRuby2 represent a population of Ly6C^{hi} monocytes with a neutrophilic gene expression.

The results also revealed that Ly6C^{hi} monocytes expressing CCR1/Clover independently in BM under sustained inflammation had markedly different gene expression profiles compared to other monocyte groups. This finding was confirmed by the PCA plot, which showed a clear separation of these cells from Ly6C^{hi} monocytes expressing both CCR1/Clover and CCR2/mRuby2 and those expressing only CCR2/mRuby2. Additionally, these cells exhibited numerous differentially expressed genes, with approximately 5000 genes differing from the transcriptomic profiles of other groups of Ly6C^{hi} monocytes. According to immGen data, the top 40 upregulated genes in Ly6C^{hi} monocytes expressing only CCR1/Clover were predominantly expressed in neutrophils across all cell types. Among these genes was GFI1, a well-known transcription factor that plays a role in neutrophil development. Thus, these cells do not appear to belong to the monocyte population but rather represent a population of neutrophils that express Ly6C and are Ly6G-negative.

To validate these results, we merged the transcriptional profiles of the Ly6C^{hi} monocyte group from the BM of mice at rest and during sustained inflammation with a recently published single-cell RNA sequencing (scRNA-seq) datasets. Xie et al.,[419] isolated cells from the BM, peripheral blood, and spleen of mice under both resting and bacterial infection conditions and identified them based on their expression levels of GR1 or Ly6G. Additionally, the datasets included hematopoietic stem progenitor cells (HSPCs) but only from the BM of resting mice.

According to Xie et al.,[419] neutrophils are classified into eight clusters based on their maturation process, with the first five clusters (G0-G4) mainly originating in the bone marrow and the remaining three clusters (G5a-G5c) present in both the peripheral blood and tissue. Our analysis reveals a high degree of alignment between the transcriptome of Ly6C^{hi} monocytes that only express CCR1/Clover and the signature genes characterising the G0, G1, and G2 neutrophil clusters (Figure 5-20 Aiii). Notably, the G0 cluster is characterised by the expression of typical genes of granulocyte-monocyte progenitor cells, as well as other myeloid precursor cells, which explains why we observed alignment between Ly6C^{hi} monocytes co-expressing CCR1/Clover and CCR2/mRuby2, as well as those expressing only CCR2/mRuby2 and this G0 cluster (Figure 5-20 Ai, ii). In contrast, the G1 cluster is where the commitment to a specific neutrophil lineage begins, and correlation analysis conducted by Xie et al.,[419] indicates its association with a morphological development stage of pro-neutrophil in BM. The G2 cluster is associated with further neutrophil differentiation and corresponds to the morphological development stage of the pre-neutrophil, which is characterized by the beginning of specific neutrophil granule formation, such as CAMP. The

expression of CAMP was found to be higher in Ly6Chi monocytes expressing CCR1-Clover than in other Ly6Chi monocyte groups.

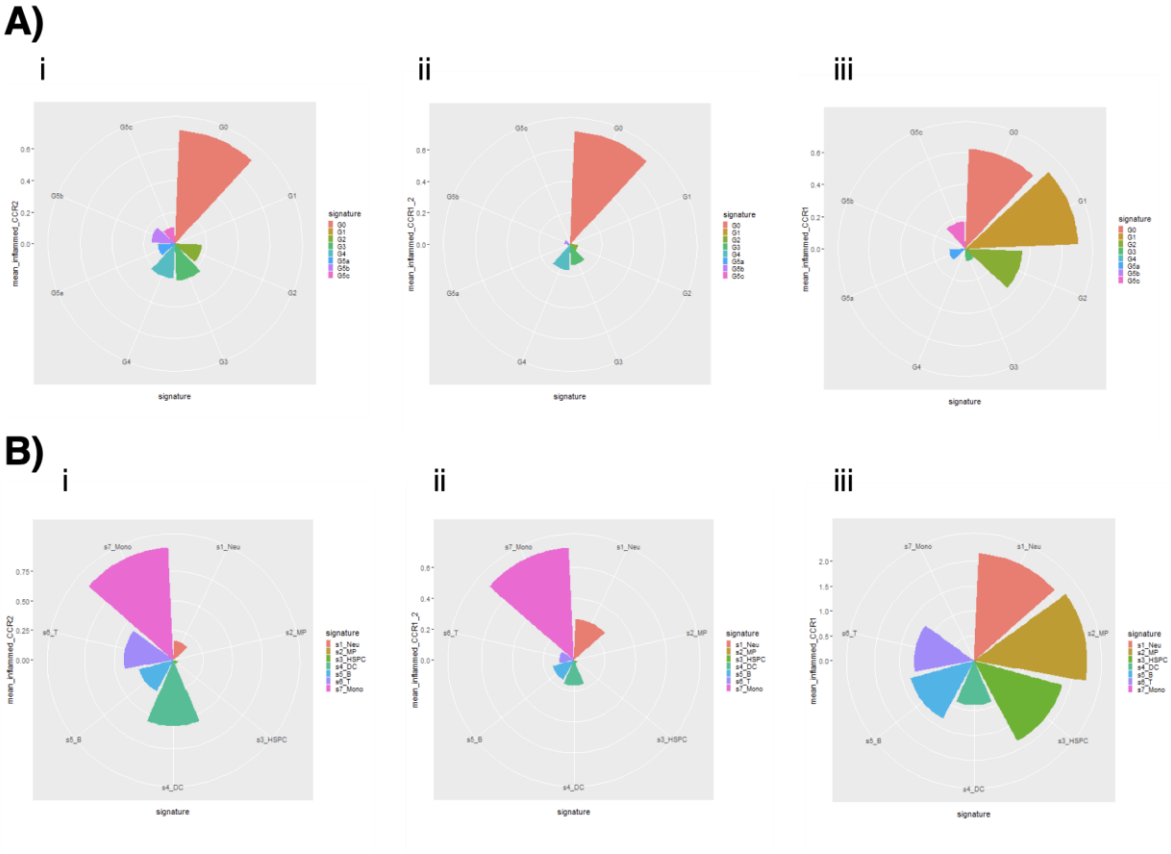

Figure 5-20 Transcribed genes of CCR1+ly6chi monocytes aligned with previously published neutrophil signature genes. A previous study identified 8 clusters of neutrophils arising during differentiation and maturation. Xie et al., (2020) performed single RNA sequencing on HSPCs from BM and GR1+(Ly6G+) neutrophils from different inflamed mice tissues. Our data of ly6chi monocytes in inflamed BM was compared to the published raw dataset. Pie charts showing the relation of the gene expression profile for i) CCR2+, ii) CCR1+CCR2+, and iii) CCR1+ ly6chi monocytes related to the signature genes for each A) neutrophil clusters and B) HSPCs.

In addition, when we merged our raw data with the signature gene for each HSPC population, the analysis revealed that Ly6C^{hi} monocytes expressing both CCR1/Clover and CCR2/mRuby2, and those expressing only CCR2/mRuby2, were clearly identified as monocytes (Figure 5-20 Bi, ii). However, some Ly6C^{hi} monocytes expressing both CCR1/Clover and CCR2/mRuby2 were aligned with neutrophils, supporting our previous conclusion that these cells represent a subpopulation of monocytes with some neutrophilic characteristics. On the other

hand, Ly6C^{hi} monocytes that only express CCR1/Clover were found to be highly associated with neutrophils and not monocytes (Figure 5-20 Biii). Therefore, our findings indicate that Ly6C^{hi} "monocytes" expressing only CCR1/Clover have transcriptional profiles similar to neutrophil clusters' signature genes, indicating that these cells may not belong to the monocyte but rather to a population of neutrophils that express Ly6C but not Ly6G.

Huang et al., conducted scRNA-seq to analyse circulating neutrophils from both healthy individuals and those with burn injuries[420]. Their study showed that human neutrophil maturation followed the same trajectory as observed in Xie et al.,'s mouse study(the one to which our data was compared), identifying five different subgroups of neutrophils that aligned with clusters (G3, G4, G5a, G5b, and G5c) previously defined in that study[419]. This alignment highlights a shared maturation or development process of neutrophils across two species. This finding is in line with another study that observed human neutrophils exhibit transcriptomic data broadly aligns with the developmental pattern identified in mice[406].

As our transcriptome of Ly6C^{hi} monocytes expressing only CCR1/Clover aligns with the signature genes in Xie et al.,'s study [419] characterising early neutrophil maturation clusters (G0, G1, and G2) in BM, this suggests that Ly6C^{hi} monocytes expressing only CCR1/Clover are undergoing a maturation process of neutrophils, which has found to be parallel between humans and mice. The lack of Ly6G expression in monocytes that only express CCR1/Clover could be explained by Zhu et al.,'s study[421]. Their study identified two clusters, C1 and C2, within the neutrophil developmental lineage[421]. Subsequently, Xie et al.,[419] performed an analysis by comparing their transcriptomic data to Zhu et al.,'s study[421], finding that C1 corresponds to early stages (G0/G1), the same cluster

in which we found Ly6C^{hi} monocytes expressing only CCR1/Clover to be aligned. In contrast, C2 corresponds to later stages (G2/G3) in neutrophil maturation. Importantly, Zhu et al., observed that this maturation transition is accompanied by fluctuations in Ly6G expression levels, starting with negative expression in C1 to intermediate in C2 and eventually reaching high levels in mature BM neutrophils[421]. This observation suggests that Ly6C^{hi} monocytes expressing solely CCR1/Clover initially appear Ly6G-negative as they develop into mature neutrophils, and that later, as they become more mature, they might induce a subsequent increase in Ly6G expression, reflecting this developmental process.

Despite the two species sharing a similar neutrophil maturation pattern, mice exhibit only three subsets[419], while humans show five subsets[420] in peripheral blood and tissues. To gain deeper insights into the specific mature subsets aligned with Ly6C^{hi} "monocytes" expressing only CCR1/Clover, sorting these cells from the blood, as they represent a more mature neutrophil, and conducting an overlay analysis with both human and mice transcription data could provide valuable insights into their alignment with relevant subsets across both species.

In summary, the transcriptomic analysis shows that Ly6C^{hi} monocytes expressing both reporter CCR1 and CCR2 exhibit distinct transcriptomic profiles compared to the majority of monocytes expressing only reporter CCR2. These discrepancies indicate that they are a subset of Ly6C^{hi} monocytes, further highlighting the specificity of CCR2 in monocyte migration and excluding the possibility of redundancy with other receptors. However, Ly6C^{hi} monocytes expressing only reporter CCR1 mostly do not belong to the monocyte population and are primarily neutrophils.

Chapter 6 Discussion

6.1 Overview

This study focuses on the role of inflammatory iCCRs; CCR1, CCR2, CCR3, and CCR5, which are crucial in mediating the recruitment of leukocytes to inflamed and damaged sites in vivo. These receptors play significant roles in the pathogenesis of various inflammatory and autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis, where excessive inflammation is the primary cause of pathology. Despite over 25 years of pharmaceutical industry efforts to develop chemokine receptor antagonists, only two have been licensed: Mozobil™, which targets CXCR4, and Maraviroc, which targets CCR5[291]. However, neither of these drugs is used to treat inflammatory and autoimmune diseases[291]. Therefore, an understanding of the individual and combined roles of these receptors in orchestrating the inflammatory response is needed. However, this task is complicated by the promiscuity of receptor-ligand interactions[286], incomplete phenotype observation from KO-mouse studies indicating possible redundancy within the system[309, 330, 422], and the need for high-quality commercially available antibodies to target these receptors[198].

To address these challenges and advance our understanding, this study used a novel mouse strain, the iCCR fluorescent reporter (iREP)[198], to investigate the role of iCCRs in inflammation. The iREP strain is a powerful tool that enables us to precisely define temporal and spatial patterns of receptor expression in response to inflammatory conditions. The study began by using in vitro BMDM to determine whether specific inflammatory conditions induce the expression of certain iCCRs. Next, the iREP strain was used in a model of sustained inflammation to assess iCCR expression on monocytes and differentiated macrophages in vivo. Finally, transcriptomic analysis was performed to gain further insights into the nature of cells expressing these receptors.

6.2 Transcriptional regulation of iCCRs in BMDM

The qPCR analysis of iCCR transcriptional expression in BMDMs indicates that the mRNA levels of CCR2 and CCR3 did not show any significant changes in response to various cytokines and TLR ligands. This lack of significant changes may be attributed to the essential roles that CCR2 and CCR3 play in monocytes and eosinophils, respectively, rather than in mature macrophages[194].

In contrast, both CCR1 and CCR5 were primarily regulated in BMDMs in response to the stimulating agents. However, the data did not identify any specific inflammatory conditions that exclusively or selectively regulate the expression of CCR1 or CCR5 over the other. Instead, their expression was found to increase to varying degrees with different agents, with CCR1 generally showing a higher induction level than CCR5, which was induced to a lesser extent.

Additionally, our findings show that BMDMs exhibited the highest transcriptional induction of CCR5 in response to viral stimuli, especially when stimulated with IFNα and viral-derived TLR3 agonists, whereas CCR1 induction is higher in response to bacterial-derived TLR agonists than viral ones. These results suggest that these receptors may play distinct roles in specific contexts. In our experiments, we treated BMDMs with bacterial LPS in combination with other pro-inflammatory cytokines and found an additive increase in CCR1 expression but no further increase in CCR5 expression. This supports the idea that activated macrophages express higher levels of both receptors, with CCR1 possibly being further enhanced in response to bacterial infections and CCR5 being activated in response to viral infections.

To further test the potential regulation of these two receptors under specific contexts, it would be beneficial to examine them using an appropriate murine model of viral or bacterial infection. This could reveal a more

comprehensive picture of these receptors in monocyte recruitment and differentiated macrophages and confirm these findings.

6.3 Dynamics of iCCR expression in inflamed monocytes/macrophages and their characterisation.

To evaluate the temporal changes in the expression of iCCRs in monocytes and differentiated macrophages during sustained inflammation in vivo, iREP mice were implanted with a subcutaneous osmotic pump loaded with either a cytokine cocktail or vehicle (PBS). Subsequent analysis of reporter mice showed that most inflammatory monocytes expressed only reporter CCR2 at rest and during sustained inflammation in both BM and blood. However, a small fraction of these inflammatory monocytes also co-expressed reporter CCR1 and CCR2 in BM and blood regardless of inflammation, with a higher proportion observed in mice under sustained inflammation. Furthermore, these inflammatory monocytes expressed less reporter CCR2 after infiltrating the membrane surrounding the minipump than in BM and blood, and even less in monocytes from mice under sustained inflammation, suggesting that monocytes rapidly downregulate CCR2 expression once they enter an inflamed site to facilitate their differentiation into macrophages, a process that has been extensively documented in previous studies[198, 355], and is thought to be a mechanism to prevent them from re-entering circulation[354].

Sustained inflammation also increased the fraction of inflammatory monocytes co-expressing reporter CCR5 and CCR2, although this fraction remained relatively low in BM, blood, and membrane. This suggests that CCR5 may play a less significant role than CCR1 in monocyte function under both inflammatory and resting conditions. The small shift in reporter CCR5 expression seen in

inflammatory monocytes from mice under sustained inflammation (2% in BM and 3% in blood and membrane) compared to mice at rest (0.3% in BM and 1% in blood and membrane) could be due to increased autofluorescence, which may be caused by increased production of proteins, vacuoles, and exosomes in inflamed monocytes.

In contrast, there was a clear shift in the differentiated macrophages in the inflamed membrane expressing reporter CCR5 as compared to resting membranes (approximately 40% versus 20%, respectively), and the number of these cells expressing reporter CCR1 was also increased, but to a lesser extent than CCR5. These findings align with the results from the first chapter, which showed that bacterial-derived TLR agonists induce CCR5 transcription in BMDMs; however, this induction of CCR5 takes longer than CCR1, which suggests that BMDMs may regulate the expression of CCR1 and CCR5 in response to bacterial-derived TLR by first increasing CCR1 expression, followed by CCR5. As the inflammation becomes sustained, macrophages switch from expressing CCR1 to expressing CCR5, and CCR5 becomes more prominently expressed than CCR1.

As noted previously, a relatively small subset of inflammatory monocytes exhibited upregulation of reporter CCR1 expression during both inflammation and rest. This observation suggests that CCR1 may play a role in monocyte recruitment, although its exact function remains unclear. While CCR1 expression in inflammatory monocytes has been previously reported[373-375, 423] and regarded as an example of redundancy in the chemokine system, our findings suggest that CCR1 may have a specific function in these cells rather than simply acting as a backup for CCR2. If for example, if CCR1 were truly redundant, we would expect to observe more inflammatory monocytes expressing it.

To better understand the role of CCR1 in inflammatory monocytes, we aimed to determine the nature of the cells expressing it and whether Ly6Chi monocytes with different iCCR expression represent phenotypically distinct cell types or distinct populations of monocytes. Therefore, inflammatory monocytes expressing mRuby2/CCR2, both mRuby2/CCR2 and Clover/CCR1, and Clover/CCR1 only were isolated from the BM of iREP mice at rest and during sustained inflammation for RNAseq. Our transcriptomic analysis of these monocyte populations revealed some significant differences between those expressing only reporter CCR2 and those co-expressing reporter CCR2 and CCR1. Notably, the top 40 upregulated genes in the latter group were mainly associated with neutrophils, suggesting that these cells are a distinct population of Ly6Chi monocytes with neutrophilic characteristics.

On the other hand, our transcriptomic analysis showed that inflammatory monocytes expressing reporter CCR1 independently in BM under sustained inflammation had markedly different gene expression profiles compared to other monocyte groups. Furthermore, our merged analysis with the Xie et al.,[419] study revealed that these cells have transcriptional profiles similar to the signature genes of neutrophil clusters. This suggests that they may not belong to monocytes, but rather to a distinct population of neutrophils that express Ly6C but not Ly6G.

6.4 Future work and Limitations

Inflammatory monocytes expressing both CCR1 and CCR2 are transcriptionally distinct from monocytes expressing only CCR2. However, it is unclear why only a small subset of monocytes co-express CCR1 and CCR2, while others do not express CCR1. Additionally, the role of CCR1 in the differentiation and effector function of macrophages still needs to be clarified. Can monocytes

co-expressing CCR1 and CCR2 differentiate directly into CCR5-expressing macrophages without first expressing CCR1 and then CCR5? In vitro analysis of CCR1 and CCR5 expression in sorted cells co-expressing CCR1 and CCR5 that have differentiated into macrophages may provide initial insights. Furthermore, it is unknown whether these monocytes migrate to different sites of inflammation than inflammatory monocytes that express CCR2 alone. To gain insight into this, imaging studies using an air pouch inflammation model in iREP mice could be performed to determine the localisation of these monocyte subsets.

To comprehensively address the research questions outlined above, a novel murine strain with reversible iCCR knockout (RiKO) is currently underway at the CRG under the guidance of Dr. Schutte. This strain will enable the expression of individual receptors in different combinations (e.g., CCR1+CCR2+CCR3-CCR5-, CCR1+CCR2-CCR3-CCR5+, and CCR1-CCR2+CCR3-CCR5+), allowing for cutting-edge experiments to provide valuable insights into the impact of CCR1 expression on macrophage function, as well as monocyte migration to specific sites of inflammation.

Using this novel mouse model in a disease-specific context other than the mini pump implantation model employed in our study could better replicate the natural complexity of inflammatory processes observed in human diseases and provide a more comprehensive understanding of the dynamic shifts in iCCR expression within leukocytes and the potential regulation of CCR1 and CCR5 within macrophages in complex scenarios. While the mini pump model provides valuable insights into the basic mechanisms of cell migration during an inflammatory response, it generates artificial membranes that do not fully capture the complexity of inflammatory processes seen in actual human biological tissues or organs. Finally, as Ly6Chi monocytes exclusively expressing CCR1/Clover do not

appear to belong to the monocyte population but rather represent a population of neutrophils, the question of which specific neutrophil subset they align with remains unclear. Therefore, conducting a further overlay analysis using single-cell transcriptomic data from both humans and mice could clarify this alignment in both species and highlight its relevance to human biology.

6.5 Conclusion

The study has enhanced our understanding of how macrophages regulate inflammatory iCCRs in response to diverse inflammatory stimuli. The findings suggest that CCR1 and CCR5 expression levels vary depending on the type of inflammatory condition, with CCR1 being highly responsive to bacterial stimuli and CCR5 typically associated with viral stimuli. Furthermore, sustained inflammation increases the fraction of inflammatory monocytes expressing both CCR1 and CCR2 or only CCR1. However, these monocyte subsets have different transcriptional profiles from the majority of monocytes that only express CCR2, indicating that this is not redundant expression. Future studies using RiKO mice are expected to clarify uncertainties regarding the co-expression and redundancy of iCCRs. These findings will provide crucial insights into how chemokine receptors coordinate inflammatory responses, advancing our understanding of basic chemokine biology and potentially informing the development of new drugs that target the chemokine system.

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