The role of inflammatory chemokine receptors in hypertension and blood pressure regulation

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

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Author's declaration

I declare that the work described in this thesis is original and, except where explicit reference is made to the contribution of others, was generated entirely as a result of my own efforts. None of the data included in this thesis have been submitted for any other degree, either at the University of Glasgow or at any other institution.

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Abstract

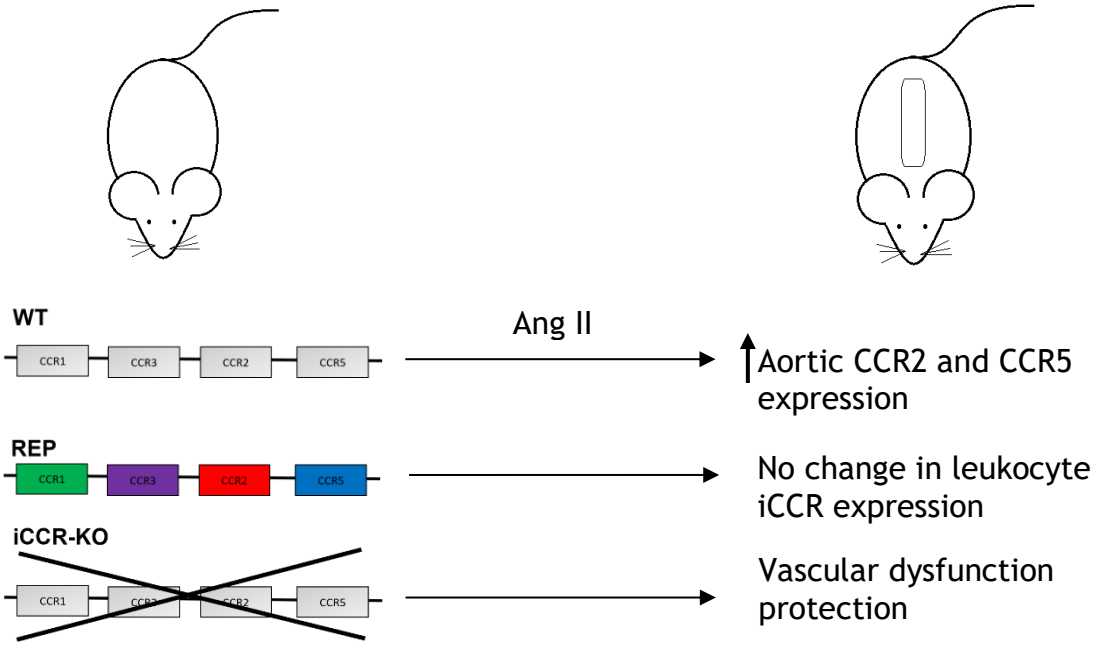
Chemokines regulate the migration of leukocytes through binding to chemokine receptors. The inflammatory CC chemokine receptors CCR1, CCR2, CCR3 and CCR5 (iCCRs) are expressed by several different leukocytes and are important in orchestrating inflammatory responses. These receptors can bind to multiple CC chemokines and many of these chemokines share receptors, resulting in a highly complex system in which the exact role of each receptor is not understood. To aid understanding of these receptors, two unique mouse strains have been developed. These are mice that are deficient for all four iCCRs (iCCR-KO) and mice that express fluorescent reporter proteins for each of the iCCRs (REP mice).

Hypertension is when blood pressure (BP) is elevated above 140/90 mmHg. There is evidence for a role of inflammation in the development of hypertension and the consequent end organ damage which increases the risk of cardiovascular disease. Several chemokines and chemokine receptors have been implicated in hypertension but due to the complexity of the chemokine system, the role they play in the pathogenesis of the disease is unclear. Therefore the role of iCCRs in hypertension was investigated using iCCR-KO and REP mice.

WT, REP and iCCR-KO mice were subject to 7 or 14 days of Angiotensin (Ang) II induced hypertension. iCCR expression was characterised in REP and WT mice and the effect of iCCR deficiency on BP, vascular function, inflammation and cardiac and vascular remodelling was assessed using the iCCR-KO mice. Aortic CCR2 and CCR5 expression increased in Ang II treated WT mice compared to control WT mice. Further, iCCR-KO mice were protected from Ang II induced vascular dysfunction but iCCR deficiency did not influence BP or remodelling. iCCR-KO mice were also shown to have altered circulating leukocyte populations in Ang II induced hypertension.

Control iCCR-KO mice tended to have a lower BP than WT mice so the effect of iCCR deficiency on regulators of BP in the kidneys and kidney leukocyte infiltration was investigated. iCCR-KO mice had fewer inflammatory monocytes and reduced mineralocorticoid receptor mRNA expression. This could influence BP but further studies are needed.

Overall, novel mouse models have been used to identify how iCCRs are involved in hypertension. The results described here suggest that iCCRs, in particular CCR2 and CCR5, are involved in regulating vascular dysfunction in hypertension. Through improving understanding of these receptors in the disease, there is increased potential to target them as treatments that would ultimately reduce the risk of cardiovascular disease.



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List of abbreviations

3Rs	Replacement, reduction and refinement
ACE	Angiotensin converting enzyme
ACE2	Angiotensin converting enzyme 2
ACh	Acetylcholine
ACKR	Atypical chemokine receptor
AMP	Antimicrobial peptide
Ang	Angiotensin
ANP	Atrial natriuretic peptide
APC	Antigen presenting cell
ARB	Angiotensin II receptor blocker
ARRIVE	Animal Research: Reporting *in vivo* experiments
ASMC	Airway smooth muscle cell
AT1	Angiotensin type 1
AT2	Angiotensin type 2
BAC	Bacterial artificial chromosome
BAFF-R	B cell activating factor receptor
BP	Blood pressure
BSA	Bovine serum albumin
COPD	Chronic obstructive pulmonary disease
CVD	Cardiovascular disease
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DHP	Dihydropyridine
DOCA	Deoxycorticosterone acetate
EC	Endothelial cell
EMP	Erythro-myeloid progenitor
ENaC	Epithelial sodium channel

eNOS	Endothelial nitric oxide synthase
FB	Flow cytometry buffer
FCS	Foetal calf serum
FMO	Fluorescence minus one
FSC	Forward scatter
GC	Germinal centre
GM-CSF	Granulocyte macrophage colony stimulating factor
GPCR	G protein-coupled receptor
H&E	Haematoxylin & Eosin
HPC	Haematopoietic progenitor cell
HSC	Haematopoietic stem cell
IBD	Inflammatory bowel disease
iCCR	Inflammatory chemokine receptor
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
LPS	Lipopolysaccharide
L-NAME	Nω-nitro-L-arginine methyl ester
MBP	Major basic protein
MC3/4-R	Melanocortin receptor types 3 and 4
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MI	Myocardial infarction
MMF	Mycophenolate mofetil
MR	Mineralocorticoid receptor
NHE3	Sodium/hydrogen exchanger 3
NK	Natural killer

NO	Nitric oxide
NPR	Natriuretic peptide receptor
PAH	Pulmonary arterial hypertension
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PRR	Pattern recognition receptor
PSS	Physiological salt solution
PVAT	Perivascular adipose tissue
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
Rag-1	Recombination activating gene 1
RAS	Renin angiotensin system
RBC	Red blood cell
REP	iCCR reporter
RLU	Relative light units
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute – 1640 medium
RT	Room temperature
SHR	Spontaneously hypertensive rat
SD	Standard deviation
SGK1	Serum/glucocorticoid-regulated kinase 1
SLE	Systemic lupus erythematosus
SNP	Sodium nitroprusside
SSC	Side scatter
TAM	Tumour associated macrophage
Tc	Cytotoxic T
TCR	T cell receptor

TGF	Transforming growth factor
Th	Helper T
TNF	Tumour necrosis factor
Treg	Regulatory T
VSMC	Vascular smooth muscle cell
WKY	Wistar Kyoto rat
WT	Wild type

Chapter 1

Introduction

1 Introduction

1.1 The immune system and inflammation

The immune system is a complex defence system that acts to protect against damage and pathogens. This is split into the innate and adaptive immune responses in which the innate system provides a quick, but non-specific, response whilst the adaptive response involves a more specific, coordinated defence. Inflammation occurs when leukocytes infiltrate a tissue to protect against an infection or damage. This is a necessary response to maintain a healthy state but if not resolved, inflammation can become chronic and cause damage to healthy tissues.

1.1.1 Innate immunity

The innate immune response is the first stage of defence against pathogens and also activates cells of the adaptive immune response. There are several components to innate immunity. These include physical barriers, such as the skin, which separate the internal environment from pathogens in the external environment, humoral factors found in the plasma and mucosal secretions and cellular mechanisms (Riera Romo et al., 2016).

There are various humoral factors that possess antimicrobial activity or modulate the immune response. Antimicrobial peptides (AMPs), such as defensins, have antimicrobial, antifungal or antiparasitic activity. Defensins have been shown to be elevated in Hepatitis C infections (Mattar et al., 2016), suggesting antiviral activity as well. The complement system is a cascade of proteins found in the circulation and tissues that results in lysis of microbes and enhances the inflammatory response (Harboe et al., 2011; Ricklin et al., 2010).

Cytokines are small proteins that mediate communication between cells to modulate the immune response. Cytokines can be proinflammatory or anti-inflammatory and include interferons (IFNs), interleukins (ILs), tumour necrosis factors (TNFs) and transforming growth factors (TGFs) (Riera Romo et al., 2016). IFNs are grouped into type I (IFN-α and IFN-ß), type II (IFN-γ) and type III (IFN-λ) and have antiviral activity (Takaoka and Yanai, 2006). As well as this, IFN-λ has

recently been reported to have antifungal activity (Espinosa et al., 2017). There are many different ILs which have a wide range of functions. IL-1α and IL-1β are proinflammatory and activate leukocytes such as neutrophils, B cells and T cells (Keyel, 2014). IL-1β induces the differentiation of Th_{17} cells and can inhibit IL-10 production by these cells (Acosta-Rodriguez et al., 2007; Zielinski et al., 2012). IL-1β also mediates neutrophil recruitment and is found at many sites of inflammation, for example, in the liver during Hepatitis C infection (Miller et al., 2007; Negash et al., 2013). IL-10 is generally thought to be anti-inflammatory but has been reported to have proinflammatory functions, depending on the cellular context. In macrophages, IL-10 exerts an anti-inflammatory effect as it inhibits lipopolysaccharide (LPS)-induced NFκB activation and TNF-α production (Dokka et al., 2001). However, following LPS stimulation in humans, IL-10 enhances the production of IFN-γ (Lauw et al., 2000). Another proinflammatory IL is IL-17, which can induce expression of the genes for IL-1, TNFα and defensins (Amatya et al., 2017).

The cells of the innate immune system recognise pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) via pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs). PAMPs are molecules that are common to pathogens, while DAMPs are endogenous molecules released by necrotic cells. These allow the immune cells to make a quick response to infected or damaged tissues (Bianchi, 2007; Elliott et al., 2014). The innate immune system utilises many cell types, each with different functions, including neutrophils, monocytes and dendritic cells (DCs).

1.1.1.1 Neutrophils

Neutrophils are typically involved in acute inflammation and are the first leukocytes recruited to an inflammatory site. They release oxidants, proteases and antimicrobial proteins to remove pathogens and can also stimulate inflammatory monocyte recruitment (Kolaczkowska and Kubes, 2013; Soehnlein et al., 2008). It has been reported that there are different subsets of neutrophils with varying surface antigen expression and macrophage activation potential (Tsuda et al., 2004). Another role of neutrophils is to regulate the adaptive immune response. It has been suggested that a subset of neutrophils can suppress T cell proliferation after LPS stimulation in humans (Pillay et al., 2012)

and that splenic neutrophils can stimulate B cells (Puga et al., 2012). Neutrophils also produce cytokines such as IFN-γ (Yin and Ferguson, 2009).

1.1.1.2 Monocytes

Monocytes are phagocytic cells that circulate in the blood, are recruited to sites of inflammation and function in wound healing and tissue repair. In mice, monocytes are characterised based on expression of the marker Ly6C as either classical/inflammatory ($Ly6C^{hi}$) or alternative/patrolling ($Ly6C^{low}$) monocytes. $Ly6C^{hi}$ monocytes are pro-inflammatory whilst $Ly6C^{low}$ monocytes have roles in tissue repair (Geissmann et al., 2003; Kratofil et al., 2017). Once recruited to an inflamed tissue, monocytes can differentiate into macrophages or DCs, depending on the cytokine environment (Crane et al., 2014; Nakano et al., 2009). Monocyte phenotype can also switch between classical and alternative states, depending on the local cytokine environment (Dal-Secco et al., 2015). During homeostasis, monocytes are involved in replacement of tissue-resident macrophages (Mitchell et al., 2014; Tamoutounour et al., 2013).

1.1.1.3 Macrophages

Macrophages are a heterogenous population of cells with various functions, including phagocytosis of pathogens and cell debris. They can be broadly classed as either inflammatory (M1) or anti-inflammatory (M2) macrophages. Polarisation towards an M1 or M2 phenotype depends on the cytokines the cell is exposed to. IFN-γ tends to induce an M1 phenotype whereas IL-4 will stimulate an M2 phenotype (Murray, 2017). M1 macrophages produce proinflammatory cytokines, proteases and reactive oxygen species (ROS), providing defence against pathogens. M2 macrophages are involved in resolving the immune response, angiogenesis and tissue repair. M1 and M2 macrophages secrete distinct cytokine patterns, orchestrating distinct immune responses (Sica et al., 2015).

Many tissues are host to tissue-resident macrophages. Examples of these are alveolar macrophages in the lung, Kupffer cells in the liver and microglia in the brain. These cells are established during embryonic development and can self-renew independently of monocytes. (Schlitzer and Schultze, 2017; Yona et al., 2013). They differentiate from erythro-myeloid progenitors (EMPs) which

originate in the yolk sac and then colonise the fetal liver, a hematopoietic organ, during embryonic development. Microglia exclusively originate from the yolk sac whilst other resident macrophages have fetal liver origins (Ginhoux et al., 2010; Hoeffel and Ginhoux, 2015; McGrath et al., 2015). Tissue-resident macrophages have been reported to have functions other than immune roles. Cardiac resident macrophages modulate electrical activity of cardiomyocytes and Kupffer cells remove red blood cell (RBC)-derived haemoglobin from the circulation (Hulsmans et al., 2017; Willekens et al., 2005).

1.1.1.4 Dendritic cells

DCs are a family of antigen presenting cells (APCs) that stimulate the adaptive immune response by presenting antigens to T cells and producing cytokines. DCs can maintain T cell homeostasis by regulating T cell reactivity in the absence of foreign antigen (Hochweller et al., 2010) whilst DCs in the thymus also regulate immune tolerance by mediating negative selection of T cells (Hubert et al., 2011). Following LPS stimulation, DCs in the spleen have been shown to produce the cytokines IFN-γ, TNF-α and TGF-β (Zhang et al., 2005). DCs are characterised by expression of the markers CD11c and major histocompatibility complex (MHC)-II and can be further grouped into subsets based on expression of CD4, CD8, CD11b and CD80 (Waisman et al., 2017; Zhang et al., 2005).

1.1.1.5 Natural killer cells

Natural killer (NK) cells are cytotoxic cells that induce lysis of virally infected and transformed cells. This is mediated by the secretion of perforin and granzymes which induce target cell apoptosis or by caspase-dependent apoptosis (Mandal and Viswanathan, 2015). NK cells have been reported to produce multiple cytokines when activated such as IFN-γ, TNF-α, IL-1β, IL-6 and IL-10, stimulating cells of the adaptive response (Fauriat et al., 2010). In mice, NK cells express CD27 which can distinguish two NK cell subsets, $CD27^{hi}$ and $CD27^{low}$. $CD27^{hi}$ NK cells are reported to have greater effector activity compared to $CD27^{low}$ NK cells (Hayakawa and Smyth, 2006). Although part of the innate immune system, NK cells can be primed by DCs to acquire effector functions (Lucas et al., 2007).

1.1.1.6 Mast cells

Mast cells are granular leukocytes found at the interface between the external and internal environments such as the skin and lungs and respond to pathogens via PRRs. Activation of mast cells results in degranulation and the release of AMPs, proteases and cytokines (da Silva et al., 2014; Di Nardo et al., 2003; Supajatura et al., 2002). Mast cells express TLR2 which, when stimulated by the PAMP peptidoglycan, results in release of IL-4 and IL-5 (Supajatura et al., 2002). Mast cells can also be activated by viral RNA binding to TLR3, leading to release of IFN-β (Orinska et al., 2005). Mast cells express Fc receptors which bind to immunoglobulins so can be considered to be part of the adaptive immune response. Mast cells have been widely studied in the context of allergy as IgE activation of FcεR1 induces an allergic response, mediated by the release of histamine, prostaglandins and leukotrienes (da Silva et al., 2014).

1.1.1.7 Eosinophils

Eosinophils are typically thought of as cells that protect against parasitic infections but also express PRRs which allow responses to viruses, bacteria and fungi. They can secrete cytotoxic proteins such as major basic protein (MBP) and eosinophil peroxidase (Rothenberg and Hogan, 2006). They express TLR7 and TLR9 which bind to viral ligands, leading to CXCL8 secretion and superoxide generation (Mansson and Cardell, 2009; Nagase et al., 2003). Eosinophils can function as APCs, presenting antigens to activate T cells (Del Pozo et al., 1992). They store preformed cytokines, including IL-4, IL-10 and IFN-γ, which enables rapid release following activation (Spencer et al., 2008). Eosinophils have been strongly implicated in asthma and allergy. This is partly due to cross-talk between eosinophils and mast cells. Mast cells secrete IL-5, promoting eosinophil activation, and eosinophils can activate mast cells via production of MBP (Rothenberg and Hogan, 2006).

1.1.2 Adaptive immunity

The adaptive immune response is characterised as being specific and provides immunological memory. Upon first exposure to a pathogen, a slow primary response is formed. Any subsequent exposure elicits a quicker and more specific secondary response. The adaptive system involves lymphoid organs and the

response is mediated by lymphocytes (T cells and B cells). Bone marrow and the thymus are primary lymphoid organs, where B cells and T cells mature, respectively. Secondary lymphoid organs coordinate the interaction between APCs and lymphocytes and include the spleen and lymph nodes. T cells and B cells migrate to secondary lymphoid organs where they can be activated and then migrate to other areas and exert effector functions (Boehm and Swann, 2013; Bonilla and Oettgen, 2010). Traditionally it was thought that only lymphocytes could produce a memory response but there is evidence of innate cells, such as monocytes, developing a trained response following an initial infection (Quintin et al., 2012).

1.1.2.1 T cells

T cells express the T cell receptor (TCR) and can express one of the co-stimulatory molecules CD4 or CD8. Activation of T cells requires the interaction of the TCR with a specific antigen complexed with an MHC molecule and the interaction of CD8 or CD4 with the MHC-I or MHC-II molecule, respectively. CD8+ T cells are cytotoxic (Tc) cells which can remove infected or transformed cells. CD4+ T cells are helper (Th) cells which regulate the immune response through the production of cytokines (Castellino and Germain, 2006; Germain, 2002).

CD4+ T cells are further grouped into subsets based on their function and cytokine production. Th_1 cells produce IL-2 and IFN-γ and can stimulate monocytes, NK cells and Tc cells in response to viral, bacterial and parasitic infections (Bonilla and Oettgen, 2010; Caza and Landas, 2015). Th_2 cells facilitate antibody production by B cells and are implicated in hypersensitivity responses. These cells produce IL-4 and IL-5 (Caza and Landas, 2015). Th_{17} cells are proinflammatory, produce IL-17 and are involved in autoimmunity (Langrish et al., 2005) whilst Th_9 cells are induced by TGF-ß, and produce IL-9 in response to helminth infections (Veldhoen et al., 2008). Regulatory T (Treg) cells are also CD4+ and have a role in suppressing the immune response to maintain immune homeostasis (Josefowicz and Rudensky, 2009).

1.1.2.2 B cells

B cells are essential mediators of humoral immunity. Once activated, B cells can differentiate to antibody producing plasma cells or enter the germinal centre (GC) of a secondary lymphoid organ where they develop into memory cells. In the GC, B cells undergo somatic hypermutation and antibody class switching which allows rapid production of high affinity antibodies during a secondary immune response (Bonilla and Oettgen, 2010; Vazquez et al., 2015). Antibodies consist of a light chain and a heavy chain, which undergo gene rearrangements during B cell development, resulting in the ability to recognise an extensive repertoire of antigens. Several different immunoglobulin (Ig) isotypes can be produced. These are IgM, IgG, IgA, IgD and IgE and this enables various functions to be carried out whilst maintaining antigen specificity (Pieper et al., 2013). Naïve B cells express IgM and IgD antibodies and predominantly switch to IgG-expressing B cells once activated (Horns et al., 2016). IgM and IgD are found on the surface of B cells but can also be secreted. IgM is released as a multimer, is associated with the primary immune response and can activate the complement system. IgG class antibodies are the most abundant and are further grouped into the subclasses IgG1, IgG2, IgG3 and IgG4. IgA antibodies are found at high levels in secretions and at mucosal surfaces whilst IgE antibodies are involved in hypersensitivity and parasitic infections (Schroeder and Cavacini, 2013). B cells are important for mediating an adaptive immune response but there is some evidence for a regulatory role of B cells as they can produce IL-10, leading to suppression of the immune response (Mizoguchi et al., 2002).

1.2 Chemokines

Chemokines are cytokines that regulate the migration of leukocytes. They are therefore fundamental for immune and inflammatory processes, but also play a role in many pathologies. Traditionally, chemokines are described as being secreted by infected or damaged tissues. Cells with the appropriate chemokine receptors can then migrate to the tissue and carry out their effector function (Fernandez and Lolis, 2002; Figure 1-1).

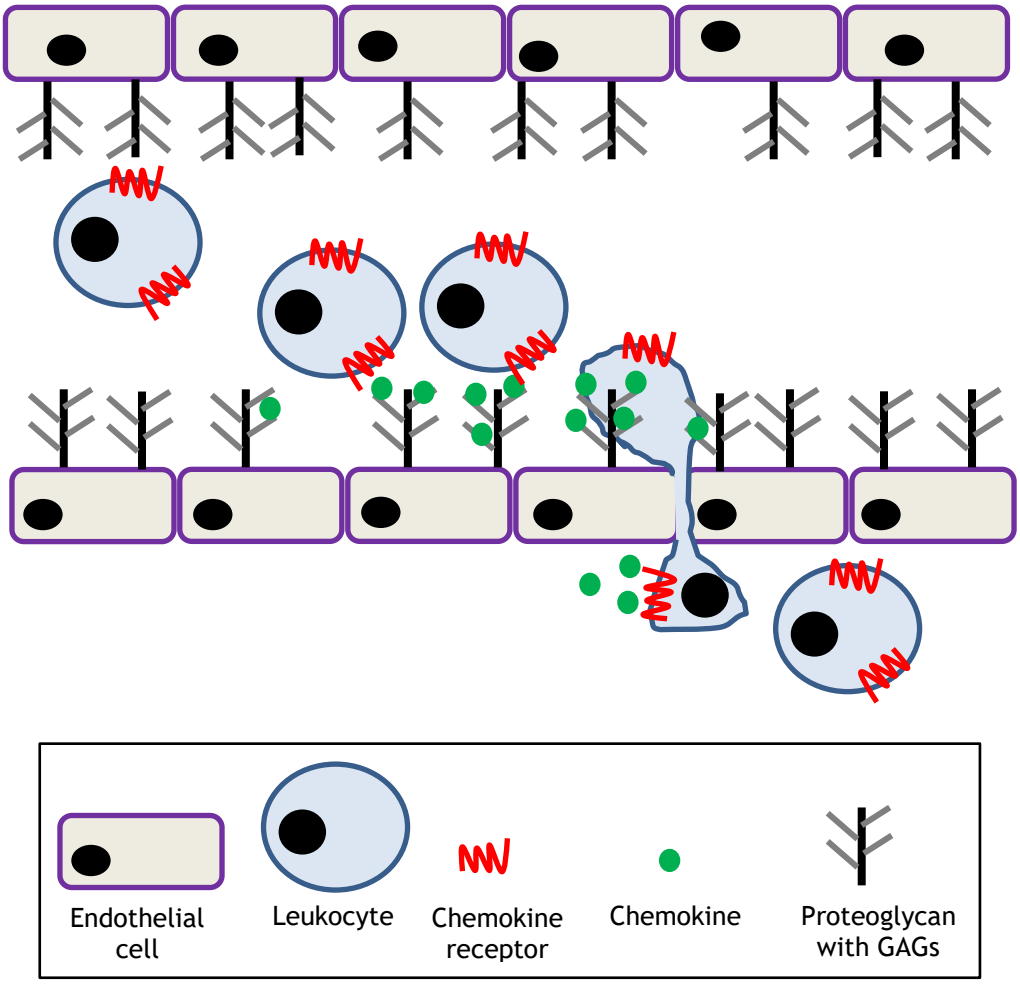


Figure 1-1. Leukocyte migration in response to chemokines
Chemokines are secreted by injured or infected tissue. Leukocytes with appropriate chemokine receptors roll along the endothelium and extravasate into the tissue.

1.2.1 Classification and structure of chemokines

Chemokines are characterised by having positionally conserved cysteine residues. They are divided into four subfamilies based on the position of the first two cysteines. These are CC, CXC, XC and CX3C. Chemokine nomenclature is based on the subfamily a chemokine is part of, for example CC, followed by L (for ligand) and a number (Table 1-1). The majority of chemokines are in the CC or CXC subfamilies whilst humans have only two XC chemokine ligands (XCL1 and XCL2) and one CX3C ligand (CX3CL1) (Turner et al., 2014). CXC chemokines can be further characterised by the presence or absence of an ELR motif. Chemokines are typically 8–12 kDa and generally all chemokines have a similar structure. An example of this is CCL2 which has four strands of ß-sheet and two α-helices (Handel and Domaille, 1996). The N-terminus of a chemokine forms the key signalling domains (Allen et al., 2007).

1.2.2 Homeostatic and inflammatory chemokines

Chemokines can be classed as being homeostatic or inflammatory. Homeostatic chemokines are constitutively expressed and regulate baseline immune function, immune system development and immune surveillance. These chemokines include CCL19 and CCL21, which bind to CCR7, and direct T cells and DCs to secondary lymphoid organs (Förster et al., 2008). CCL25 binds to CCR9 on T cell progenitors and plasmacytoid DCs, directing these cells to the thymus and small intestine, respectively (Forster et al., 2009; Wendland et al., 2007). CCL27 is expressed by keratinocytes and mediates T cell recruitment through CCR10 to the skin (Homey et al., 2002). CXCL12/CXCR4 signalling is required for haematopoiesis and bone marrow recruitment and retention of haemopoietic stem cells (HSCs), as well as development of B cells, monocytes, macrophages and NK cells (Sokol and Luster, 2016). CXCL13 regulates B cell migration and localisation within lymph nodes (Förster et al., 1996).

The majority of chemokines are inflammatory. Their expression by immune and non-immune cells is induced by infection or tissue damage and they have a role in innate and adaptive immune responses (Allen et al., 2007). Inflammatory chemokines include CCL2, CCL3, CCL4, CCL5, CXCL1, CXCL2, CXCL3 and CXCL5. Some chemokines can function in both inflammatory and homeostatic ways. Examples of these are CCL17, CCL20, CCL22, CXCL9, CXCL10 and CXCL11 (Palomino and Marti, 2015).

1.3 Chemokine receptors

Chemokine receptors are G protein-coupled receptors (GPCRs). Nomenclature of these receptors consists of the subfamily of ligands the receptor binds followed by an R and a number (Table 1-1). They are expressed on many different cell types including monocytes, macrophages, T cells and DCs. Chemokines are usually promiscuous, binding to more than one receptor, and each receptor tends to have more than one chemokine ligand. This makes the chemokine system highly complex (Zlotnik et al., 2006). The majority of chemokine receptors are involved in cell migration but a subset of atypical chemokine receptors (ACKRs) do not signal through G proteins and have a role in chemokine scavenging (Bonecchi and Graham, 2016). These are ACKR1, ACKR2, ACKR3 and

ACKR4. ACKR1 has been shown to regulate neutrophil recruitment whilst ACKR2 has a role in regulating lymphangiogenesis (Lee et al., 2014; Zarbock et al., 2007). ACKR3 binds to CXCL11 and CXCL12 and is important in cardiac development as ACKR3 deficiency in mice is lethal due to heart defects (Sierro et al., 2007). ACKR4 binds to the homeostatic chemokines CCL19 and CCL21 and is important for establishing gradients of these chemokines in lymph nodes (Ulvmar et al., 2014).

Chemokine receptor	*Ligands*
CCR Subfamily	
CCR1	CCL3, CCL3L1, CCL5, CCL7, CCL8, CCL13, CCL14, CCL15, CCL16, CCL23
CCR2	CCL2, CCL7, CCL8, CCL13, CCL16, ß-defensin 2, 3
CCR3	CCL3L1, CCL5, CCL7, CCL11, CCL13, CCL14, CCL15, CCL24, CCL26, CCL28
CCR4	CCL17, CCL22
CCR5	CCL3, CCL3L1, CCL4, CCL4L1, CCL5, CCL8, CCL11, CCL16
CCR6	CCL20, ß-defensin 2
CCR7	CCL19, CCL21
CCR8	CCL1
CCR9	CCL25
CCR10	CCL27, CCL28
CXCR Subfamily	
CXCR1	CXCL6, CXCL7, CXCL8, acPGP
CXCR2	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, acPGP, MIF
CXCR3	CXCL4, CXCL9, CXCL10, CXCL11, CXCL14
CXCR4	CXCL12, MIF, ubiquitin
CXCR5	CXCL13
CXCR6	CXCL16
XCR Subfamily	
XCR1	XCL1, XCL2
CX3CR Subfamily	
CX3CR1	CX3CL1
ACKR Subfamily	
ACKR1	CCL2, CCL5, CCL11, CCL13, CCL14, CCL17, CXCL1, CXCL2, CXCL3, CXCL7, CXCL8
ACKR2	CCL2, CCL3, CCL3L1, CCL4, CCL4L1, CCL5, CCL7, CCL8, CCL11, CCL12, CCL13, CCL14, CCL17, CCL22, CCL23, CCL24
ACKR3	CXCL11, CXCL12
ACKR4	CCL19, CCL21, CCL25, CXCL13
ACKR5	CCL19, chemerin

Table 1-1. Chemokine receptors and ligands in humans
The chemokine receptors identified in humans and the ligands that bind to the receptors (Zlotnik and Yoshie, 2012).

1.3.1 Chemokine receptor structure and signalling

GPCRs consist of seven transmembrane helices linked by extracellular and intracellular loops. The extracellular N-terminus of chemokine receptors is involved in ligand binding and the intracellular C-terminus regulates signalling. A key modulator of chemokine signalling is the DRYLAIV motif in the second intracellular loop which is lacking in ACKRs (Nibbs and Graham, 2013; Nomiyama and Yoshie, 2015). GPCRs are coupled to heterotrimeric G proteins (αßγ) and in the inactive state, the α subunit is coupled to GDP. Upon ligand binding, GDP is displaced by GTP, leading to dissociation between the α- and ßγ-subunits which then activate downstream signalling pathways. During resolution of the response, the C-terminal tail is phosphorylated, leading to ß-arrestin recruitment. This prevents interaction of the receptor and the G protein and results in receptor internalisation (Hanlon and Andrew, 2015). Chemokine receptors appear to form homodimers and heterodimers which may influence the downstream signalling pathways and affect chemokine function (Mellado et al., 2001).

1.3.2 Inflammatory chemokine receptors

Humans and mice have a cluster of genes for the inflammatory chemokine receptors CCR1, CCR2, CCR3 and CCR5 (collectively referred to from now on as iCCRs). This cluster is found on chromosome 9 and chromosome 3 in mice and humans, respectively (Figure 1-2). These receptors are important in orchestrating the inflammatory response to various stimuli (Dyer et al., 2019; Shi and Pamer, 2011). They are expressed on multiple cell types and expression pattern changes with different disease states and pathologies. These receptors share multiple ligands which has made understanding the role of these receptors challenging (Figure 1-3).

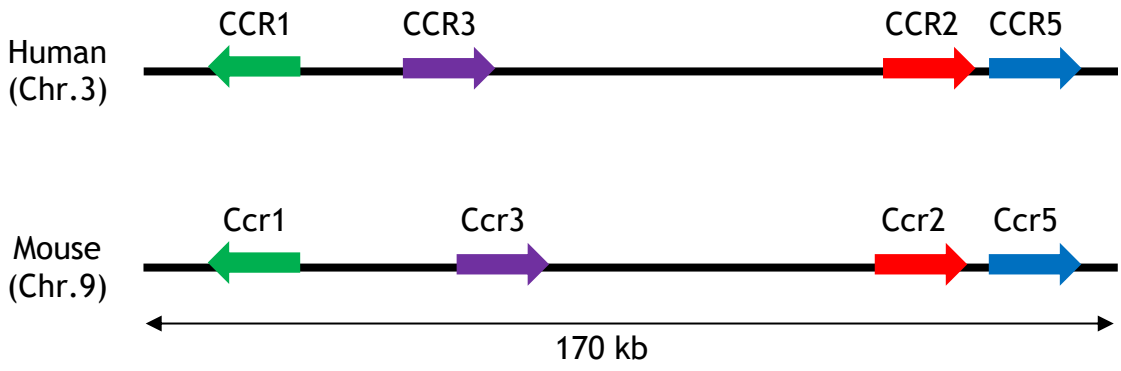


Figure 1-2. Physical map of iCCR gene cluster
Gene position and orientation of CCR1, CCR2, CCR3 and CCR5 on chromosome 3 in humans and chromosome 9 in mice.

1.3.2.1 CCR1

The ligands for CCR1 include CCL3, CCL5, CCL7, CCL8, CCL14, CCL15, CCL16 and CCL23 and CCR1 has been reported to be expressed by several different cell types. These include T cells, B cells, DCs and neurons (Armas-González et al., 2018; Coates et al., 2004; Llorián-Salvador et al., 2016; Mikolajczyk et al., 2016). CCR1 is also expressed by immature monocyte-derived DCs and expression is downregulated as these cells mature (Gouwy et al., 2014). In the pathology of haemolytic uraemic syndrome (HUS), monocytes can express CCR1 and the level of CCR1 correlates with severity of renal dysfunction in HUS patients (Ramos et al., 2015). Basophils can express CCR1 and this increases in patients with systemic lupus erythematosus (SLE) (Pan et al., 2017). The ligands for CCR1, CCL3 and CCL5, increase in the kidney after ischaemia-reperfusion injury (Furuichi et al., 2008).

1.3.2.2 CCR2

CCR2 binds to ligands including CCL2, CCL7 and CCL8 and is expressed by monocytes and macrophages. CCR2 is required for the migration of monocytes from the bone marrow to the blood and then from the blood to inflamed tissues (Dyer et al., 2019; Tsou et al., 2007). CCR2 is also expressed on HSCs and haematopoietic progenitor cells (HPCs) and mediates the migration of these cells to sites of inflammation (Si et al., 2010). NK cells can express CCR2 which correlates with the tumour killing activity of these cells (Hansell et al., 2018).

CCR2 has been demonstrated to have a role in several cancers. For example, CCR2 is expressed in the tumours of patients with renal cell carcinoma and this expression correlates with survival time (Wang et al., 2016). As well as this, CCR2+ macrophages are found in the tumours of lung cancer patients and CCR2 antagonism reduces tumour burden and metastasis in a mouse model of lung cancer (Schmall et al., 2015).

1.3.2.3 CCR3

CCR3 is expressed on eosinophils, mast cells, basophils and some T cells (Brightling et al., 2005b; Danilova et al., 2015; Hausmann et al., 2011). The ligands for CCR3 include CCL11, CCL24, CCL26, CCL5, CCL7, CCL8 and CCL13. This receptor has been implicated in asthma and atopic dermatitis (AD). Airway smooth muscle cells (ASMCs) and airway epithelial cells can express CCR3 and ASMC CCR3 expression is greater in asthmatic patients than healthy controls (Joubert et al., 2005; Stellato et al., 2001). CCR3 expression has also been reported in keratinocytes and dermal fibroblasts and epidermal CCR3 expression is higher in AD lesions than non-lesional areas or healthy controls (Gaspar et al., 2013; Wakugawa et al., 2001). An increase in CCR3 expression has also been reported in synovial cells of rheumatoid arthritis (RA) patients (Liu et al., 2017).

1.3.2.4 CCR5

CCR5 is expressed on monocytes, macrophages, NK cells and T cells and binds to ligands including CCL3, CCL4 and CCL5. Expression of CCR5 increases as monocytes mature into monocyte-derived macrophages (Fox et al., 2015; Lima et al., 2015). CCR5 is most widely studied in the context of HIV infection as it is a coreceptor for viral entry into target cells (Deng et al., 1996) but it has also been implicated in pulmonary arterial hypertension (PAH) and cancer. CCR5 expression increases in the lungs of PAH patients and is found on smooth muscle cells and endothelial cells (ECs) (Amsellem et al., 2014). In skin squamous cell carcinoma, Tregs express CCR5 which directs these cells to the tumour. CCR5 deficiency reduces the number of Tregs in the tumour which results in increased anti-tumour activity of CD8+ T cells (de Oliveira et al., 2017). CCR5 has also been reported to be expressed by osteoclasts and to regulate osteoclast function (Lee et al., 2017).

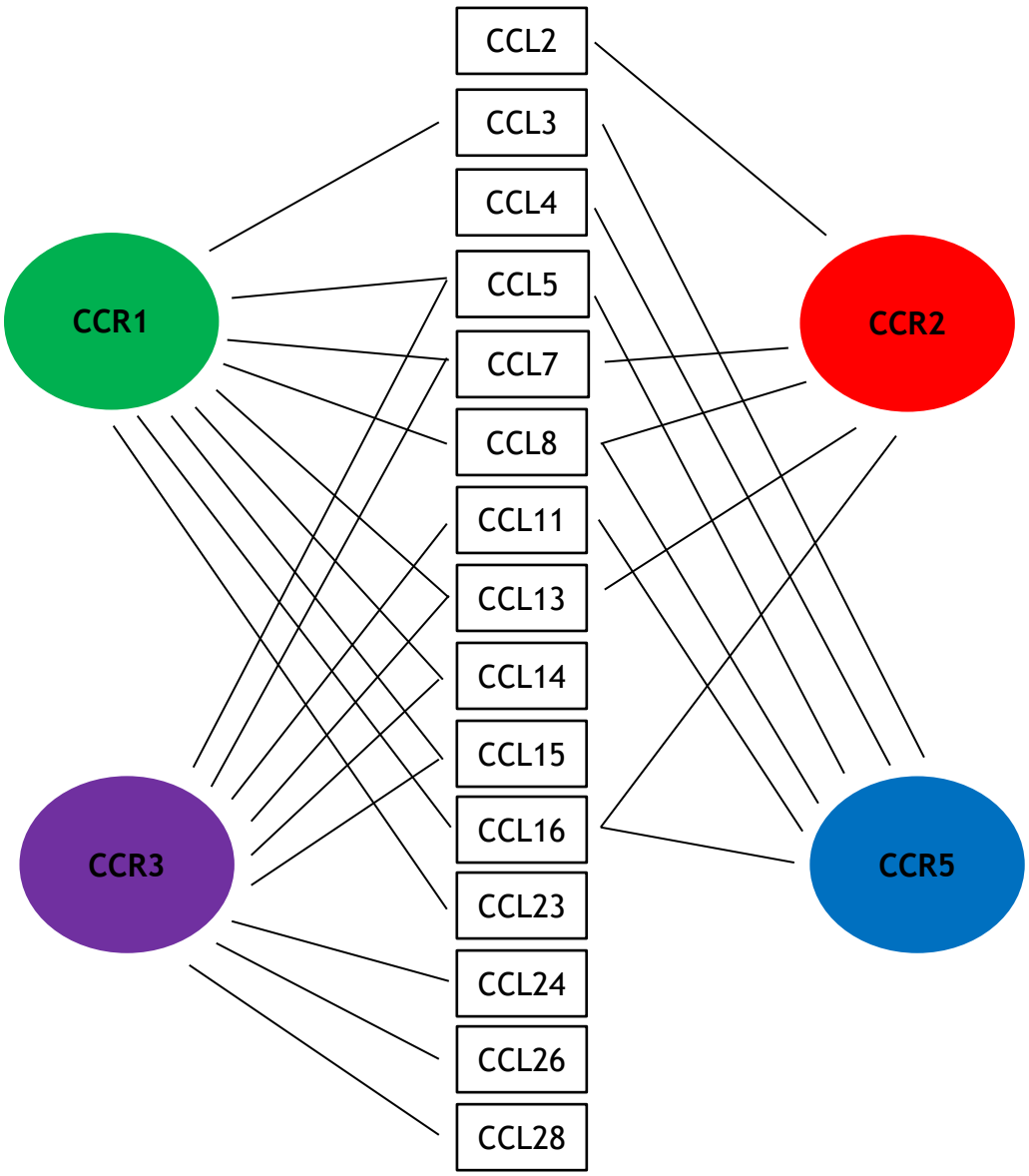


Figure 1-3. iCCR and chemokine interactions
CCR1, CCR2, CCR3 and CCR5 bind to multiple CC chemokines whilst the majority of these chemokines also bind to more than one iCCR, resulting in a highly complex system.

1.4 Evolution of chemokines and chemokine receptors

Chemokine receptors originated at the time vertebrates first evolved. These receptors can be classed into two phylogenetic groups. One group binds mainly homeostatic chemokines and appeared first, in jawless fish, while the other group mainly binds inflammatory chemokines and appeared later in jawed vertebrates (Nomiyama et al., 2011). It is likely that these genes evolved as complex vertebrate systems that increased exposure to pathogens developed, such as blood based oxygen transport, and a more complex immune response

was required. It may also suggest a role for chemokines and cell migration in the development of these systems. The number of receptors and chemokines varies widely between species. For example the zebrafish has 24 receptor genes and 63 chemokine genes whilst chickens have only 14 receptor genes and 23 chemokine genes (DeVries et al., 2006). This could reflect a difference in exposure to certain pathogens. Only mammals have the cluster containing the genes for the iCCRs.

Chemokine genes are mainly found in clusters, for example most human CC chemokine genes are part of two major clusters on chromosome 17 (Naruse et al., 1996). This is likely a result of tandem gene duplication where genes are copied and then independently evolve. Genes that do not appear in a cluster are more conserved between species and tend to have only one receptor. These are often genes for homeostatic chemokines (Zlotnik et al., 2006).

1.5 Non-immune roles of chemokines and receptors

Several chemokines and their receptors have been shown to have a role outside of orchestrating an immune or inflammatory response. For example, the ligand receptor pair CXCL12/CXCR4 is involved in development of the brain. These are both expressed in the embryonic nervous system and mice without functional CXCR4 have impaired hippocampal development (Lu et al., 2002; Tissir et al., 2004). More recently, a role for chemokine receptors in postnatal development has been demonstrated. The atypical chemokine receptor ACKR2 regulates epithelial branching in mammary gland development (Wilson et al., 2017). The receptor CCR8 can be expressed by vascular smooth muscle cells (VSMCs) and mediates chemotaxis of these cells (Haque et al., 2004). Many CXC chemokines are involved in regulating angiogenesis and therefore may be important in embryonic development and wound healing but also some pathologies like tumorigenesis. CXC chemokines with the ELR motif such as CXCL1, CXCL2, CXCL3 and CXCL5 are pro-angiogenic whilst those without, such as CXCL4, CXCL9, CXCL10 and CXCL11, are angiostatic (Dimberg, 2010). A balance of both types of chemokine may be important for appropriate wound healing and angiogenesis in cancer.

1.6 Chemokine receptors in disease

Chemokine receptors have been implicated in many disease states. They mediate the migration of leukocytes to inflammatory sites in various inflammatory diseases such as psoriasis and asthma (Koelink et al., 2012). They also play a role in cancer, which can be defensive or promote the disease, and are involved in the pathogenesis of HIV (Nagarsheth et al., 2017; Wang et al., 2017).

1.6.1 Inflammatory diseases

Inflammatory diseases are characterised by infiltration of immune cells into various tissues which is mediated by chemokine receptors. In asthma, eosinophils and mast cells are activated in the lungs. Mast cell migration to the lungs is regulated by CCR1, CCR3, CXCR1 and CXCR3 whilst eosinophils are regulated by CCR3. CXCR3, CCR3 and CCL11, the ligand for CCR3, are increased in bronchial biopsies from asthma patients (Brightling et al., 2005a; Koelink et al., 2012; Ying et al., 1997). Chronic obstructive pulmonary disease (COPD) is another disease involving lung inflammation. In COPD, CCR2 and CXCR2 mediate monocyte and neutrophil recruitment to the lungs, respectively. CXCR2 is increased in bronchial biopsies of COPD patients (Donnelly and Barnes, 2006; Qiu et al., 2003).

RA is characterised by chronic inflammation of the joints in the hands and feet. CXCR3 has been implicated in RA as blocking CXCR3 can reduce T cell infiltration into the inflamed joints and disease severity of arthritic mice. CCR1 and CCR5 are also expressed in the synovial tissue of RA patients, suggesting accumulation of CCR1+ and CCR5+ immune cells (Haringman et al., 2006; Mohan and Issekutz, 2014). The inflammatory skin disease psoriasis involves T cell infiltration, mediated by CCR4, CCR10 and CXCR3, as well as neutrophil recruitment via CXCR2. CXCR2 and CXCR3 are elevated in psoriatic lesions (Chen et al., 2010; Koelink et al., 2012; Kulke et al., 1998). CXCR2 is also implicated in inflammatory bowel disease (IBD). Antibodies against CXCR2 can inhibit intestinal inflammation in mouse IBD models (Farooq et al., 2009).

1.6.2 Cancer

Chemokines can be expressed by tumour and stromal cells. Chemokine and chemokine receptor signalling can drive anti-tumour immunity but has also been widely reported to promote tumour survival and metastasis. The effect of chemokine receptor signalling depends on the cancer type and the chemokines involved (Nagarsheth et al., 2017). CD8+ T cell infiltration into a tumour is associated with decreased metastasis and increased survival in colon and ovarian cancer. This infiltration can be mediated by CXCR3 and CXCL9 and CXCL10 (Nagarsheth et al., 2017; Pagès et al., 2005; Sato et al., 2005). CCR2 is involved in the recruitment of tumour associated macrophages (TAMs) and CCL2 correlates with the number of TAMs and poor prognosis in some cancers. TAMs promote tumour progression and metastasis and may inhibit T cell activation (Nagarsheth et al., 2017; Wan et al., 2014).

Tumour cells can also express chemokine receptors which can be associated with severity of the cancer. CCR2 is upregulated in breast cancer and interaction with CCL2 promotes cancer cell migration and survival (Fang et al., 2012). CCR9 is expressed by ovarian cancer cells. CCL25-CCR9 signalling can prevent apoptosis of these cells in response to chemotherapy (Johnson et al., 2010). Expression of CXCR1 in pancreatic duct adenocarcinoma and CXCR4 in prostate cancer correlates with metastasis. CXCR4 antibodies in prostate cancer may inhibit tumour growth and vascularisation (Chen et al., 2014; Darash-Yahana et al., 2004).

1.6.3 HIV

The chemokine receptors CCR5 and CXCR4 are co-receptors for the infection of CD4+ T cells by HIV. A 32 base pair deletion of the CCR5 gene (CCR5Δ32) confers resistance to HIV infection (Dean et al., 1996; Deng et al., 1996; Wang et al., 2017). CXCL10 is elevated in HIV infection and is associated with disease progression. It has been suggested that CXCR3+ T cells are recruited to the HIV-infected lymph node via CXCL10 where they are retained, disrupting the peripheral T cell response and aiding infection between T cells (Foley et al., 2014; Jiao et al., 2012). CCR6+ T cells are also susceptible to HIV infection and CCR6 may be a weaker co-receptor for HIV *in vitro* (Islam et al., 2013).

1.6.4 Targeting chemokine receptors

There is a lot of evidence for a role for chemokine receptors in many disease states, making them potential therapeutic targets. GPCRs are the most common pharmacological targets but there has been very limited success in developing drugs that target chemokine receptors. One reason for this is the complexity of the chemokine system as well as a limited understanding of the function of chemokines in distinct receptor and cell contexts (Solari et al., 2015). As well as this, GPCRs with multiple ligands can show signalling bias which needs to be further understood in order to develop better drugs (Liu et al., 2012). There have only been three drugs that target chemokine receptors that have made it to market. Maraviroc, a CCR5 antagonist, is used to treat HIV and the CXCR4 antagonist Plerixafor is used to treat cancer patients as it mobilises stem cells from the bone marrow to the blood (Dorr et al., 2005; Teusink et al., 2016). More recently Mogamulizumab, a monoclonal antibody to CCR4, has been used to treat certain types of cutaneous T cell lymphoma (Duvic et al., 2015).

1.7 Mice used to study iCCRs

To improve understanding of iCCRs, unique mouse strains have been developed. These mice can be used to identify the individual, combinatorial and redundant roles of iCCRs in various contexts which will hopefully aid the development of drugs that target iCCRs.

1.7.1 iCCR-KO mice

iCCR-KO mice are deficient for the gene cluster on chromosome 9 containing the genes for CCR1, CCR2, CCR3 and CCR5. These mice show no overt developmental abnormalities and can be used alongside individual receptor KO mice to determine the roles of the iCCRs (Dyer et al., 2019). These are particularly useful as only single receptor deficient mice have been generated so far, due to the close proximity of the iCCR genes.

1.7.2 iCCR reporter mice

iCCR reporter (REP) mice have been generated using a bacterial artificial chromosome (BAC) in which genes for fluorescent reporter proteins are under

control of the promoters for each of the iCCR genes. Therefore, cells that express a particular iCCR can be detected by the reporter protein, enabling individual and combinatorial iCCR expression to be determined in a temporal and spatial manner.

1.8 The cardiovascular system and cardiovascular diseases

The cardiovascular system consists of the heart, arteries, veins and capillaries and functions to deliver blood to tissues. Blood vessels are comprised of three layers. These are a thin inner intima layer of ECs, a medial layer consisting of VSMCs and an outer adventitia layer made up mostly of fibroblasts (McCurley and Jaffe, 2012). Cardiovascular diseases (CVDs) are a class of diseases that involve the cardiovascular system and are a leading cause of morbidity and mortality (Benjamin et al., 2019). These include atherosclerosis, hypertension, PAH, myocardial infarction (MI), cardiomyopathy and stroke. Atherosclerosis is the narrowing of an artery due to the build up of lipid in the arterial wall and formation of a fibrous plaque. If the plaque ruptures, a blood clot forms which can cause a blockage to an artery. If this occurs in the coronary artery it can result in MI, or a stroke if the blockage occurs in the brain (Bergheanu et al., 2017).

1.9 Hypertension and blood pressure regulation

Hypertension is when blood pressure (BP) is elevated above 140/90 mmHg. This is a risk factor for atherosclerosis, and therefore MI and stroke, as well as aneurysms, cardiomyopathy and kidney disease (Kannel, 1996; Lip et al., 2000; Mennuni et al., 2014; Wong et al., 2007). Most cases of hypertension have an unknown cause. These are classed as essential hypertension and are thought to have multiple genetic and environmental factors contributing to the disease (Tanira and Al Balushi, 2005). There are several systems in place that regulate BP which can be altered in hypertension but also represent targets to lower BP. Despite current treatments, BP remains uncontrolled in about a third of hypertensive patients (Hales et al., 2017), showing a need for new treatments that lower BP or prevent end organ damage.

1.9.1 The renin-angiotensin system

The renin-angiotensin system (RAS) is a key regulator of BP but also has a role in the pathogenesis of CVD (Figure 1-4). The RAS is initiated by the kidneys which release renin into the blood when a reduction in BP is detected. Renin is an enzyme which cleaves circulating angiotensinogen to the decapeptide angiotensin (Ang) I. This is then further cleaved to the octapeptide Ang II by angiotensin converting enzyme (ACE). Ang II causes an increase in BP by various mechanisms and binds to the GPCRs, angiotensin type 1 (AT1) or type 2 (AT2) receptors. In the adrenal glands, Ang II acts on the AT1 receptor which results in the release of aldosterone. Aldosterone is a steroid hormone that binds to the mineralocorticoid receptor (MR). MR activation in the kidney nephrons leads to increased activation of the epithelial sodium channel (ENaC). This results in an increase in sodium retention, followed by retention of water, and an increase in BP (Soundararajan et al., 2010; Sparks et al., 2014). Ang II also acts on the vasculature via the AT1 receptor, regulating vasoconstriction as well as migration and proliferation of VSMCs (Benigni et al., 2010; Lee et al., 2018; Wang et al., 2017). It is thought that the AT2 receptor acts to antagonise the functions of the AT1 receptor, mediating vasodilation (Benigni et al., 2010). Ang II also induces vascular inflammation and is involved in vascular fibrosis, both of which are associated with hypertension induced end organ damage (Carver et al., 2015; Piqueras et al., 2000).

There is a counter-regulatory axis to the RAS, mediated by angiotensin converting enzyme 2 (ACE2). This enzyme cleaves the C-terminal amino acids from Ang I and Ang II to form Ang-(1-9) and Ang-(1-7), respectively. Ang-(1-7) acts on the Mas receptor to inhibit the proliferative and migrative effects mediated by Ang II and can also bind to the AT2 receptor (McKinney et al., 2014). Ang-(1-7) can inhibit the vascular fibrosis induced by Ang II (Carver et al., 2015).

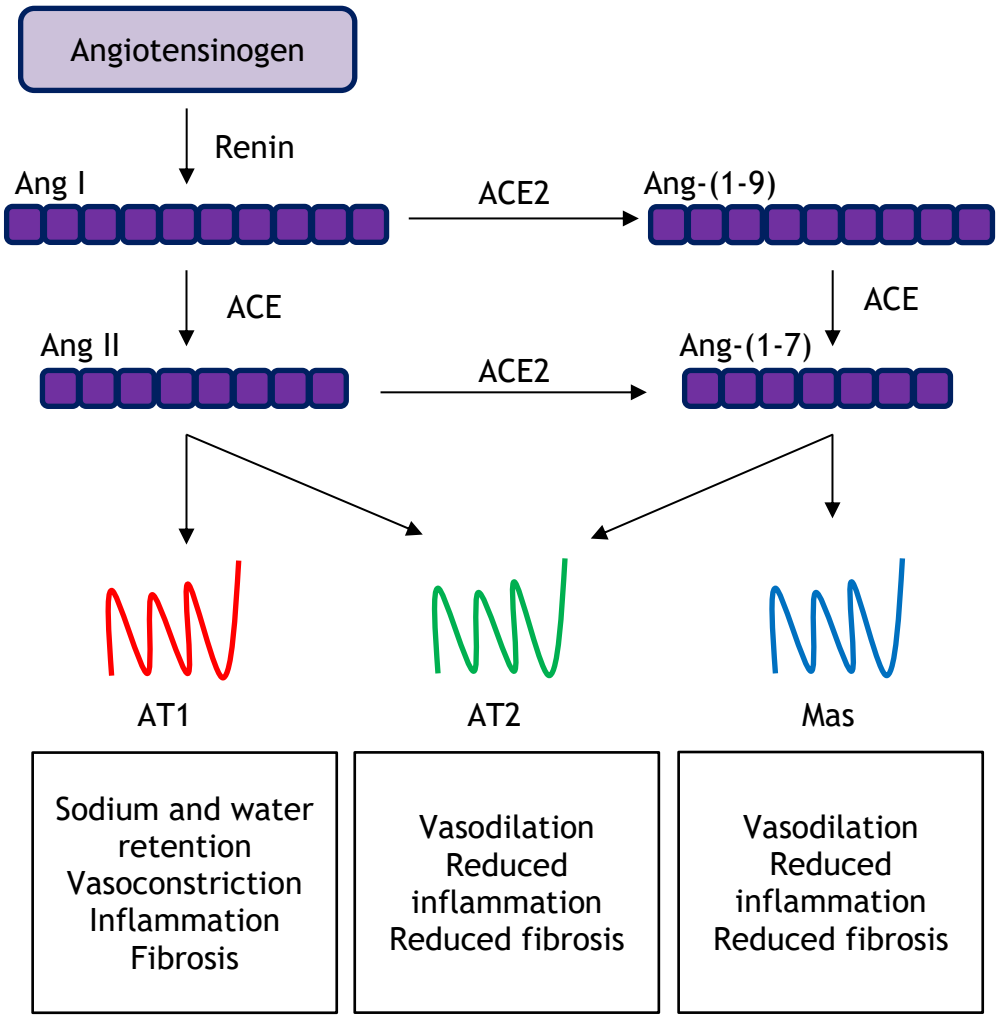


Figure 1-4. The renin angiotensin system
Circulating angiotensinogen is converted to Ang I by renin which is cleaved to Ang II by ACE. Ang II mediates BP increasing effects via the AT1 receptor and BP lowering effects via the AT2 receptor. ACE2 converts Ang I to Ang-(1-9) and Ang II to Ang-(1-7) in the counter regulatory RAS axis in which Ang-(1-7) mediates BP lowering and cardioprotective effects via the Mas and AT2 receptors.

1.9.2 Atrial natriuretic peptide

Atrial natriuretic peptide (ANP) is a 28 amino acid peptide that induces BP lowering effects and is secreted by the heart in response to increased atrial tension. ANP binds to the natriuretic peptide receptors (NPR) NPR-A, NPR-B and NPR-C which are expressed in the kidney, heart, brain and vasculature (Wong et al., 2017). In the kidney, ANP can reduce sodium reabsorption and increase urine production as well as inhibit the release of renin (Kurtz et al., 1986; Stevens et al., 1994; Wong et al., 2017). In the vasculature, ANP stimulates the production of nitric oxide (NO) which induces VSMC relaxation (Elesgaray et al., 2008). ANP

can also regulate the sympathetic nervous system, inducing vasodilation (Wong et al., 2017).

1.9.3 The sympathetic nervous system

Sympathetic neurons innervate the heart, blood vessels, kidneys and adrenal medulla, all of which contribute to BP regulation. The combination of vascular resistance and cardiac output affect BP, as does renal sodium excretion. These can all be regulated by the sympathetic nervous system (Guyenet, 2006). Some cases of hypertension are associated with elevated sympathetic nerve activity (Schlaich et al., 2003) and animal models have been used to demonstrate a role of sympathetic nerve activity in arterial pressure control. In the hypothalamus, the melanocortin receptor types 3 and 4 (MC3/4-R) are involved in BP regulation. Chronic MC3/4-R activation in rats increases arterial pressure whilst blockade of MC3/4-R lowers BP in spontaneously hypertensive rats (SHRs) (Da Silva et al., 2008; Kuo et al., 2003). In addition, both renal denervation and adrenergic blockade reduce arterial pressure in a hyperandrogenemic female rat model (Maranon et al., 2015).

1.9.4 Perivascular adipose tissue

Perivascular adipose tissue (PVAT) surrounds the outer adventitia layer of blood vessels and is comprised of adipocytes and immune cells. PVAT is a regulator of vascular function and BP by releasing paracrine factors that act on the blood vessel. These can be relaxing factors or contractile factors (Ramirez et al., 2017). Relaxing factors include adiponectin, Ang-(1-7), hydrogen peroxide and hydrogen sulphide and can mediate the relaxation response through NO release, potassium channel modulation or cGMP generation (Deng et al., 2010; Gao et al., 2007; Lee et al., 2009; Ramirez et al., 2017; Schleifenbaum et al., 2010). Production of chemerin and superoxide by PVAT have been shown to induce a contractile response, along with certain cytokines such as TNF-α (Gao et al., 2006; Virdis et al., 2015; Watts et al., 2013). PVAT also expresses components of the RAS (Gálvez-Prieto et al., 2008). Inflammation of PVAT can result in dysregulation of vascular function and is associated with obesity, hypertension and atherosclerosis (Ramirez et al., 2017).

1.9.5 Vascular function and oxidative stress

Vascular function involves the role of the endothelium in modulating vascular tone as well as the role of ECs in coagulation, angiogenesis and platelet adhesion. ECs release relaxing factors such as NO and prostaglandins and endothelial dysfunction occurs when endothelium-dependent relaxation is impaired (Frey et al., 2008; Godo and Shimokawa, 2017). This is associated with reduced NO bioavailability or reduced NO production by endothelial NO synthase (eNOS). Oxidative stress contributes to endothelial dysfunction as superoxide, derived from NADPH oxidase, reacts with NO to form peroxynitrite, reducing NO bioavailability (Frey et al., 2008; La Favor et al., 2016; Mordi et al., 2016). Endothelial dysfunction is associated with hypertension, atherosclerosis and obesity (Guzik et al., 2000; La Favor et al., 2016; Panza et al., 1994).

1.9.6 Current treatments of hypertension

There are five classes of antihypertensive agents. These are thiazide diuretics, beta-blockers, ACE inhibitors, Ang II receptor blockers (ARBs) and calcium channel blockers. Thiazide diuretics inhibit kidney sodium reabsorption through inhibition of the electroneutral sodium chloride cotransporter and have also been suggested to induce vasodilation. Adverse effects of thiazide diuretics include hyponatraemia, hypokalaemia and hypovolaemia (Blowey, 2016; Laurent, 2017). Beta-blockers target beta-adrenergic receptors and lower BP by reducing cardiac output and possibly reducing sympathetic activity. Some beta-blockers are non-vasodilating, such as atenolol and metoprolol, whilst others like nebivolol can induce vasodilation. Beta-blockers can induce adverse effects such as hallucinations, insomnia and depression (Laurent, 2017). ACE inhibitors reduce the production of circulating and tissue Ang II, preventing the Ang II mediated mechanisms that increase BP. ACE inhibitors have also been shown to reduce arterial stiffness but have a few reported adverse effects, the main one being cough (Laurent, 2017; Law et al., 2003; Tropeano et al., 2006). ARBs antagonise the AT1 receptor, preventing the effects of Ang II. These drugs have similar efficacy to ACE inhibitors but do not produce adverse effects (Schmidt et al., 2004). Calcium channel blockers are vasodilatory drugs and classed as dihydropyridines (DHPs) and non-DHPs. DHPs block L-type calcium channels, inhibiting depolarisation of VSMCs and cardiomyocytes. Calcium channel blockers

can induce headaches, hypotension and pedal oedema (Eisenberg et al., 2004; Laurent, 2017). Often these treatments are combined and it has been shown that combining low dose drug treatments can increase efficacy and reduce adverse effects (Law et al., 2003).

1.9.7 Models of hypertension

Animal models of diseases enable the study of disease development as well as screening of therapeutics. Several models have been used to study hypertension. and these can be genetic models or based on endocrine or diet factors. The SHR is an inbred rat strain that has multiple genetic pathways contributing to the disease. Details of these genes are still to be elucidated, similar to human essential hypertension (Doris, 2017). SHRs develop hypertension at the age of 4-6 weeks and this can be influenced by environmental factors. SHRs have been shown to develop renal inflammation and oxidative stress, suggesting they can be used to study end organ damage, as well as hypertension development (Dornas and Silva, 2011; Durante et al., 2010). Dahl salt-sensitive rats are another genetic model of hypertension that develop the disease with a high salt diet. These rats have reduced urinary sodium excretion and increased urinary potassium excretion (Jo et al., 2017). They are also prone to glomerulosclerosis, possibly due to podocyte injury (Nagase et al., 2006). The deoxycorticosterone acetate (DOCA)-salt rodent model of hypertension involves the administration of DOCA and a high salt diet and is sometimes combined with a uninephrectomy. DOCA increases sodium reabsorption in the kidney, leading to hypervolemia, whilst the high salt diet increases the onset of hypertension (Basting and Lazartigues, 2017). Nω-nitro-L-arginine methyl ester (L-NAME) is an eNOS inhibitor, administered to rodents to induce hypertension. This results in increased vasoconstriction and the development of glomerulosclerosis and cardiac hypertrophy (Dornas and Silva, 2011; Francois et al., 2008). Administration of Ang II is another rodent model of hypertension, resulting in increased BP, endothelial dysfunction and vascular oxidative stress (Mikolajczyk et al., 2016). Other hypertensive models include induction by environmental stress or neurogenic hypertension, such as denervation of sinoaortic baroreceptors (Dornas and Silva, 2011).

1.10 The immune system in hypertension

There is a lot of evidence for a role of the immune system in hypertension and end organ damage. Leukocytes have been shown to infiltrate the kidneys and vasculature of hypertensive patients and animal models (Moore et al., 2015; Ozawa et al., 2006; Youn et al., 2013). Mice and rats lacking recombination activating gene 1 (Rag-1) have reduced T and B cell numbers and these have an attenuated hypertensive response to Ang II, DOCA or a high salt diet (Guzik et al., 2007; Mattson et al., 2013). Immunosuppressive treatments have been shown to reduce BP in animal models of hypertension. For example, mycophenolate mofetil (MMF) reduces BP, renal lymphocyte infiltration and renal oxidative stress in SHRs (Rodríguez-Iturbe et al., 2015). Immunosuppression with dexamethasone does not reduce BP but reduces renal macrophage and T cell infiltration (Muller et al., 2011). Targeting the immune system represents a possible strategy to treat hypertension. Patients with psoriasis or RA were treated with MMF and this was associated with reduced systolic and diastolic BP (Herrera et al., 2006). However, highly active antiretroviral therapy for HIV patients was associated with increased prevalence of hypertension (Seaberg et al., 2005). An improved understanding of the role of the immune system in hypertension is required to develop hypertensive treatments that target the immune system.

1.10.1 Monocytes and macrophages

Monocytes and macrophages have been reported to accumulate in the aorta, mesenteric arteries, PVAT, kidney and heart in various models of hypertension and are likely to contribute to end organ damage (Mikolajczyk et al., 2016; Moore et al., 2015; Ozawa et al., 2006; Thang et al., 2015; Wang et al., 2017). Depleting macrophages with clodronate liposomes in mice with DOCA-salt induced hypertension results in lower BP and reduced superoxide in mesenteric arteries compared to control DOCA-salt mice (Thang et al., 2015). A similar effect is seen in mice with Ang II induced hypertension and depletion of LysM+ monocytes. LysM+ monocyte depletion also attenuates endothelial dysfunction which is restored, along with an increase in BP, when mice are reconstituted with monocytes (Wenzel et al., 2011). Op/Op mice, which have reduced macrophage numbers, also respond to Ang II with a lower BP and reduced

endothelial dysfunction, superoxide and NADPH oxidase activation compared to WT mice. These mice also do not show accumulation of macrophages in the mesenteric arteries after Ang II treatment (De Ciuceis et al., 2005). Together these studies suggest that macrophages accumulate in the vasculature and modulate vascular function. This could be through NADPH oxidase activity and superoxide production. Macrophages from DOCA-salt treated mice express higher levels of the NADPH oxidase subunit $p22^{phox}$ than control normotensive mice (Thang et al., 2015). It has also been suggested that an increase in $Ly6C^{hi}$ monocytes in the aorta following Ang II treatment impairs eNOS function (Kossmann et al., 2014). Contrary to these studies, clodronate liposome depletion of macrophages in rats on a high salt diet increased BP compared to control rats (Machnik et al., 2009). This could be due to differences between salt and Ang II induced hypertension.

Macrophages that accumulate in the heart are thought to regulate cardiac remodelling. Rats given a splenectomy prior to Ang II treatment had fewer macrophages in the heart than control rats and this was associated with reduced cardiac fibrosis. Rats that had undergone a splenectomy also responded to Ang II with an increase in BP, but this was not as high as the rats that did not have a splenectomy (Wang et al., 2017). As macrophages accumulate in the kidney in hypertension, it is possible they mediate kidney fibrosis and collagen deposition observed in hypertensive mice (Xiao et al., 2015).

1.10.2 Dendritic Cells

DCs have been implicated in hypertension as they activate T cells. The interaction of T cell CD28 and B7 ligands on DCs is required for activation. Blocking this interaction with CTLA4-Ig attenuates both Ang II and DOCA-salt induced hypertension (Vinh et al., 2010). This effect is also seen in $B7^{-/-}$ mice treated with Ang II whilst reconstituting $B7^{-/-}$ mice with WT bone marrow restores the response to Ang II (Vinh et al., 2010). Ang II increases the production of superoxide from DCs as well as the production of IL-6, IL-1ß and IL-23 from splenic DCs (Kirabo et al., 2014). DCs accumulate isoketal protein adducts, a product of oxidative stress, in Ang II and DOCA-salt induced hypertension. It has been suggested that these DCs regulate hypertension, as treatment with the isoketal scavenger 2-HOBA prevents isoketal protein adduct

accumulation and reduces BP (Kirabo et al., 2014). DCs from hypertensive mice can support the proliferation and survival of T cells but DCs from normotensive mice do not. In addition, transfer of DCs from hypertensive, but not normotensive mice, to WT mice induces susceptibility to a low dose of Ang II that would not normally elicit a hypertensive response. This is not seen when the DCs are transferred to Rag-$1^{-/-}$ mice, suggesting that T cells are required for DC priming of hypertension (Kirabo et al., 2014). Expression of CD80 and CD86 by DCs increases with Ang II induced hypertension, showing activation of DCs. This is prevented, along with a blunted hypertensive response and reduced DC isoketal protein adduct accumulation, in mice subject to renal denervation (Xiao et al., 2015). Recently, a study has indicated that DCs could have a more essential role in hypertension as ablation of CD11c+ APCs, which includes DCs, prevents an increase in BP and cardiac hypertrophy following Ang II and salt treatment. Adoptive transfer of CD11c+ APCs to these mice restores the hypertensive response and it has been suggested that these effects are mediated through altered sodium excretion (Hevia et al., 2018). DCs have also been shown to increase in the PVAT of Ang II treated mice (Mikolajczyk et al., 2016).

1.10.3 NK cells

NK cells accumulate in the aorta and mesenteric arteries of hypertensive mice (Kossmann et al., 2013; Taherzadeh et al., 2010). Depletion of NK cells in Ang II treated mice can attenuate the Ang II induced vascular dysfunction (Kossmann et al., 2013). C57BL/6 mice are more susceptible to L-NAME induced hypertension than BALB/c mice and NK cells only accumulate in the arteries of C57BL/6 mice after L-NAME treatment, not BALB/c mice. This could be mediated by the NK gene complex as BALB/c mice expressing the C57BL/6 NK gene complex show a similar hypertensive response to L-NAME as C57BL/6 mice (Taherzadeh et al., 2010).

1.10.4 Neutrophils

Depletion of neutrophils attenuates the increase in BP that occurs in the reduced utero-placental perfusion pressure and Sabra hypertension prone rat models of hypertension (Mazor et al., 2007; Regal et al., 2015). The number of neutrophils has also been shown to increase in the aortas of Ang II treated mice (Wenzel et

al., 2011). Neutrophils may contribute to hypertension through superoxide production. Neutrophils from pregnant women with hypertension produce more superoxide than neutrophils from healthy pregnant women (Tsukimori et al., 2007). In addition, neutrophils from SHRs produce more superoxide than Wistar Kyoto rat (WKY) controls. However, this is not seen in the L-NAME or DOCA-salt models of hypertension (Ohmori et al., 2000). An elevated blood neutrophil to lymphocyte ratio correlates with risk of hypertension (Liu et al., 2015).

1.10.5 T cells

An increase in the number of circulating CD4+ and CD8+ T cells has been reported in hypertensive patients compared to normotensive controls and these T cells produce more IL-17A, IFNγ and TNFα (Itani et al., 2016). T cells also accumulate in the kidneys and aortas of hypertensive mice (Guzik et al., 2007; Itani et al., 2016; Krebs et al., 2014). Rag-$1^{-/-}$ mice, which have a blunted hypertensive response to Ang II, undergo an increase in BP, vascular oxidative stress and endothelial dysfunction after the adoptive transfer of T cells. This suggests that T cells are required for Ang II induced hypertension (Guzik et al., 2007). Further studies showed that adoptive transfer of CD8+ T cells, but not CD4+ T cells, to Rag-$1^{-/-}$ mice restores the blunted response to Ang II and that $CD8^{-/-}$ mice also have an attenuated response to Ang II (Trott et al., 2014). $CD247^{-/-}$ rats have a reduced number of T cells. When given a high salt diet, BP of $CD247^{-/-}$ rats is lower than $CD247^{+/+}$ rats and they also have reduced renal damage (Rudemiller et al., 2014).

Th17 cells may augment hypertension as circulating Th17 cells increase following Ang II treatment, along with kidney Th17 cell infiltration and production of IL-17 by T cells. IL-$17^{-/-}$ mice also have lower BP and improved vascular function compared to WT mice when treated with Ang II. However, IL-$17^{-/-}$ mice have worse renal damage when hypertension is induced by DOCA and Ang II (Krebs et al., 2014; Madhur et al., 2010). Treg cells could be protective in hypertension. Adoptive transfer of Tregs to Ang II treated WT mice prevents an increase in systolic BP, endothelial dysfunction and aortic oxidative stress whilst adoptive transfer of Treg-deficient T cells to hypertensive Rag-$1^{-/-}$ mice exaggerates endothelial dysfunction and oxidative stress (Barhoumi et al., 2011; Mian et al., 2015).

1.10.6 B cells

Fewer studies have focused on the role of B cells in hypertension but some recent studies have started to reveal how B cells may contribute to the disease. Hypertensive patients and animal models have elevated circulating IgG antibodies (Chan et al., 2015, 2014) and when perfusion pressure in the kidney is increased, B cells have been shown to accumulate in the kidney (Evans et al., 2017). Mice with depleted B cells, through B cell-activating factor receptor (BAFF-R) deficiency or treatment with anti-CD20 antibody, have an attenuated response to Ang II whilst adoptive transfer of B cells to BAFF-$R^{-/-}$ mice restores the response to Ang II. BAFF-$R^{-/-}$ mice also do not show an increase in serum IgG or aortic macrophage accumulation after Ang II treatment (Chan et al., 2015). Recently c-$myb^{h/h}$ mice, which have reduced circulating and kidney B cells, have been shown to have reduced baseline BP and a reduced response to DOCA-salt induced hypertension. This could possibly be due to increased sodium excretion (Dingwell et al., 2019). SLE is an autoimmune disease associated with an increase in the prevalence of hypertension. The proteasome inhibitor Bortezomib reduces bone marrow plasma cells and plasma IgG in mice with SLE as well as reducing mean arterial pressure and renal injury, suggesting IgG antibodies may be involved in hypertension in SLE (Taylor et al., 2018). Several targets of antibodies in hypertension have been suggested. These are AT1 receptor, alpha1-adrenergic receptor and beta1-adrenergic receptor, L-type voltage gated calcium channels and HSP-70 (Chan et al., 2014).

1.10.7 Immune cell summary

Many different immune cells have been implicated in hypertension. Depletion of monocytes, macrophages, T cells or B cells have been shown to be protective in models of hypertension (Chan et al., 2015; Guzik et al., 2007; Thang et al., 2015; Wenzel et al., 2011). DCs also contribute to the disease, possibly through activation of T cells, whilst it has been suggested that NK cells regulate vascular dysfunction and neutrophils contribute to oxidative stress in hypertension (Kirabo et al., 2014; Kossmann et al., 2013; Tsukimori et al., 2007).

1.10.8 Cytokines

Various cytokines have been implicated in hypertension. T cells from Ang II treated mice have increased production of TNF-α and IFN-γ whilst production of IL-2, IL-4 and IL-5 does not change after Ang II treatment (Guzik et al., 2007). Treatment of hypertensive patients with the AT1 receptor blocker Losartan reduces production of IFN-γ by peripheral blood lymphocytes (Weidanz et al., 2005). In a mouse model of Ang II induced hypertension, TNF-α deficiency attenuates the hypertensive response to Ang II as well as cardiac hypertrophy and renal injury. This is possibly due to increased eNOS expression and NO bioavailability in the kidneys of TNF-$\alpha^{-/-}$ mice. By contrast, IFN-γ deficiency does not affect BP or renal injury in this model (Zhang et al., 2014). However, IFN-$\gamma^{-/-}$ mice are protected from Ang II induced vascular dysfunction (Kossmann et al., 2013). Mice lacking the IFN-γ receptor respond to Ang II with an increase in BP similar to WT mice but have reduced cardiac hypertrophy and renal injury (Markó et al., 2012). Treatment of Ang II infused mice with anti-IFN-γ antibody does not affect the response to Ang II but treatment with Etanercept, a soluble TNF-α receptor, reduces vascular superoxide production (Guzik et al., 2007).

Ang II increases the production of IL-17 by circulating T cells and IL-$17^{-/-}$ mice have a lower BP than WT mice after 4 weeks of Ang II infusion. IL-$17^{-/-}$ mice are also protected from Ang II induced vascular dysfunction and superoxide generation as well as aortic T cell infiltration (Madhur et al., 2010). However IL-$17^{-/-}$ mice show increased renal injury and impaired renal function compared to WT mice when treated with Ang II and DOCA. When this model is used for 14 days, IL-$17^{-/-}$ mice also have a similar BP to WT mice (Krebs et al., 2014). This suggests that IL-17 has different roles in different tissues and this is affected by the model of hypertension used. IL-23 is important for expansion of Th17 cells. Mice deficient for IL-23p19 also show increased renal injury compared to WT mice after Ang II and DOCA treatment (Krebs et al., 2014). Anti-IL-23 receptor or anti-IL-17A antibodies have no effect on BP in Ang II treated mice (Markó et al., 2012).

The proinflammatory cytokine IL-6 may regulate BP in hypertension. Anti-IL-6 antibody attenuates the BP elevation induced by high salt diet in Dahl salt

sensitive rats and IL-$6^{-/-}$ mice do not respond to Ang II with increased BP (Brands et al., 2010; Hashmat et al., 2016).

Overall, deficiency of the cytokines TNF-α, IFN-γ, IL-17 or IL-6 may protect from an elevation in BP, inflammation or vascular dysfunction and oxidative stress in hypertension (Brands et al., 2010; Kossmann et al., 2013; Madhur et al., 2010; Zhang et al., 2014). This demonstrates an important role for the immune system in the disease. However, the roles of these cytokines may vary between models and tissues.

1.11 Chemokines and chemokine receptors in hypertension

As important regulators of immune cells, it is likely that chemokines contribute to the pathogenesis of hypertension. Several chemokines and chemokine receptors have been reported to be upregulated in the vasculature and kidneys of hypertensive animal models and it has been suggested that this can regulate BP and end organ damage (Mikolajczyk et al., 2016; Moore et al., 2015; Xia et al., 2013). Most research has focused on CC or CXC chemokines and receptors whilst no studies on XC chemokines in the context of hypertension have been carried out to date.

1.11.1 CC chemokines and receptors

The role of CCR2 and its ligand CCL2 has been widely studied in hypertension. Circulating CCL2 is elevated in recently diagnosed, untreated, hypertensive patients (Antonelli et al., 2012; Madej et al., 2005) and CCR2 expression increases in the aorta of both Ang II treated mice and DOCA/salt treated mice (Chan et al., 2012; Moore et al., 2015). CCR2 expression by circulating monocytes is also increased in SHRs, Ang II treated mice and L-NAME treated rats (Ishibashi et al., 2004). CCR2 deficiency does not have an effect on BP in Ang II induced hypertension (Ishibashi et al., 2004) although treatment with a CCR2 inhibitor can affect BP. Mice given the CCR2 antagonist INCB3344 after Ang II or DOCA-salt treatment showed a reduction in BP and aortic macrophage accumulation (Chan et al., 2012; Moore et al., 2015). However, the CCR2 antagonist RS102895 did not affect the BP of rats treated with Ang II and a high

salt diet and resulted in a higher BP than controls in a mouse model of renovascular hypertension. RS102895 can however reduce renal fibrosis and macrophage accumulation in hypertension (Elmarakby et al., 2007; Kashyap et al., 2015). Despite having no effect on BP, CCR2 deficiency is protective against Ang II induced vascular remodelling (Ishibashi et al., 2004). In renovascular hypertension, CCL2 deficiency has no effect on BP but CCL2 deficiency does prevent an increase in BP in response to DOCA-salt. $CCL2^{-/-}$ mice may also be protected from renal injury and cardiac inflammation in hypertension (Kashyap et al., 2018; Shen et al., 2014).

CCR5 and its ligands may have a role in hypertension. The CCR5Δ32 mutation has been associated with increased prevalence of hypertension but some studies have also suggested that CCR5Δ32 is not associated with the disease (Mettimano et al., 2006; Zhang et al., 2006). Renal, aortic and cardiac expression of CCR5 can increase in hypertension as well as renal expression of CCL3 and CCL5 (Chan et al., 2012; Krebs et al., 2012). CCR5 deficiency has no effect on BP elevation, renal function, injury and inflammation or cardiac fibrosis in a DOCA and Ang II model of hypertension (Krebs et al., 2012). The role of CCL5 in hypertension may vary between tissues. CCL5 has been suggested to have a negative effect in the vasculature as $CCL5^{-/-}$ mice are protected from Ang II induced vascular dysfunction and superoxide production. Similar effects are seen when the CCL5 inhibitor met-RANTES is given to Ang II treated mice (Mikolajczyk et al., 2016). In contrast, CCL5 may be protective in the kidney as $CCL5^{-/-}$ mice show worse renal injury and fibrosis compared to WT mice when treated with Ang II (Rudemiller et al., 2016). CCL5 deficiency does not affect BP in hypertension (Mikolajczyk et al., 2016; Rudemiller et al., 2016). VSMCs can express CCL5. VSMCs from SHRs have reduced CCL5 expression compared to WKY VSMCs and Ang II reduces the expression of CCL5 by SHR VSMCs (Yun et al., 2011). SHRs treated with CCL5 also show reduced BP (Kim et al., 2015).

The percentage of T cells expressing CCR1, CCR3 and CCR5 in the PVAT increases in Ang II induced hypertension (Mikolajczyk et al., 2016). An increase of CCL7, CCL8 and CCL12 is also observed in the aortas of DOCA-salt treated mice (Chan et al., 2012). Studies of hypertension using $CCR1^{-/-}$ and $CCR3^{-/-}$ animals or CCR1 and CCR3 inhibitors have not been carried out.

1.11.2 CXC chemokines and receptors

Aortic and cardiac expression of CXCR2 increases in hypertensive models (Chan et al., 2012; Wang et al., 2016; Zhang et al., 2019). When treated with Ang II or DOCA-salt, $CXCR2^{-/-}$ mice have a blunted BP response compared to WT mice and are protected from vascular dysfunction and superoxide production. They are also protected from aortic wall thickening, fibrosis and macrophage and T cell infiltration. The same effect is seen when hypertensive mice are treated with the CXCR2 inhibitor SB265610. This protective effect is likely a result of disrupting CXCR2 signalling in myeloid cells (Wang et al., 2016). Reparixin, an inhibitor of CXCR2 activation, can reduce blood pressure and aortic wall thickness of SHRs. Reparixin can also increase the production of NO by VSMCs (Kim et al., 2011). Further, the CXCR2 inhibitor SB225002 reduces BP and attenuates cardiac remodelling, oxidative stress and inflammation in SHRs (Zhang et al., 2019). CXCL8 is a ligand for CXCR2 and expression of CXCL8 is higher in the aortas of SHRs than WKY controls. CXCL8 expression by VSMCs can be induced by Ang II (Kim et al., 2008).

CXCR6 and its ligand CXCL16 may regulate hypertensive end organ damage. T cells and macrophages that accumulate in the kidneys of Ang II treated mice can express CXCR6. When treated with Ang II, CXCR6-GFP mice have a similar increase in BP to WT mice but are protected from kidney injury and fibrosis (Xia et al., 2014). CXCL16 expression is induced in the kidneys of Ang II and DOCA-salt treated mice. CXCL16 deficiency does not protect from elevated BP in these models but does protect from renal injury, fibrosis and T cell and macrophage infiltration (Liang et al., 2016; Xia et al., 2013).

The ligands for CXCR3, CXCL9, CXCL10 and CXCL11, have been reported to be elevated in the circulation of hypertensive patients (Antonelli et al., 2012; Youn et al., 2013). $CXCR3^{-/-}$ mice have a higher BP than WT mice at baseline and after a high salt diet (Hsu et al., 2009). CXCR7 has been implicated in endothelial progenitor cell mediated repair of the endothelium in hypertensive patients (Zhang et al., 2014).

1.11.3 CX3CL1 and CX3CR1

Few studies have focused on CX3CL1 and CX3CR1 in hypertension but both have been shown to be upregulated in the kidneys during DOCA induced hypertension. BP increases in CX3CR1$^{-/-}$ mice when treated with DOCA to the same extent as WT mice but CX3CR1 deficiency attenuates renal fibrosis and macrophage infiltration (Shimizu et al., 2011). CX3CL1 expression has been reported to be higher in the mesenteric arteries of female SHRs than male SHRs, although it is unlikely that this affects BP (Sullivan et al., 2009).

1.11.4 Chemokines and receptors summary

Several chemokines and chemokine receptors have been implicated in hypertension. CCR2, CCR5 and CXCR2 expression is upregulated in the vasculature of hypertensive animal models whilst renal expression of CCR5 and CX3CR1 also increases in hypertension. Deficiency of these receptors and their ligands may protect against BP elevation, inflammation or end organ damage in hypertension but this could be model and tissue dependent (Krebs et al., 2012; Mikolajczyk et al., 2016; Moore et al., 2015; Shen et al., 2014; Shimizu et al., 2011; Wang et al., 2016).

1.12 Aims

Chemokine receptors have a role in the pathogenesis of hypertension and consequent end organ damage but the exact role they play is unclear. Disrupting chemokine-chemokine receptor interactions may reduce BP and protect from end organ damage, as is seen in some animal studies (Mikolajczyk et al., 2016; Moore et al., 2015). The complexity of the chemokine system has limited our understanding of how these receptors contribute to the disease. As chemokine receptors have multiple ligands and share ligands with other receptors (Figure 1-3), knocking out or blocking a receptor may not reveal the whole role of the receptor as it is compensated for by other receptors and chemokines. It is hypothesised that the iCCRs are important in regulating leukocyte migration in hypertension and that targeting these receptors will protect from elevated BP and end organ damage in the disease. The aim of this study was to investigate

this, increasing our understanding of how iCCRs contribute to hypertension and BP regulation. This was broken down into three aims.

1. Characterisation of iCCR expression in Ang II induced hypertension.

2. Investigation of how deficiency of all iCCRs affects Ang II induced hypertension.

3. Investigation of how iCCR deficiency affects immune cells and molecules that are involved in BP regulation in the kidney.

The results described here have added to our understanding of the role of iCCRs in hypertension. In doing this, potential chemokine receptors have been identified which can be targeted to reduce BP and hypertensive end organ damage.

Chapter 2

Materials and methods

2 Materials and methods

2.1 Mice

All genetically modified mice were bred at the biological services unit at the Beatson Institute for Cancer Research and then housed at the cardiovascular research unit or central research facility at the University of Glasgow. Mice were allowed to acclimatise for at least 7 days after arrival before carrying out any procedures. Once on procedure, mice were monitored for weight loss as a way to monitor their health. At the end of experiments, mice were euthanased by carbon dioxide asphyxiation or cervical dislocation. All experiments were approved and performed under the project licence of Professor Gerard Graham (70/8377) or Dr Delyth Graham (70/9021).

2.1.1 C57BL/6 mice

8-11 wk old male C57BL/6 WT mice were purchased from Charles River or bred in house.

2.1.2 iCCR-reporter mice

REP mice expressing fluorescent proteins under the control of iCCR gene promoters were generated on a C57BL/6 background by Dr Laura Medina-Ruiz. The reporter proteins used were Clover (CCR1), mRuby2 (CCR2), mTagBFP2 (CCR3) and iRFP682 (CCR5).

2.1.3 iCCR-KO mice

The locus on mouse chromosome 9 containing the iCCR genes was deleted to produce iCCR deficient mice on a C57BL/6 background. These mice have no overt developmental abnormalities but have impaired inflammatory leukocyte recruitment (Dyer et al., 2019). WT littermates were used as controls.

2.1.4 Individual receptor-KO mice

CCR1 deficient, CCR2 deficient and CCR5 deficient mice were maintained on a C57BL/6 background and CCR3 deficient mice were maintained on a BALB/c background. The appropriate WT littermates were used as controls.

2.2 Ang II induced hypertension

REP, WT and iCCR-KO mice were subjected to Ang II induced hypertension. 12-15 wk old mice were given Ang II (Sigma-Aldrich) at a dose of 490 ng/min/kg in Ang II buffer (150 mM NaCl, 1 % acetic acid in distilled water) for 7 or 14 days via subcutaneous osmotic minipump (ALZET). Control mice underwent sham surgery. Mice were anaesthetised with 3-5 % isoflurane and oxygen at a flow rate of 2 L/min. An area of skin on the back was shaved and wiped with povidone-iodine (Betadine). A small incision was made at the top of the back, below the neck, and forceps were used to make a pocket under the skin. The osmotic minipump was inserted into the pocket and the incision was closed with 2-3 sutures. Sham surgery involved creating the pocket but no minipump was implanted. All surgery was performed with aseptic technique and mice were given a subcutaneous injection of 100 µl of 1 mg/ml carprofen (Rimadyl) as analgesia before the incision was made. Before surgery, mice were housed with their littermates. After surgery, mice were housed individually to avoid fighting which could open the surgical wound. To demonstrate that the osmotic minipump had delivered the Ang II, BP of the mice was measured.

2.3 Blood pressure measurements

BP was measured non-invasively by tail cuff plethysmography or invasively using telemetry.

2.3.1 Tail cuff measurements

Systolic BP was measured with a BP 2000 Series II analysis system (Visitech Systems) at 37 °C. 5 preliminary measurements were performed before recording 10 measurements and the average of all valid measurements was taken. A measurement was excluded if the mouse was moving around during recording. If a mouse consistently moved during the measurements, the system was paused until the mouse was still again. Daily BP measurements were started 7 days before recording BP to train the mice for tail cuff BP measurements and measurements were taken at the same time each day.

2.3.2 Telemetry

Telemetry probe implantation was carried out by Dr Ryszard Nosalski or Wendy Beattie. Mice were anaesthetised with 3-5 % isoflurane and oxygen at a flow rate of 2 L/min. An area of skin on the neck was shaved and wiped with povidone-iodine (Betadine). Mice were given a subcutaneous injection of 100 µl of 1 mg/ml carprofen (Rimadyl) before a small incision was made and the left common carotid artery was isolated. The artery was ligated and a catheter connected to a transducer was inserted into the artery, towards the aorta. 2 sutures were used to hold the catheter in place. The transducer was positioned under the skin on the left flank of the mouse. Mice recovered for 1 wk before baseline BP was measured. After 7 days of baseline BP measurements, mice were treated with Ang II as in section 2.2 and BP continued to be measured for 14 days. Telemetry measurements were taken every 10 minutes and the average for each day and night was calculated.

2.4 Replacement, reduction and refinement (3Rs)

When designing the *in vivo* experiments the 3Rs were taken into account. Replacing animal experiments was not possible as hypertension cannot be induced or measured using *in vitro* techniques. To reduce the number of mice used, the number of mice per group was decided based on previously published data (Mikolajczyk et al., 2016). Refinement, improving animal welfare, was considered and mice were given analgesia before surgery to minimise suffering.

The Animal Research: Reporting *in vivo* experiments (ARRIVE) guidelines were also considered when designing experiments (Kilkenny et al., 2010). For example, mice were randomly assigned Ang II or sham treatments to remove subjective bias. This could not be done by blinding when analysing the results as it is obvious which mice had the osmotic minipump implant and which mice had the sham surgery. Further, the strain, sex and number of mice used for each experiment has been reported, along with details of the procedures used.

2.5 Lucigenin chemiluminescence assay

2 mm segments of thoracic aorta were isolated from sham and Ang II treated mice and superoxide production was assessed using a lucigenin assay. Baseline luminescence of 5 μM lucigenin (Sigma-Aldrich) in Krebs-HEPES buffer (99 mM NaCl, 25 mM $NaHCO_3$, 11.1 mM glucose, 20 mM HEPES, 4.7 mM KCl, 1.9 mM $CaCl_2$, 1 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$; pH 7.35) was monitored at 37 ºC by a FB12 Luminometer (Titertek-Berthold) using FB12 Sirius software for 15 min. The aorta was added and luminescence was monitored for a further 15 min. The difference of average relative light units (RLU)/s before and after the addition of the aorta was calculated. The aorta was left to dry and weighed so that superoxide production could be expressed as RLU/s/mg of aorta.

2.6 Myography

Wire myography was carried out by Dr Dominik Skiba or Dr Ryszard Nosalski to determine endothelium-dependent and endothelium-independent relaxation of thoracic aorta and mesenteric artery segments from sham and Ang II treated mice. The PVAT was removed from the vessels before mounting them in myograph baths containing PSS and incubating for 30 min at 37 ºC. Vessels were pre-constricted with 3 x 10^{-8} M U46619 and cumulative concentration response curves were conducted by incrementally increasing the concentration of acetylcholine (ACh) or sodium nitroprusside (SNP). The concentration of ACh or SNP started at 1 x 10^{-10} M and gradually increased to 3 x 10^{-5} M.

2.7 Flow cytometry

2.7.1 Tissue processing

Bone marrow, blood, thymus, spleen, kidney and PVAT were taken and processed using flow cytometry buffer (FB; 1 % FCS 2 mM EDTA in PBS) for flow cytometry with individual, optimised protocols.

2.7.1.1 Bone marrow

Legs were isolated and the fur and muscle were removed from the femur. The ends of the femur were cut off and bone marrow was isolated one of two ways.

1. A hole was pierced in the bottom of a 500 µl Eppendorf tube and this was placed in a 1.5 ml Eppendorf tube. The femur was put in the 500 µl tube, 200 µl of PBS was added and this was centrifuged for 5 – 10 s. Cells pelleted in the 1.5 ml tube. 500 µl of ACK lysis buffer (Gibco) was added to the cells for 1 min before adding 500 µl of FB and centrifuging for 5 min at 400g and 4 °C. Cells were resuspended in 0.3 – 0.5 ml of FB.

2. The bone marrow was flushed out of the femur using a 5 ml syringe with a 23 G needle with 5 ml of RPMI-1640 (Sigma-Aldrich)-10 % FCS. The cell suspension was then passed through a 70 µm cell strainer and centrifuged for 5 min at 400 g and 4 °C. The supernatant was discarded and the cells were resuspended in 1 ml of ACK lysis buffer (Gibco) for 1 min. FB was added and the cells were centrifuged again for 5 min at 400 g and 4 °C. The supernatant was discarded and cells were resuspended in 0.3 – 0.5 ml of FB.

2.7.1.2 Blood

Blood was collected via cardiac puncture or from the vena cava into 1 ml syringes containing 50 µl of 0.2 M EDTA. 10 ml of RBC lysis buffer (eBioscience) was added per 1 ml of blood and incubated for 5 min at room temperature (RT) on a shaker. Up to 10 ml of PBS was added and samples were centrifuged at 400 g and 4 °C for 5 min. The supernatant was discarded and cells were resuspended in 0.5 – 1 ml of FB or the lysis was repeated for 1 min if a lot of RBCs remained.

2.7.1.3 Thymus

The thymus was cut into small pieces in FB and passed through a 70 µm strainer into a 50 ml falcon tube. Up to 20 ml of FB was added and samples were centrifuged for 5 min at 400 g and 4 °C. The supernatant was discarded and cells were resuspended in 0.5 ml of FB.

2.7.1.4 Spleen

Spleens were cut up into 3 pieces in FB and passed through a 70 µm strainer into a 50 ml falcon tube. Up to 20 ml of FB was added and samples were centrifuged for 5 min at 400 g and 4 °C. The supernatant was discarded and cells were

resuspended in 5 ml of RBC lysis buffer (Biolegend) for 5 min at RT. Up to 20 ml of FB was added and samples were passed through another 70 µm strainer and centrifuged again for 5 min at 400 g and 4 °C. The supernatant was discarded and cells were resuspended in 0.5 - 5 ml of FB.

2.7.1.5 Kidney

An enzyme mix of 73.4 mg Collagenase I (Sigma-Aldrich), 6.2 mg Collagenase XI (Sigma-Aldrich) and 5.3 mg Hyaluronidase (Sigma-Aldrich) in 20 ml of 20 mM Hepes-PBS containing calcium and magnesium was made on the day of use and kept on ice. Kidneys were cut into small pieces in a 12 well plate containing 1 ml of 20 mM Hepes-PBS containing calcium and magnesium and 1 ml of the enzyme mix was added. Final enzyme concentrations were 450 U/ml Collagenase I, 125 U/ml Collagenase XI and 60 U/ml Hyaluronidase. Samples were incubated at 37 °C for 10 min, agitated with a pipette, and incubated for another 10 min at 37 °C. 500 µl of 10 % FCS-PBS was added to stop the enzyme reaction and samples were passed through a 70 µm strainer into a 50 ml falcon tube. Up to 20 ml of FB was added and samples were centrifuged for 5 min at 400 g and 4 °C. The supernatant was discarded and cells were resuspended in 5 ml of RBC lysis buffer (Biolegend) for 5 min at RT. Up to 20 ml of FB was added and samples were passed through another 70 µm strainer and centrifuged again for 5 min at 400 g and 4 °C. The supernatant was discarded and cells were resuspended in 0.3 - 5 ml of FB.

2.7.1.6 PVAT

PVAT was isolated from the thoracic and abdominal aorta and was weighed. The PVAT was placed in 1 ml of 20 mM Hepes-PBS containing calcium and magnesium and cut into small pieces. 1 ml of the enzyme mix from section 2.7.1.5 was added. Samples were incubated at 37 °C for 10 min, agitated with a pipette, and incubated for another 10 min at 37 °C. 500 µl of 10 % FCS-PBS was added to stop the enzyme reaction and samples were passed through a 70 µm strainer into a 50 ml falcon tube. Up to 20 ml of FB was added and samples were centrifuged for 5 min at 400 g and 4 °C. The supernatant was discarded and cells were resuspended in 0.3 - 0.5 ml of FB.

2.7.2 Cell staining

Zombie violet fixable viability dye (Biolegend) was prepared by diluting 1/2000 in PBS or fixable viability dye eFluor 506 (eBioscience) was prepared by diluting 1/1000 in PBS. The appropriate antibody mix was prepared in FB with Fc block (Miltenyi Biotec), diluted 1/100. Antibodies used are detailed in Table 2-1. Cell suspensions, prepared as detailed above, were added to FACS tubes or a 96 well plate and washed in FB by centrifuging for 5 min at 400 g and 4 °C. The supernatant was discarded and 100 µl of the appropriate viability dye was added to the cells and incubated at RT for 15 min. The cells were washed in FB, the supernatant was discarded and 100 µl of the antibody mix was added for 20 min at 4 °C. Cells were washed again in FB and resuspended in 200 µl of FB. Samples were either analysed that day or fixed for 10 min in 1 % paraformaldehyde (PFA) at 4 °C, washed in FB and stored at 4 °C for analysis the next day.

2.7.2.1 Chemokine binding assay

AF647-CCL2 (Almac) was used to measure CCR2 expression by flow cytometry instead of using an antibody (Ford et al., 2014). Cell suspensions were prepared as described above and washed in 5 % BSA-RPMI by centrifuging for 5 min at 400 g and 4 °C. The supernatant was discarded and cells were resuspended in 100 µl of 25 nM AF647-CCL2 in 5 % BSA-RPMI for 60 min at 4 °C. Cells were then washed again in 5 % BSA-RPMI and stained with the appropriate viability dye and antibodies, following the protocol above.

2.7.2.2 Intracellular staining

Intracellular staining was used to analyse MR expression. Cell suspensions were prepared and stained as detailed above and fixed in 4 % PFA for 10 min at 4 °C. Cells were then washed, resuspended in Perm/Wash (BD Bioscience) for 15 min, and washed again. The anti-MR antibody (Novus Biologicals) was diluted 1/25 in Perm/Wash and 100 µl was added to the cells for 30 min at 4 °C. Cells were washed again in Perm/Wash before adding 100 µl of anti-mouse IgG1-APC Cy7 secondary antibody (Biolegend), diluted 1/100 in Perm/Wash, for 30 min at 4 °C. Finally, cells were washed in Perm/Wash and resuspended in 200 µl of FB.

Antibody	Clone	Conjugate	Company	Catalogue number
CCR3	J073E5	PE Cy7	Biolegend	144514
CCR5	HM-CCR5(7A4)	PE	eBioscience	12-1951-82
CD3	17A2	FITC	Biolegend	100204
CD3	17A2	APC Cy7	Biolegend	100222
CD3e	145-2C11	PE	Biolegend	100308
CD4	GK1.5	APC-eF780	eBioscience	47-0041-82
CD4	RM4.5	PE Cy7	eBioscience	25-0042-82
CD8a	53-6.7	FITC	Miltenyi Biotec	130-102-490
CD8a	53-6.7	APC eF780	eBioscience	47-0081-82
CD11b	M1/70	AF700	Biolegend	101222
CD11b	M1/70	APC Cy7	Biolegend	101226
CD11b	M1/70	PE Cy7	Biolegend	101216
CD11b	M1/70	APC	eBioscience	17-0112-82
CD11c	N418	PerCP Cy5.5	Biolegend	117328
CD19	6D5	AF700	Biolegend	115528
CD19	6D5	PerCP Cy5.5	Biolegend	115534
CD44	IM7	PerCP Cy5.5	Biolegend	103032
CD45	30-F11	BV510	Biolegend	103138
CD45	30-F11	PerCP Cy5.5	Biolegend	103132
F4/80	BM8	PE Cy7	eBioscience	25-4801-82
F4/80	BM8	PE	Biolegend	123110
F4/80	BM8	BV605	Biolegend	123133
Ly6C	HK1.4	AF700	Biolegend	128024
Ly6C	HK1.4	APC	Biolegend	128016
MHC II	M5/114.15.2	BV605	Biolegend	107639

Table 2-1. Antibodies used for flow cytometry
The target, clone, conjugated fluorophore, supplier and catalogue number of the antibodies used for flow cytometry. All antibodies were used at a dilution of 1/100.

2.7.3 Flow cytometry analysis

All data were acquired using a LSR II (BD bioscience) or Fortessa (BD bioscience) flow cytometer and data were analysed using Flowjo software. All samples were analysed with the same initial gating strategy (Figure 2-1). Cells were identified based on SSC-A and FSC-A. Single cells were then selected based on FSC-H and FSC-A and live cells were identified using the viability stain.

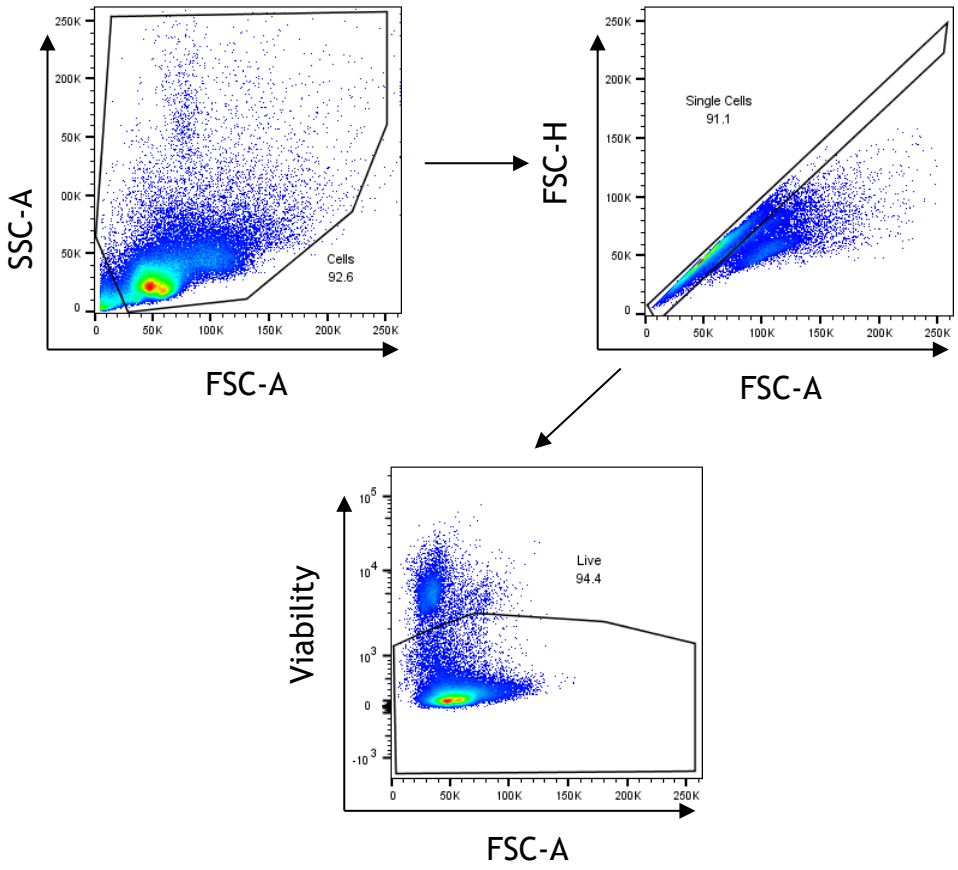


Figure 2-1. Initial gating strategy for flow cytometry analysis
Cells were identified and from these, single cells were selected. Live cells were then gated from the single cells.

2.7.4 Magnetic cell sorting

Cell suspensions from mouse kidneys were isolated as described in section 2.7.1.5. 10^7 cells were stained with an anti-F4/80-PE antibody (Biolegend), diluted 1/10 in FB, for 20 min at 4 °C. Cells were washed and stained with anti-PE Microbeads (Miltenyi Biotec) diluted 1/5 in FB, for 20 min at 4 °C. Cells were washed again and resuspended in 500 µl of FB.

F4/80+ cells were sorted using LS columns and a QuadroMACS separator (Miltenyi Biotec). The separator was attached to the magnetic multistand and the columns were placed in the separator. 1 ml of FB was run through the columns before adding the stained samples. 500 µl of FB was then run through the columns 3 times and collected in Eppendorf tubes. This was the negative fraction. The columns were then removed from the magnet and 1 ml of FB was run through and collected in new Eppendorf tubes. This was the positive fraction. Samples of

pre-sorted cells, the negative fraction and the positive fraction were analysed using a MACSQuant (Miltenyi Biotec) flow cytometer to determine how well the sort worked.

2.8 Histology

2.8.1 Tissue processing

Heart, kidney and thoracic aorta were taken from perfused mice and stored in 1 % PFA overnight at 4 °C. Tissues were then transferred to a solution of 30 % sucrose in distilled water and stored at 4 °C overnight or until the tissue had sunk to the bottom of the solution. After this the tissues were frozen in O.C.T (TissueTek) on dry ice and stored at -80 °C. 8-10 µm sections were cut with a cryostat and put onto SuperFrost slides (VWR). Sections were stored at -20 °C until used for staining.

2.8.2 Haematoxylin & Eosin (H&E) staining

Frozen tissue sections were fixed in ice cold acetone for 5 min and allowed to air dry. Sections were hydrated by incubating them for 1 min in 100 % ethanol, 1 min in 100 % ethanol again, 1 min in 70 % ethanol and washing in running water. Nuclei in the sections were stained with haematoxylin (Cell path) for 7 min before washing in running water. Sections were then dipped 12 times in 1 % acid alcohol, washed in running water and placed in Scotts tap water solution for 2 min. Sections were washed again in running water and stained with eosin (Cell path) for 4 min. Sections were washed for a final time in running water for 2 min and dehydrated by incubating for 1 min in 70 % ethanol, 1 min in 100 % ethanol and 1 min in 100 % ethanol again. Sections were cleared in 3 changes of xylene for 1 min and mounted with DPX (Leica Biosystems).

2.8.3 Picrosirius red staining

Frozen tissue sections were fixed in ice cold acetone for 5 min and allowed to air dry. Sections were hydrated by incubating them for 1 min in 100 % ethanol, 1 min in 100 % ethanol again, 1 min in 70 % ethanol and washing in running water. Sections were stained with 0.1 % picrosirius red solution (Direct Red 80 (Sigma Aldrich) in picric acid) for 60 min at RT and then washed in 2 changes of

acidified water (0.5 % acetic acid). Sections were dehydrated by incubating for 1 min in 70 % ethanol, 1 min in 100 % ethanol and 1 min in 100 % ethanol again and then cleared in 3 changes of xylene for 1 min. DPX (Leica Biosystems) was used to mount the sections.

2.8.4 Mounting sections from REP mice

Frozen tissue sections were fixed in ice cold acetone for 5 min and allowed to air dry. Slides were mounted with ProLong gold antifade mountant with DAPI (Thermofisher) and sealed with clear nail polish.

2.8.5 Microscopy

H&E and picrosirius red stained sections were imaged using brightfield microscopy with an Axio Imager M2 microscope (Zeiss) and Zen software. Images were analysed with Fiji software. Fluorescent sections were imaged by fluorescent microscopy with an Axio Imager M2 microscope (Zeiss) or LSM 880 confocal microscope (Zeiss) and Zen software.

2.9 Molecular biology

2.9.1 RNA extraction

Tissues were harvested from euthanased mice and stored in RNAlater (Thermofisher) at 4 °C for up to 7 days before RNA was extracted. Tissues were homogenised in Qiazol (Qiagen) using a TissueLyser LT (Qiagen) set at 50 oscillations/s for up to 10 min. RNA was extracted with the miRNeasy kit (Qiagen), following the manufacturer's protocol. The optional DNase digest was performed using the RNase-free DNase set (Qiagen). Once extracted, RNA was stored at -80 °C.

2.9.2 cDNA synthesis

Concentration and quality of RNA was measured using a DeNovix DS-11+ spectrophotometer. 1 µg of RNA was used to make cDNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems) following the manufacturer's protocol and stored at –20 °C.

2.9.3 Taqman qPCR

Taqman probe/primer sets (Table 2-2) were used to determine relative mRNA expression in tissues. All reagents and samples were kept on ice while the plate was set up. A mastermix of RNase-free water (3 µl per sample), Taqman universal mastermix II, no UNG (Thermofisher; 5 µl per sample) and Taqman probe/primer (0.5 µl per sample) was made and 8.5 µl of mastermix was added to the appropriate wells of a 384 well plate. 1.5 µl of cDNA (prepared as in section 2.9.2) was added to the appropriate wells. The plate was sealed, centrifuged for 1 min at 300 g and 4 °C and run on a Quant Studio 7 Flex qPCR machine (Applied Biosystems) with Quant Studio Real-Time PCR software. Samples were run in triplicate and target gene expression was calculated relative to Gapdh or Cox7a2l using the $2^{-\Delta CT}$ method (Livak and Schmittgen, 2001). Gapdh was used as a housekeeping gene to compare gene expression within the same tissue and Cox72al was used to compare gene expression between different tissues (Barber et al., 2005; Li et al., 2017). No template controls and – reverse transcriptase controls were run to check for primer contamination and sample genomic contamination, respectively.

Target gene	Target name	Catalogue number
Ccr1	CCR1	Mm00438260_s1
Ccr2	CCR2	Mm99999051_gH
Ccr3	CCR3	Mm00515543_s1
Ccr5	CCR5	Mm01963251_s1
Cox7a2l	Cytochrome c oxidase subunit 7a related protein	Mm00784451_s1
Gapdh	GAPDH	Mm99999915_g1
Nr3c2	Mineralocorticoid receptor	Mm01241596_m1
Scnn1g	Epithelial sodium channel (ENaC)	Mm00441228_m1
Sgk1	Serum/glucocorticoid-regulated kinase 1 (SGK1)	Mm00441380_m1
Slc9a3	Sodium-hydrogen exchanger 3 (NHE3)	Mm01352473_m1

Table 2-2. Taqman primer/probe sets used for qPCR
Targets and catalogue numbers of primers used for qPCR. All used the dye FAM-MGB and were purchased from Thermofisher.

2.10 Plasma chemokine and cytokine detection

2.10.1 Sample preparation

Blood was collected from euthanased mice via cardiac puncture into tubes containing 50 µl of 0.2 M EDTA. Blood samples were centrifuged at 1000 g for 10 min to isolate plasma which was collected and stored at -80 °C.

2.10.2 Luminex assay

Plasma samples were diluted 1/2 and the concentration of chemokines and cytokines was measured using a mouse premixed multi-analyte kit (R&D Systems), following the manufacturers protocol. The samples were analysed using a Bio-Plex 200 system (Bio-Rad).

2.11 Statistical analysis

All statistical analyses were carried out with GraphPad Prism software. A t test, Mann Whitney test, Two-Way ANOVA with a post hoc Tukey test or Two-way ANOVA with repeated measures test was performed as appropriate. A p value of < 0.05 was determined as statistically significant. Data are presented as the mean ± standard deviation (SD) unless otherwise stated.

Chapter 3

iCCR expression in Ang II induced hypertension

3 iCCR expression in Ang II induced hypertension

3.1 Introduction

There is a large amount of evidence for a role of the immune system in the pathogenesis of hypertension, particularly factors influencing end organ damage such as oxidative stress and vascular dysfunction (Herrera et al., 2006; Moore et al., 2015; Youn et al., 2013). Monocytes and macrophages accumulate in the kidneys and vasculature in hypertension and depleting macrophages in mice is protective against DOCA-salt induced hypertension (Moore et al., 2015; Ozawa et al., 2006; Thang et al., 2015). T cells also accumulate in the kidneys and vasculature and it has been suggested that T cells are required for Ang II induced hypertension in mice (Guzik et al., 2007; Itani et al., 2016; Krebs et al., 2014).

Chemokines and their receptors are important in orchestrating leukocyte migration and inflammatory responses. Therefore, they are likely to regulate leukocytes in the context of hypertension and influence end organ damage. $CCL5^{-/-}$ mice are protected from Ang II induced vascular dysfunction and oxidative stress (Mikolajczyk et al., 2016) whilst $CCL2^{-/-}$ mice may be protected from hypertensive renal injury (Kashyap et al., 2018). CCR2 or CCR5 deficiency does not influence BP in hypertensive models but $CCR2^{-/-}$ mice are protected from Ang II induced vascular remodelling (Ishibashi et al., 2004; Krebs et al., 2012). Inflammatory chemokine receptors influence hypertension and end organ damage but the exact role of these receptors is still unclear.

iCCRs have been reported to be expressed by various leukocytes. For example, CCR1 is expressed by T cells and B cells (Armas-González et al., 2018; Mikolajczyk et al., 2016) whilst CCR2 is classically described as being expressed by monocytes and macrophages (Sierra-Filardi et al., 2014; Tacke et al., 2007). CCR3 is expressed by eosinophils and CCR5 has been reported to be expressed by T cells and NK cells (Fox et al., 2015; Lima et al., 2015). It is hypothesised that iCCRs regulate leukocyte migration in hypertension. Circulating monocytes have elevated CCR2 expression and T cells in the PVAT increase CCR1, CCR3 and CCR5 expression in hypertension (Ishibashi et al., 2004; Mikolajczyk et al., 2016) but little else is known about which cell types in various tissues express these receptors in the disease.

The aim of this study was to characterise iCCR expression by different leukocytes in multiple tissues in mice with Ang II induced hypertension. WT or REP mice were subject to Ang II induced hypertension and iCCR expression was determined by flow cytometry, fluorescent microscopy and qPCR. Furthermore, plasma chemokine and cytokine levels were characterised. This revealed some differences in iCCR and chemokine expression between healthy and hypertensive states which may represent targets to treat elevated BP and end organ damage in hypertension.

3.2 Response of REP mice to Ang II

REP mice and WT littermate controls were treated with Ang II for 14 days and their response to Ang II was determined by measuring BP (Figure 3-1a) and assessing cardiac hypertrophy (Figure 3-1b). The ratio of heart weight to tibia length indicates how the weight of the heart changed over the experiment and therefore this was used to determine cardiac hypertrophy. The BP of both WT and REP mice increased in response to Ang II (WT sham 114 ± 1.2 vs WT Ang II 172 ± 3.3 mmHg, $p < 0.01$, Two-Way ANOVA; REP sham 109 ± 1.8 vs REP Ang II 159 ± 9.3 mmHg, $p < 0.01$, Two-Way ANOVA). Compared to Ang II treated WT mice, Ang II treated REP mice had a slightly lower BP (WT Ang II 172 ± 3.3 mmHg vs REP Ang II 159 ± 9.3 mmHg, $p < 0.05$, Two-Way ANOVA). The ratio of heart weight to tibia length increased in the WT mice treated with Ang II (WT sham 9.3 ± 0.8 vs WT Ang II 13.2 ± 1.3 mg/mm, $p < 0.05$, Two-Way ANOVA). This effect was not seen in the REP mice (REP sham 11.7 ± 2.5 vs REP Ang II 12.5 ± 0.2 mg/mm, $p = 0.88$, Two-Way ANOVA).

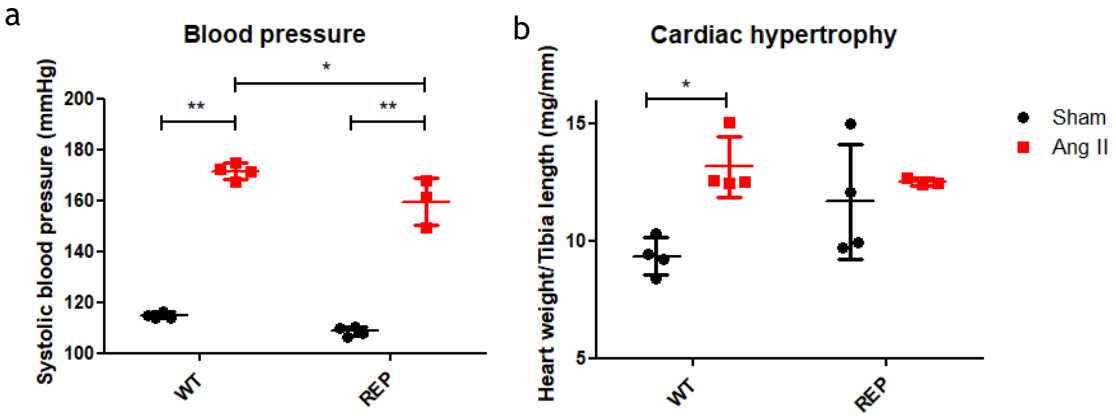


Figure 3-1. Response of WT and REP mice to Ang II
WT and REP mice were treated with Ang II for 14 days. BP (a) and the ratio of heart weight to tibia length (b), as a measure of cardiac hypertrophy, were assessed. * $p < 0.05$, ** $p < 0.01$, Two-Way ANOVA. Data are combined from two independent experiments.

3.3 iCCR expression in REP mice following 7 days of Ang II induced hypertension

To determine how iCCRs contribute to earlier stages of Ang II induced hypertension, REP mice were subject to 7 days of sham or Ang II treatment and iCCR expression by different subsets of leukocytes in the bone marrow, blood, spleen, kidney and PVAT was investigated using flow cytometry. The gating strategies used are detailed below. Fluorescent microscopy was used to determine CCR1, CCR2 and CCR5 expression in the kidneys, heart, aorta and PVAT. CCR3 was not looked at as mTagBFP2 fluoresces at the same wavelength as Dapi.

3.3.1 Bone marrow

B cells were identified as CD19+ cells from live, single CD45+ cells. CD11b+SSChi and CD11b+SSCint cells were also identified from the CD45+ cells (Figure 3-2). CD3 was used as a T cell marker but only a very small proportion of cells in the bone marrow were CD3+. These are likely to be T cell precursors (Klein et al., 2003) so bone marrow CD3+ cells were not analysed. CCR1 was expressed by CD19+ B cells in the bone marrow of mice given sham or Ang II treatment for 7 days. These cells also expressed low amounts of CCR2 (Figure 3-3a). CD11b+SSChi cells expressed high amounts of CCR3 (Figure 3-3b). CD11b+SSCint cells most highly expressed CCR2 whilst CCR1 and CCR3 could also be detected on these cells (Figure 3-3c). No differences were seen between sham and Ang II treated mice.

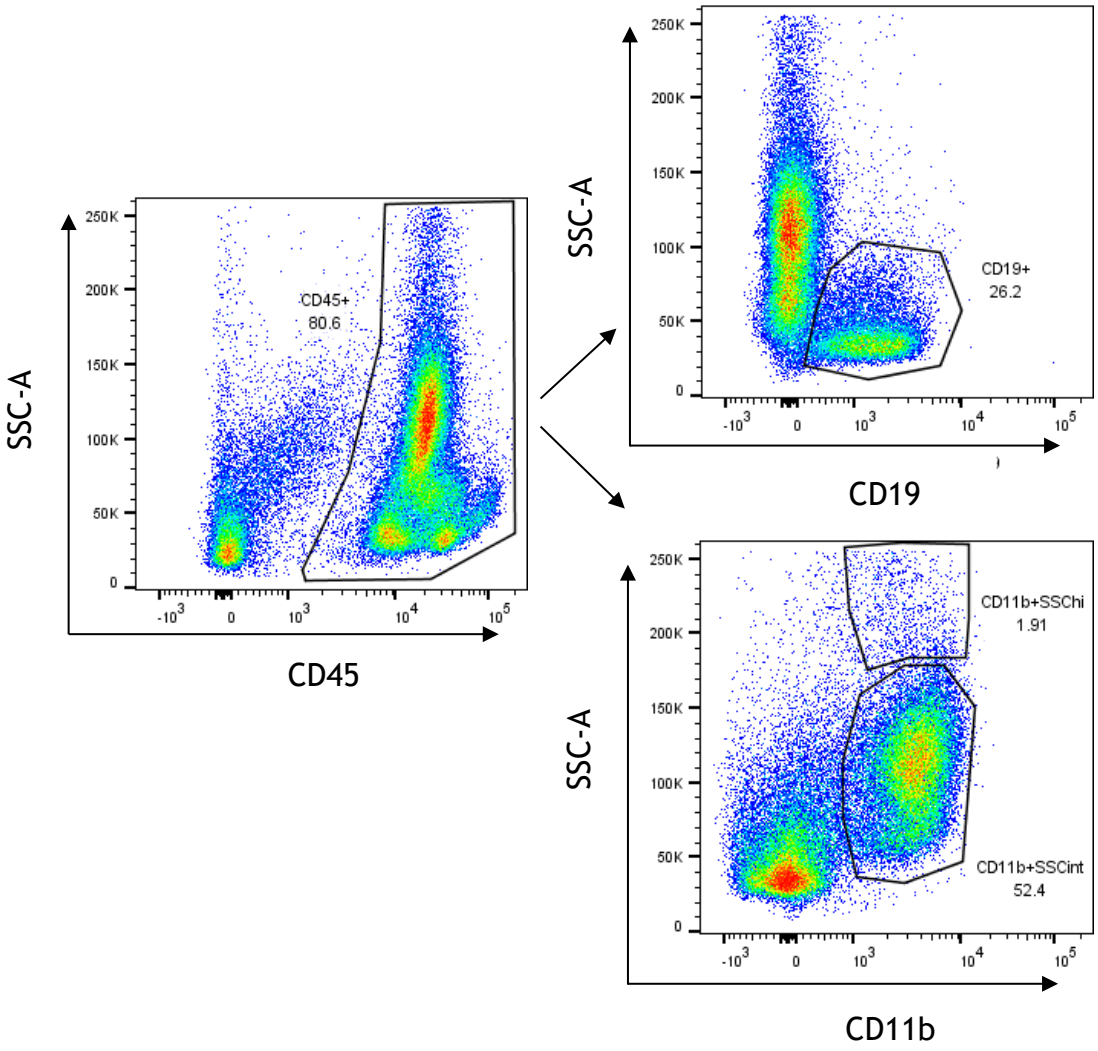


Figure 3-2. Gating strategy for the analysis of bone marrow cells from mice with Ang II induced hypertension
CD45+ cells were gated from live, single cells. CD19+, CD11b+SSCint and CD11b+SSChi cells were gated from the CD45+ cells.

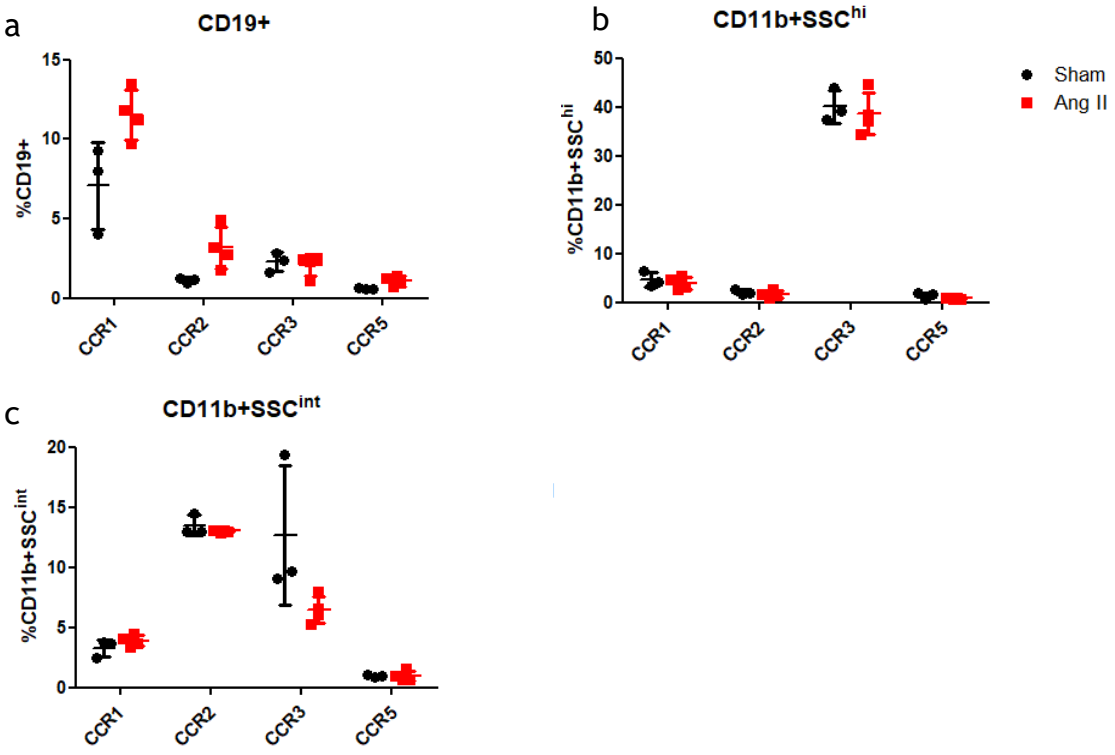


Figure 3-3. iCCR expression by leukocytes in the bone marrow of REP mice after 7 days of Ang II induced hypertension
The expression of CCR1, CCR2, CCR3 and CCR5 by CD19+ cells (a), CD11b+SSChi cells (b) and CD11b+SSCint cells (c) in the bone marrow of REP mice after sham or Ang II treatment for 7 days.

3.3.2 Blood

Blood CD19+ B cells, CD3+ T cells and CD11b+ cells were gated from live CD45+ cells (Figure 3-4). CD11b+ cells were grouped into CD11b+SSChi, CD11b+SSCint and CD11b+SSClo populations. CD19+ B cells in the blood showed high CCR1 expression after 7 days of sham or Ang II treatment (Figure 3-5a). A small proportion of CD3+ T cells expressed CCR2 and CCR5 was detected in only one sham sample (Figure 3-5b). Only CCR3 was expressed by CD11b+SSChi cells (Figure 3-5c) whilst a small proportion of CD11b+SSCint cells expressed CCR1, CCR2 or CCR3 (Figure 3-5d). CCR2 and CCR5 were expressed by CD11b+SSClo cells and some of these cells co-expressed these receptors (Figure 3-5e). No differences in iCCR expression by leukocytes in the blood were observed following 7 days of Ang II induced hypertension.

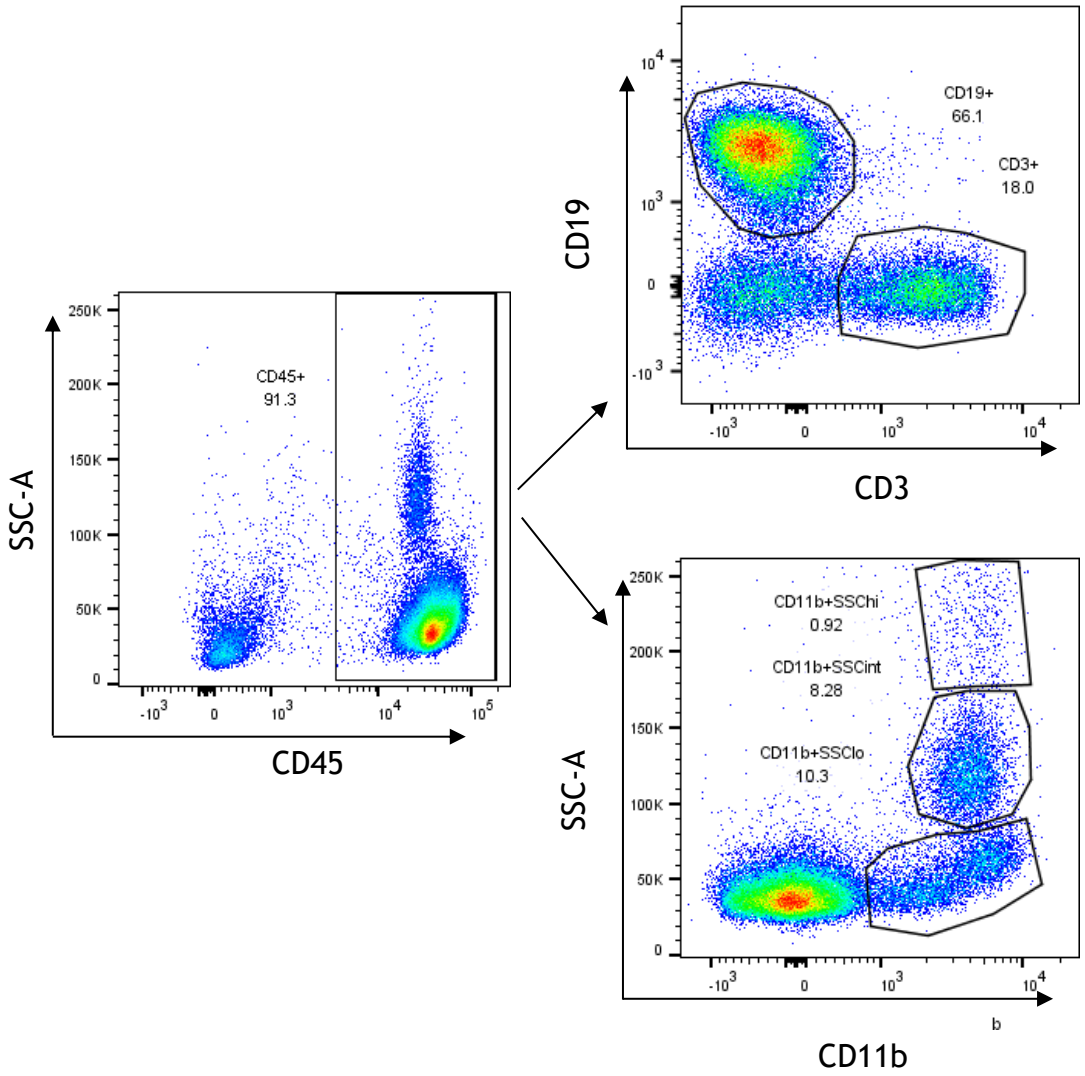


Figure 3-4. Gating strategy for the analysis of blood cells from mice with Ang II induced hypertension
CD45+ cells were gated from live, single cells. CD19+, CD3+, CD11b+SSClo, CD11b+SSCint and CD11b+SSChi cells were gated from the CD45+ cells.

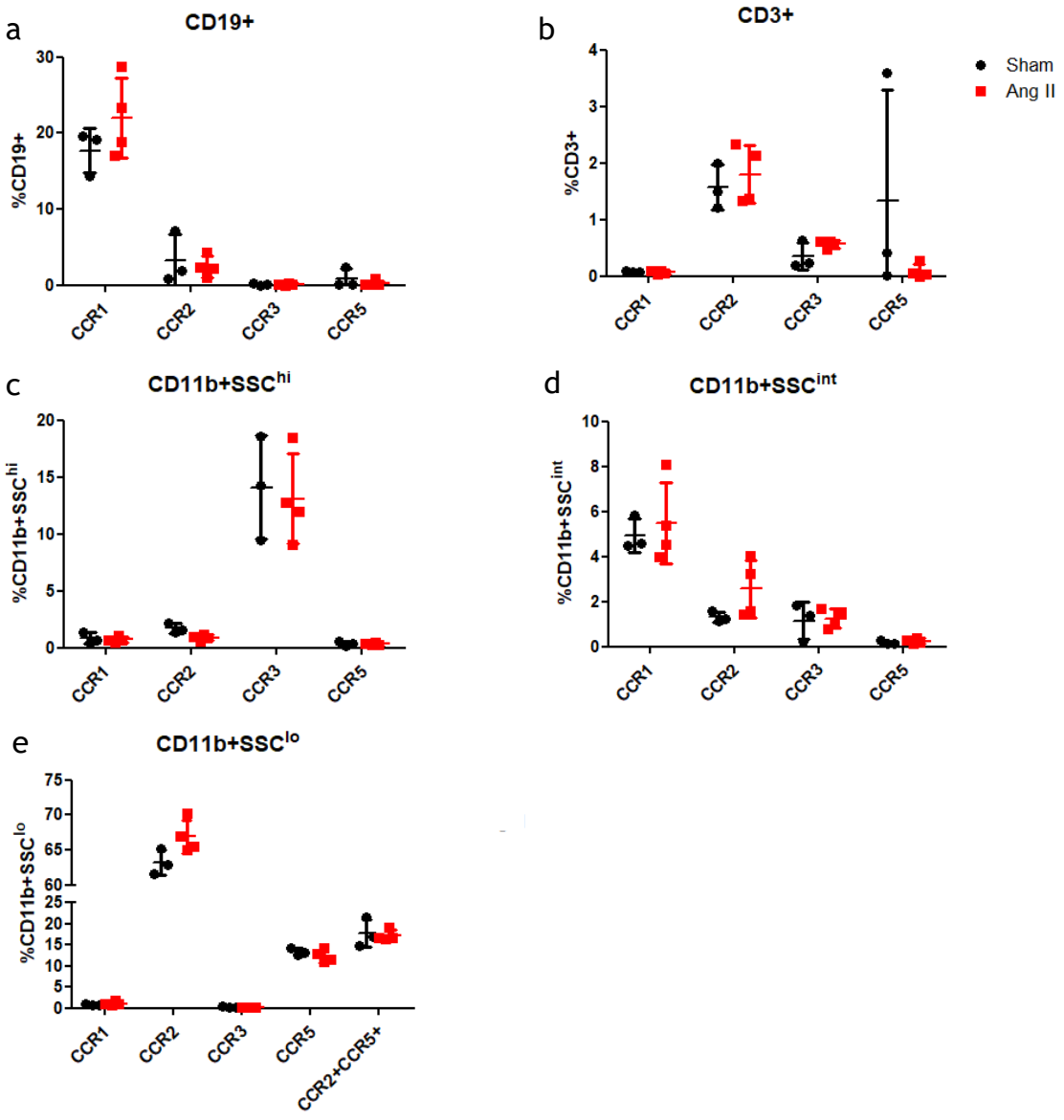


Figure 3-5. iCCR expression by leukocytes in the blood of REP mice after 7 days of Ang II induced hypertension
The expression of CCR1, CCR2, CCR3 and CCR5 by CD19+ cells (a), CD3+ cells (b), CD11b+SSChi cells (c), CD11b+SSCint cells (d) and CD11b+SSClo cells (e) in the blood of REP mice after sham or Ang II treatment for 7 days.

3.3.3 Spleen

In the spleen, live CD45+ cells were identified and from these, populations of CD19+ B cells, CD3+ T cells, F4/80+ macrophages and CD11b+ cells were determined (Figure 3-6). The CD11b+ cells were further gated into CD11b+SSChi, CD11b+SSCint and CD11b+SSClo cells. After 7 days of Ang II induced hypertension or sham treatment, CD19+ B cells expressed only CCR1 (Figure 3-7a) whilst CD3+ T cells showed low expression of CCR2 and CCR3 (Figure 3-7b). Both

CD11b+SSChi and CD11b+SSCint cells expressed CCR3 and a small amount of CD11b+SSCint cells expressed CCR1 or CCR2 (Figure 3-7c, d). CCR2 and CCR5 were expressed by CD11b+SSClo cells and these cells also showed co-expression of these receptors (Figure 3-7e, f). F4/80+ cells highly expressed CCR3 (Figure 3-7g). There were no differences in iCCR expression between sham and Ang II treatments for any of the cell types analysed.

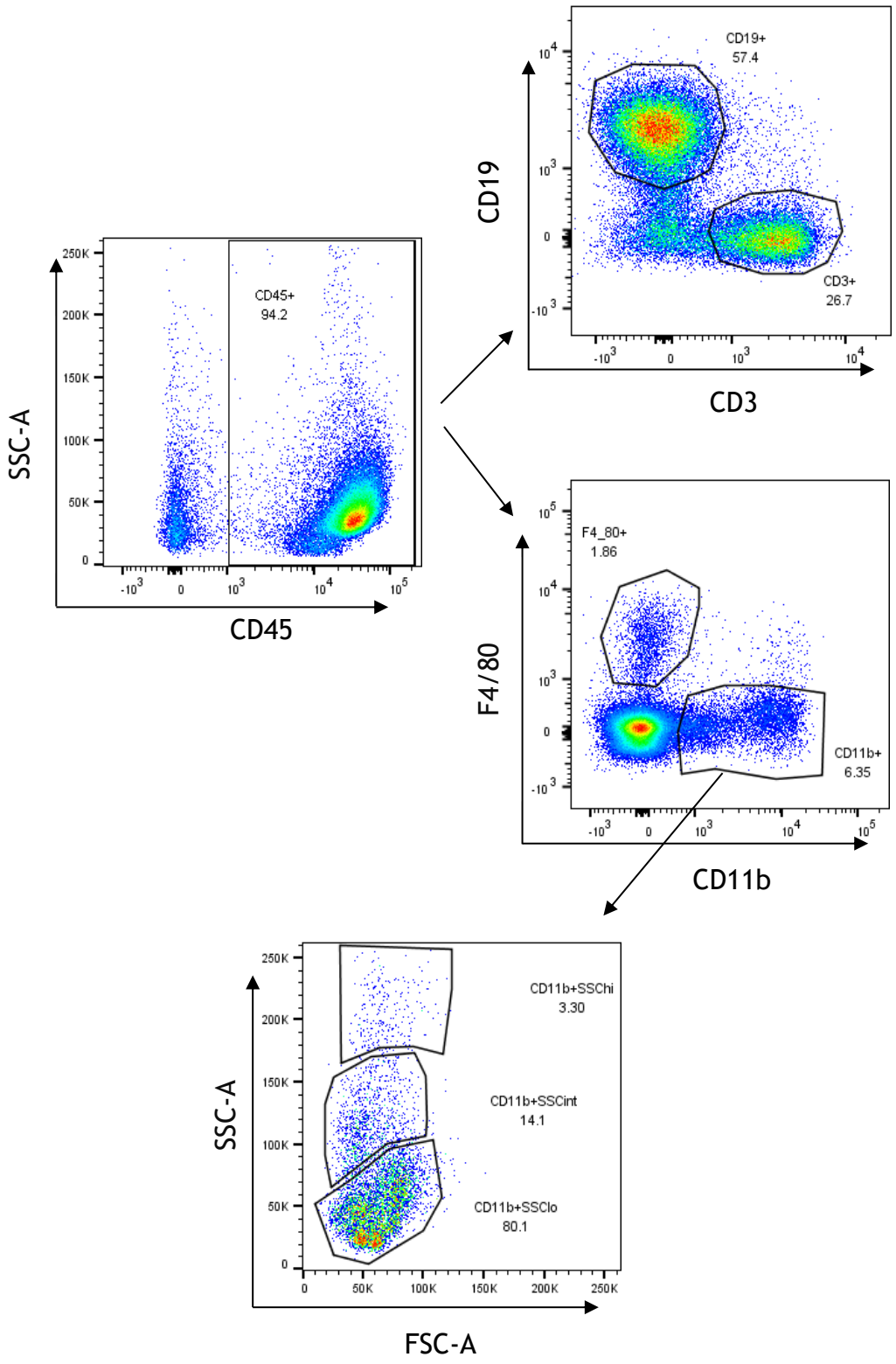


Figure 3-6. Gating strategy for the analysis of spleen cells from mice with Ang II induced hypertension
CD45+ cells were gated from live, single cells. CD19+, CD3+, F4/80+ and CD11b+ cells were gated from the CD45+ population. The CD11b+ population was then gated into CD11b+SSClo, CD11b+SSCint and CD11b+SSChi cells.

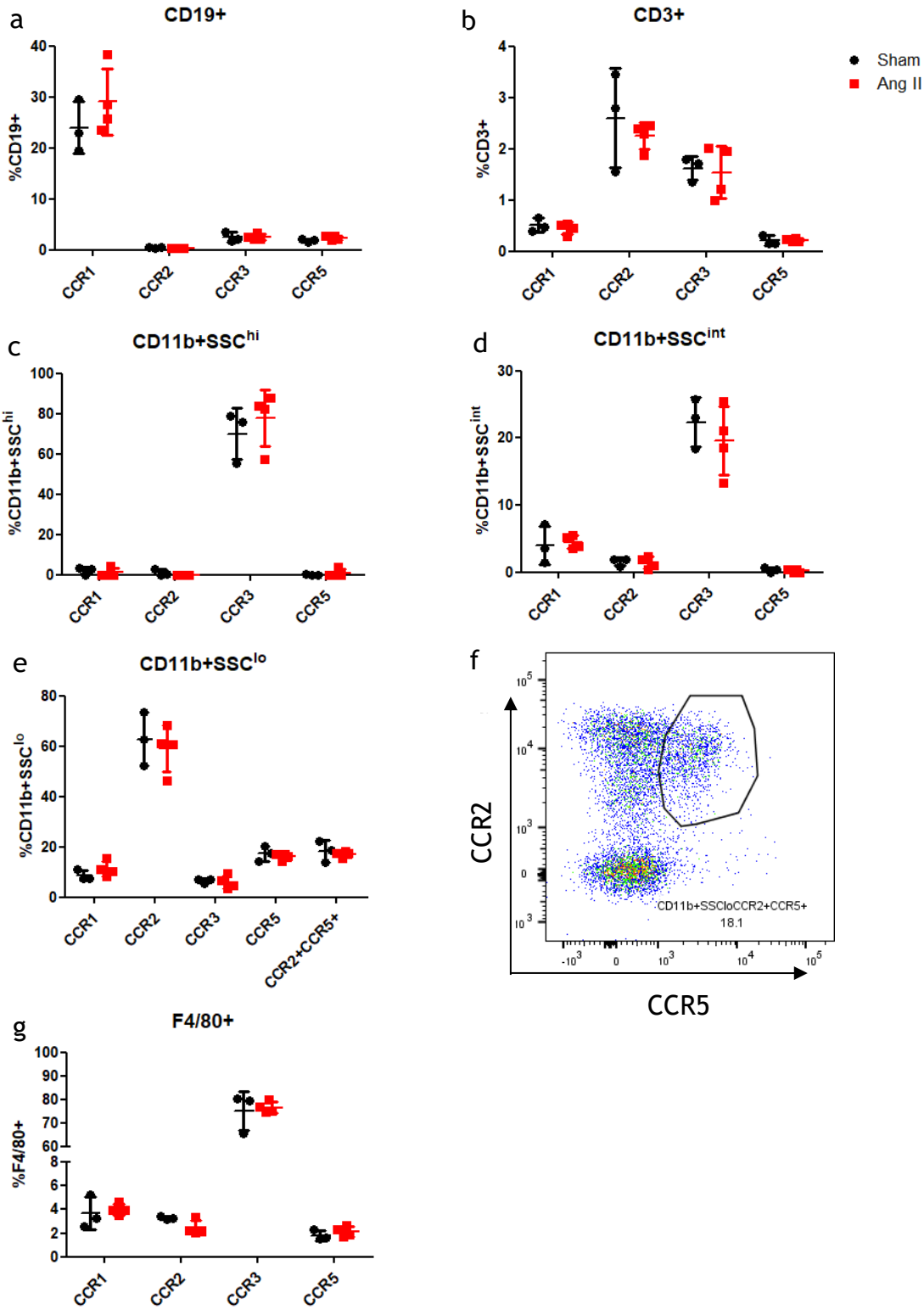


Figure 3-7. iCCR expression by leukocytes in the spleens of REP mice after 7 days of Ang II induced hypertension

The expression of CCR1, CCR2, CCR3 and CCR5 by CD19+ cells (a), CD3+ cells (b), CD11b+SSChi cells (c), CD11b+SSCint cells (d), CD11b+SSClo cells (e) and F4/80+ cells (g) in the spleens of REP mice after sham or Ang II treatment for 7 days. A representative flow cytometry plot of CD11b+SSClo cell co-expression of CCR2 and CCR5 is shown (f).

3.3.4 Kidney

Heterogeneous populations of F4/80+ cells and CD11b+ cells were identified which are likely to include macrophages, monocytes, DCs and neutrophils (Inoue et al., 2005; Misharin et al., 2013; Sahu et al., 2014). These cell types, along with CD19+ B cells and CD3+ T cells, were gated from live CD45+ cells (Figure 3-8). Following 7 days of sham or Ang II treatment, CD19+ cells expressed CCR1 and also showed low CCR5 expression (Figure 3-9a). CD3+ T cells expressed CCR2 and CCR5. A small proportion of these CD3+ cells also expressed CCR1 (Figure 3-9b). CD11b+ cells most highly expressed CCR2 and also expressed CCR5 (Figure 3-9c). Co-expression of CCR2 and CCR5 by CD11b+ cells was observed (Figure 3-10a) and revealed that all CCR5 expressing CD11b+ cells also expressed CCR2 and that the majority of these cells only expressed CCR2.

F4/80+ cells expressed CCR1, CCR2 and CCR5. Of these, CCR5 was the most highly expressed (Figure 3-9d). Co-expression of these receptors was also observed with the majority of these cells expressing both CCR1 and CCR5. About 11 % of F4/80+ cells expressed none of the iCCRs and none expressed CCR1 alone. Some expressed just CCR2 or just CCR5 and about 15 % were CCR2+CCR5+ (Figure 3-10b). No differences between sham and Ang II treated mice were observed.

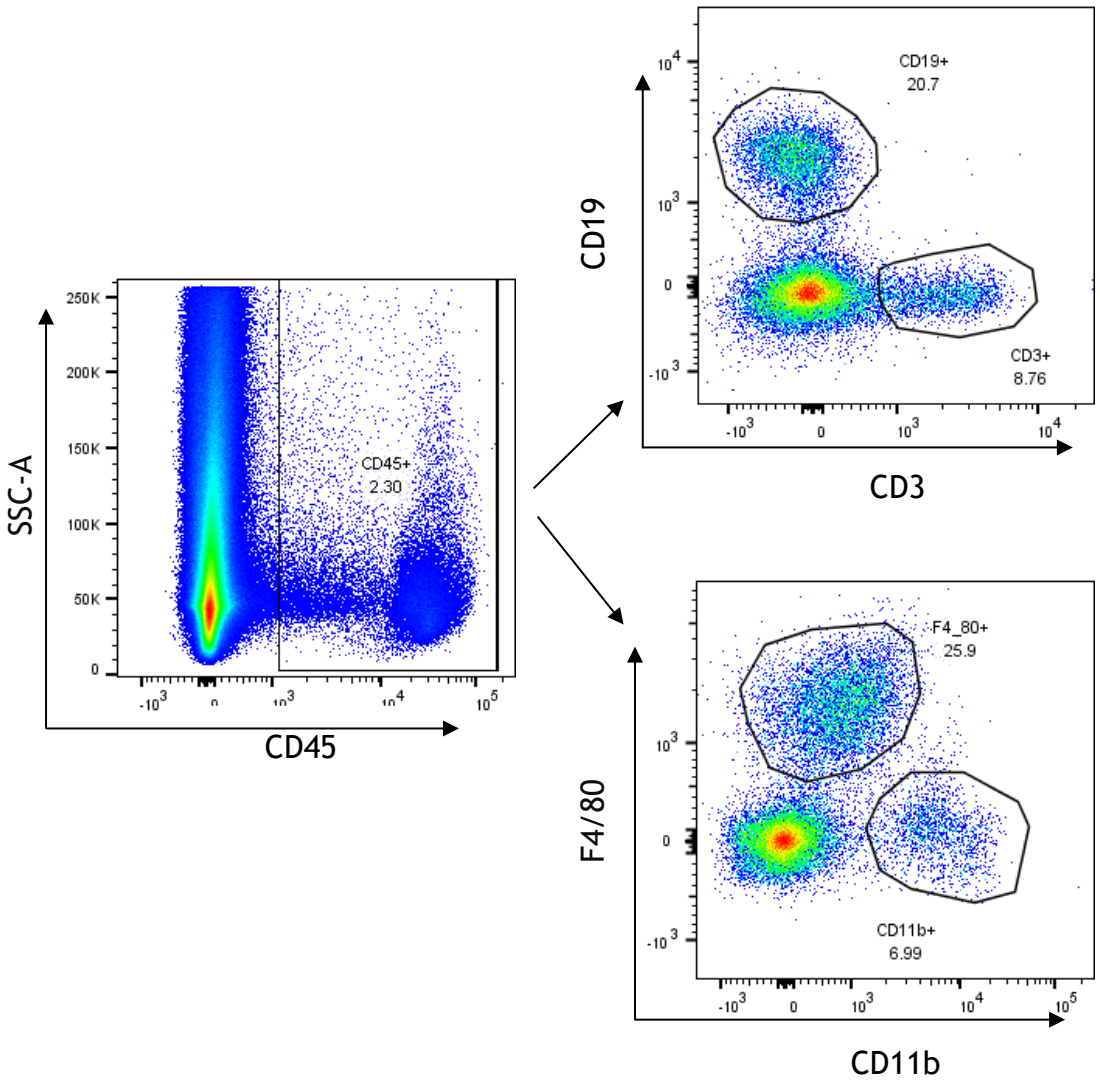


Figure 3-8. Gating strategy for the analysis of kidney leukocytes from mice with Ang II induced hypertension
CD45+ cells were gated from live, single cells. CD19+, CD3+, F4/80+ and CD11b+ cells were gated from the CD45+ cells.

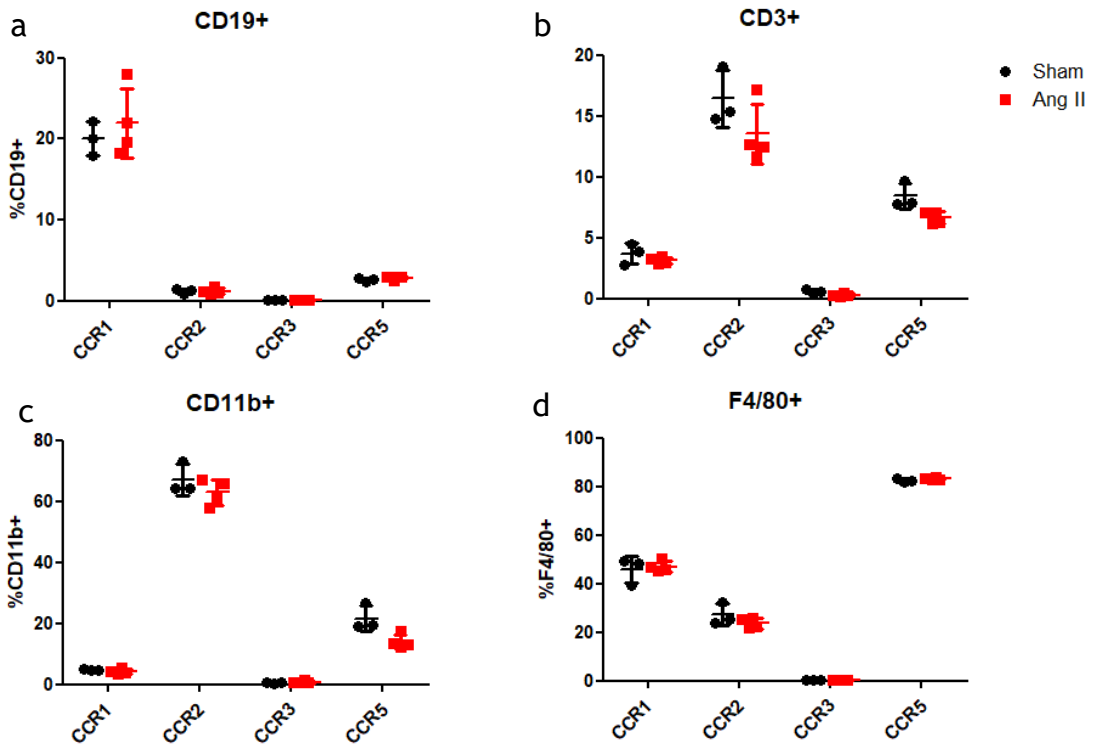


Figure 3-9. iCCR expression by leukocytes in the kidneys of REP mice after 7 days of Ang II induced hypertension

The expression of CCR1, CCR2, CCR3 and CCR5 by CD19+ cells (a), CD3+ cells (b), CD11b+ cells (c) and F4/80+ cells (d) in the kidneys of REP mice after sham or Ang II treatment for 7 days.

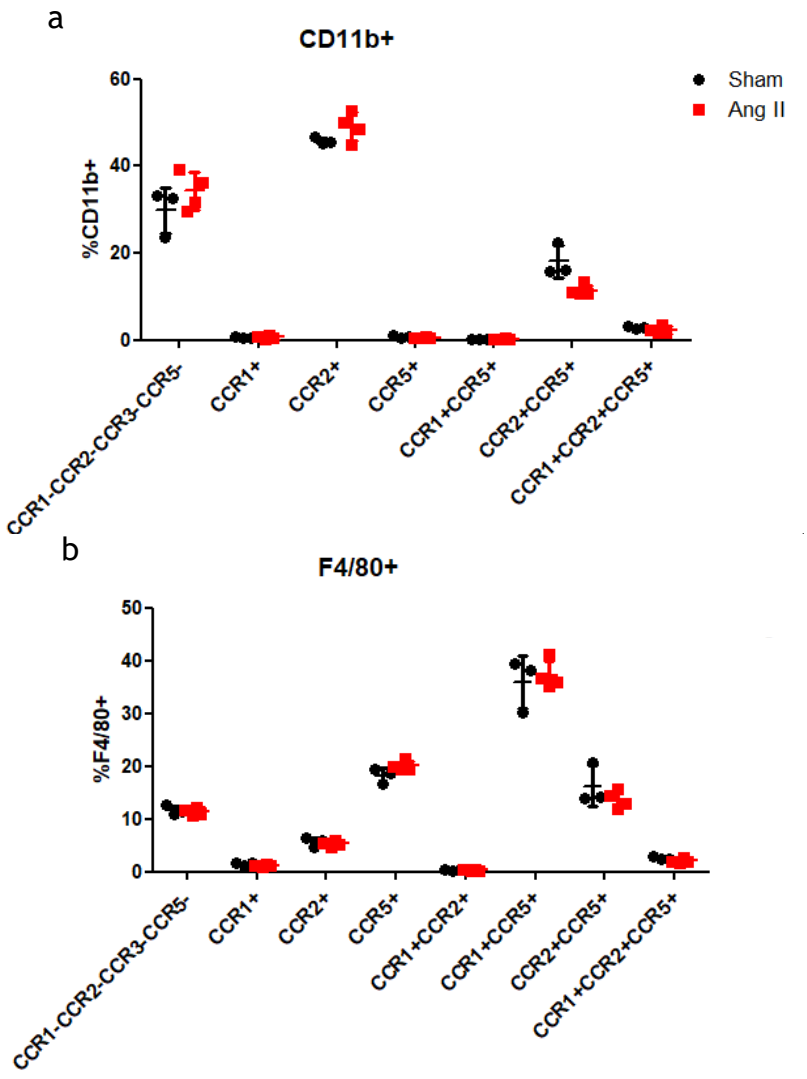


Figure 3-10. iCCR co-expression by kidney leukocytes from REP mice subject to 7 days of Ang II induced hypertension
The co-expression of CCR1, CCR2 and CCR5 by CD11b+ cells (a) and F4/80+ cells (b) in the kidneys of REP mice after 7 days of Ang II or sham treatment.

Only CCR5 could be seen after 7 days of treatment in the kidney when frozen tissue sections were imaged (Figure 3-11). These could be T cells or macrophages as CD3+, CD11b+ and F4/80+ cells were shown to express CCR5 when determined by flow cytometry. There was also no apparent difference between sham and Ang II treatments.

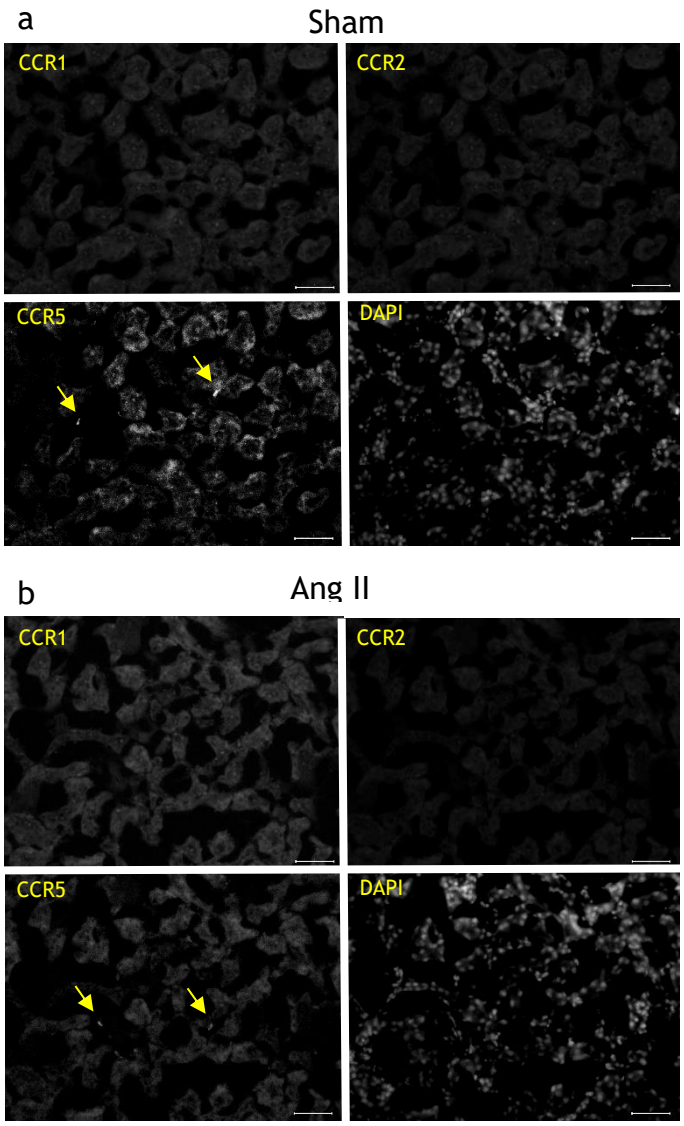


Figure 3-11. Kidney iCCR expression in REP mice after 7 days of Ang II induced hypertension
CCR1, CCR2, CCR5 and Dapi were detected in the kidneys of Rep mice by fluorescent microscopy. Representative images after 7 days of sham (a) or Ang II treatment (b) are shown. Scale bar = 50 µm. Yellow arrows point to some CCR5+ cells.

3.3.5 Aorta and PVAT

In the PVAT, CD19+ B cells, CD3+ T cells, CD11b+ cells, CD11b+F4/80+ cells and F4/80+ cells were gated from live, CD45+ cells (Figure 3-12). CD11b+, CD11b+F4/80+ and F4/80+ cells represent heterogeneous populations including macrophages and monocytes (Misharin et al., 2013; Xiong et al., 2018). Following 7 days of Ang II induced hypertension or sham treatment, PVAT CD19+ B cells expressed CCR1 and low amounts of CCR2 or CCR5 (Figure 3-13a). CD3+ cells expressed very low and variable amounts of all iCCRs (Figure 3-13b).

CD11b+ cells most highly expressed CCR2 and also expressed CCR1 and CCR5 (Figure 3-13c). When co-expression of these receptors was investigated, the majority of CD11b+ cells were found to not express any of the iCCRs and around 20 % only expressed CCR2. A small proportion of CD11b+ cells were CCR2+CCR5+ or CCR1+CCR2+CCR5+ (Figure 3-14a). CD11b+F4/80+ cells also expressed these three receptors (Figure 3-13d). Most of these cells did not express any of the iCCRs whilst some were shown to be CCR2+, CCR5+, CCR1+CCR2+ or CCR1+CCR5+ (Figure 3-14b). F4/80+ cells expressed variable amounts of all the iCCRs (Figure 3-13e). There were no differences in PVAT leukocyte iCCR expression between sham and Ang II treated mice.

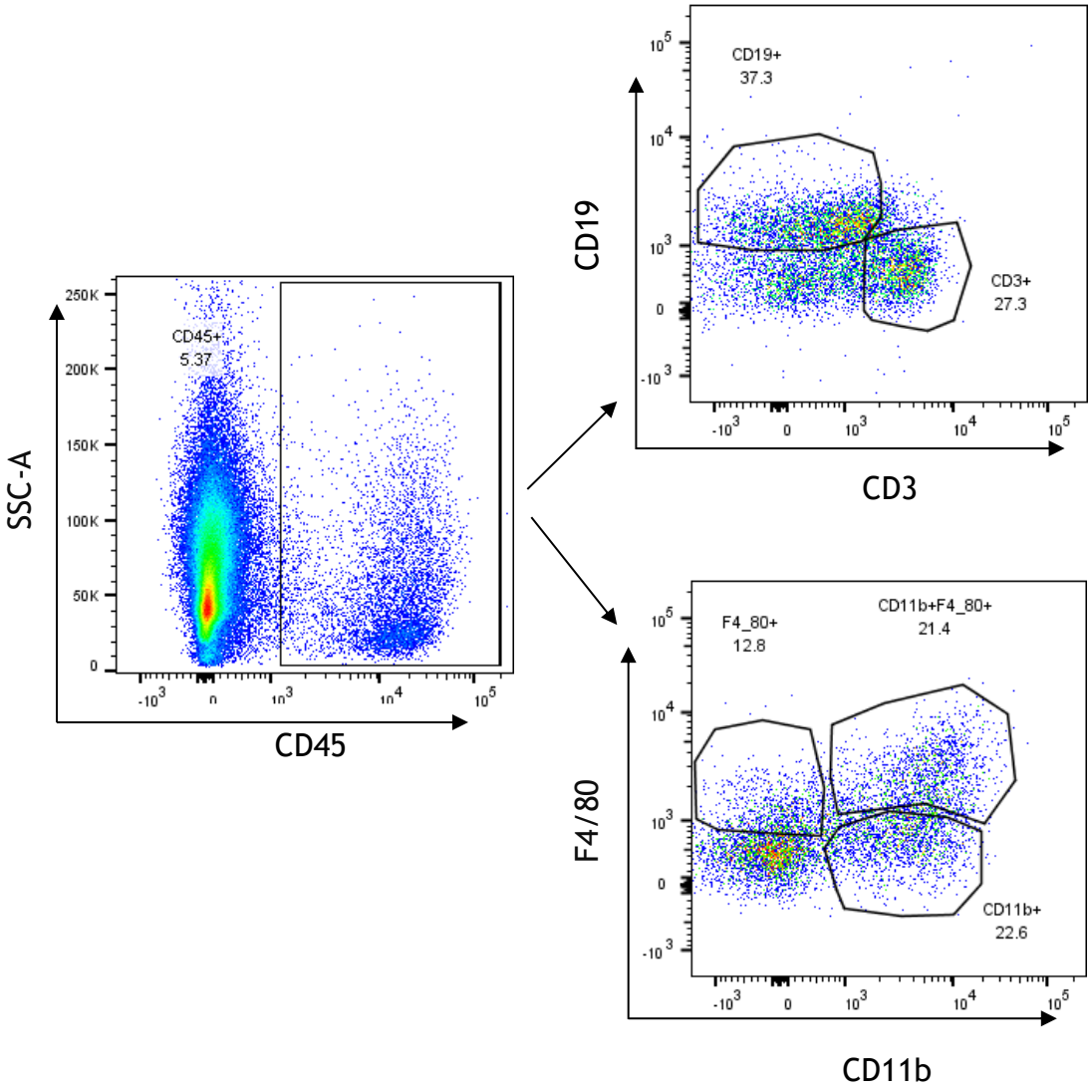


Figure 3-12. Gating strategy for the analysis of PVAT leukocytes from mice with Ang II induced hypertension

CD45+ cells were gated from live, single cells. CD19+, CD3+ and CD11b+ cells were gated from the CD45+ cells.

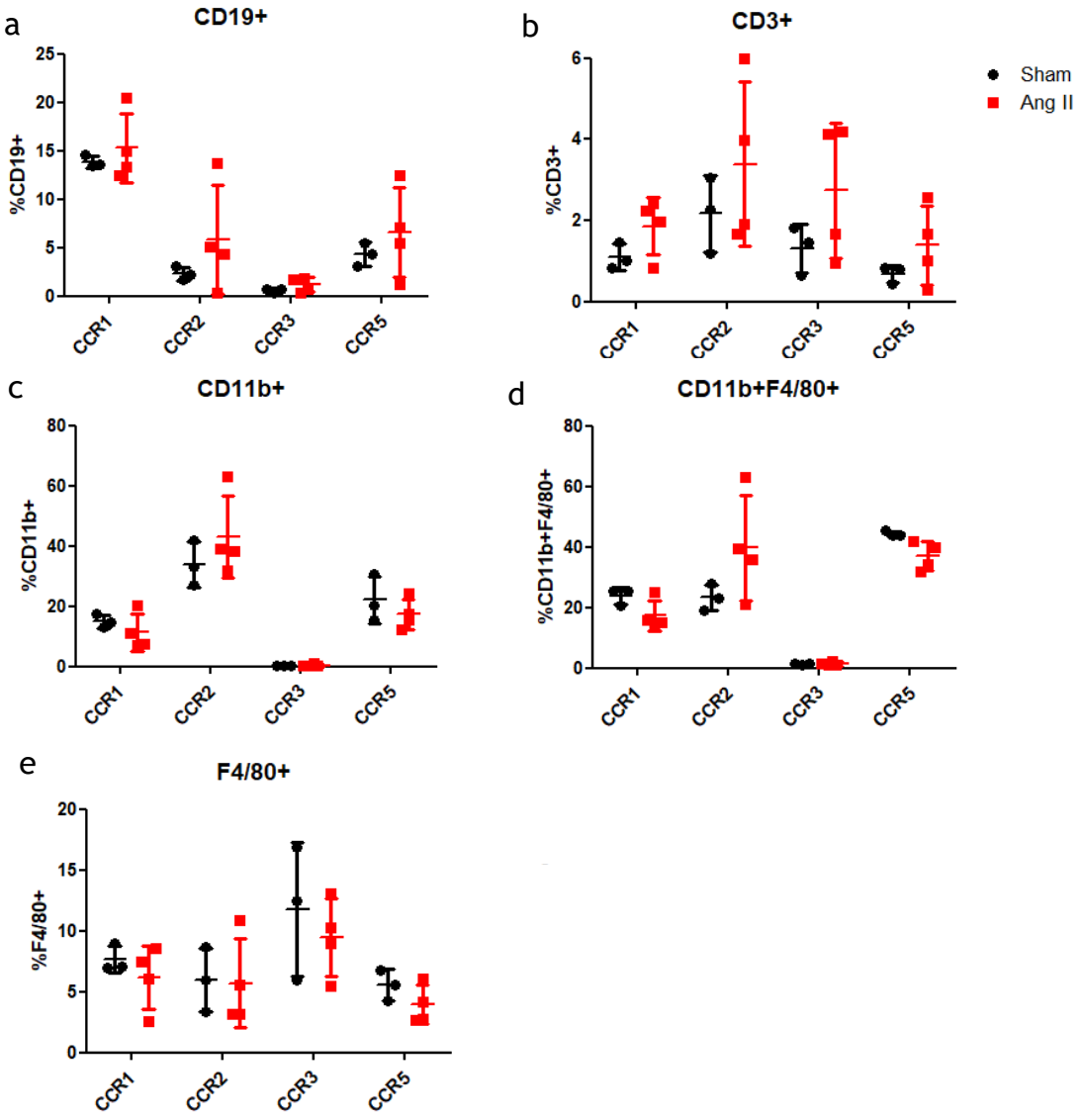


Figure 3-13. iCCR expression by leukocytes in the PVAT of Rep mice after 7 days of Ang II induced hypertension

The expression of, CCR1, CCR2, CCR3 and CCR5 by CD19+ cells (a), CD3+ cells (b), CD11b+ cells (c), CD11b+F4/80+ cells (d) and F4/80+ cells (e) in the PVAT of Rep mice after 7 days of sham or Ang II treatment.

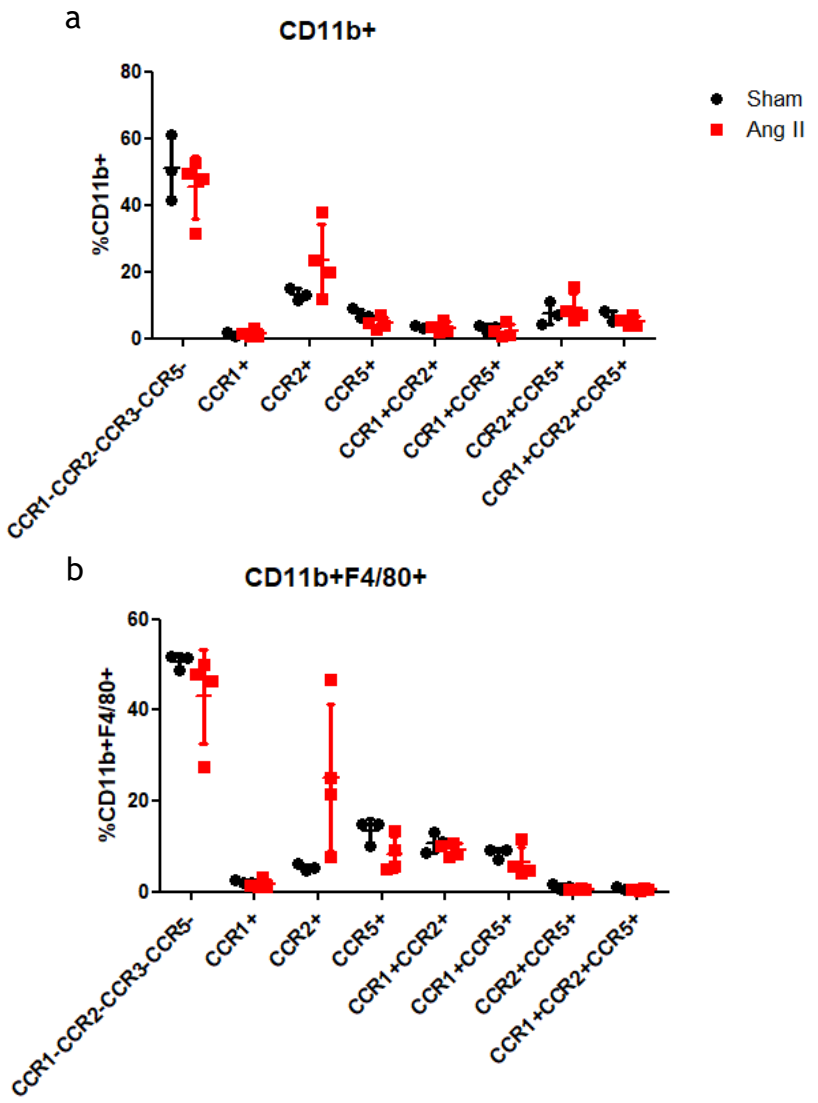


Figure 3-14. iCCR co-expression by PVAT leukocytes from REP mice subject to 7 days of Ang II induced hypertension
The co-expression of CCR1, CCR2 and CCR5 by CD11b+ cells (a) and CD11b+F4/80+ cells (b) in the PVAT of REP mice after 7 days of Ang II or sham treatment.

Clear expression of CCR5 was observed in the adventitia and PVAT of aortas from sham and Ang II treated mice. These could be CD11b+ or F4/80+ cells. CCR1 was not expressed and very low amounts of CCR2 were observed in some samples (Figure 3-15). iCCRs were not detected in the medial or endothelial layer of the aorta and CCR5 expression did not appear to be effected by Ang II treatment.

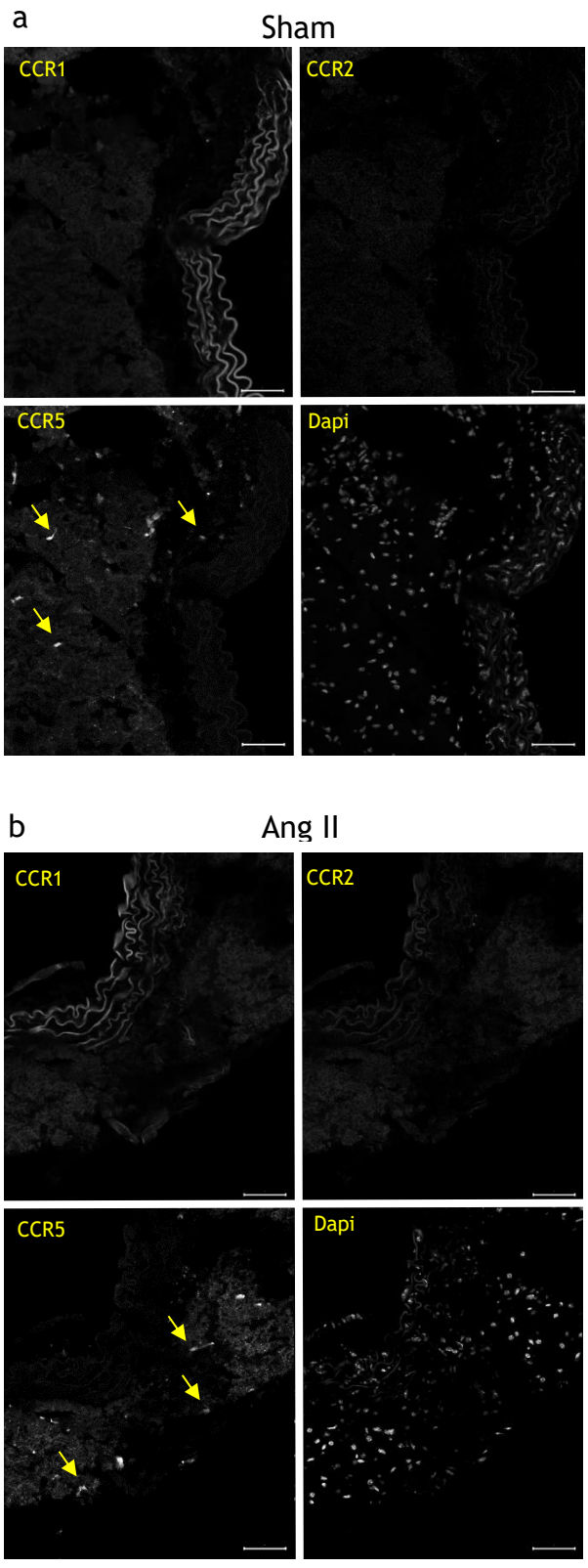


Figure 3-15. Aortic iCCR expression in REP mice after 7 days of Ang II induced hypertension
CCR1, CCR2, CCR5 and Dapi were detected in the aortas of Rep mice by fluorescent microscopy. Representative images after 7 days of sham (a) or Ang II treatment (b) are shown. Scale bar = 50 µm. Yellow arrows point to some CCR5+ cells.

3.3.6 Heart

Heart tissue was not analysed by flow cytometry as an effective cell isolation protocol had not been established but the tissue was analysed by microscopy. Low expression of CCR5 could be detected in the hearts after 7 days of sham or Ang II treatment, but it was not seen in all samples. CCR1 and CCR2 were not detected (Figure 3-16). CCR5 expression did not appear to be effected by Ang II induced hypertension. These CCR5+ cells could be macrophages or T cells as these cell types in other peripheral tissues, such as the kidney, expressed CCR5.

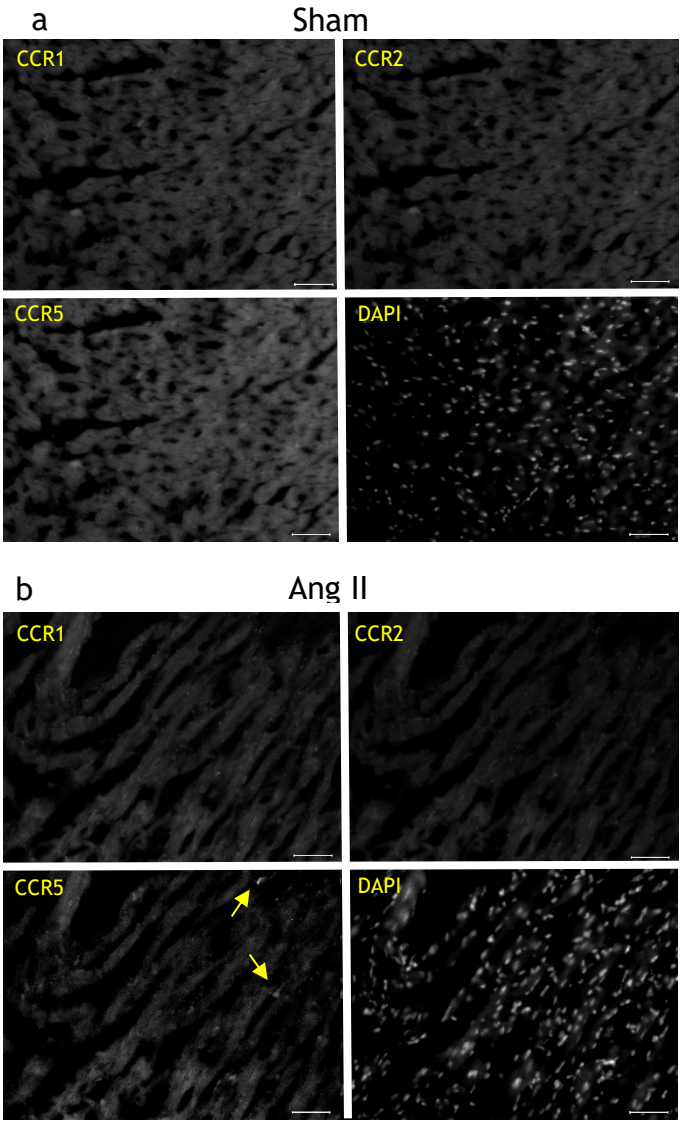


Figure 3-16. Heart iCCR expression in REP mice after 7 days of Ang II induced hypertension
CCR1, CCR2, CCR5 and Dapi were detected in the hearts of Rep mice by fluorescent microscopy. Representative images after 7 days of sham (a) or Ang II treatment (b) are shown. Scale bar = 50 µm. Yellow arrows point to some CCR5+ cells.

3.4 iCCR expression in REP mice following 14 days of Ang II induced hypertension

REP mice were subject to Ang II induced hypertension for 14 days and the expression of iCCRs by different subsets of leukocytes in the bone marrow, blood, spleen and kidneys was determined by flow cytometry. PVAT was not analysed as not all the lymph nodes in the PVAT were able to be removed during isolation. Gating strategies used were as detailed in section 3.3. CCR1, CCR2 and CCR5 expression in the kidneys, heart, aorta and PVAT was also investigated using fluorescent microscopy.

3.4.1 Bone marrow

After 14 days of Ang II induced hypertension about 15 – 20 % of CD19+ B cells expressed CCR1 (Figure 3-17a). This was slightly higher than the expression observed at the 7 day time point and CCR1 expression did not increase in the Ang II treated mice. CD11b+SSChi cells only expressed CCR3 (Figure 3-17b). CD11b+SSCint cells expressed CCR1 and CCR3, had the highest expression of CCR2 and did not express CCR5 (Figure 3-17c). These cell types showed a similar expression pattern to that seen after 7 days of sham or Ang II treatment and there were no differences in iCCR expression between sham and Ang II treated mice.

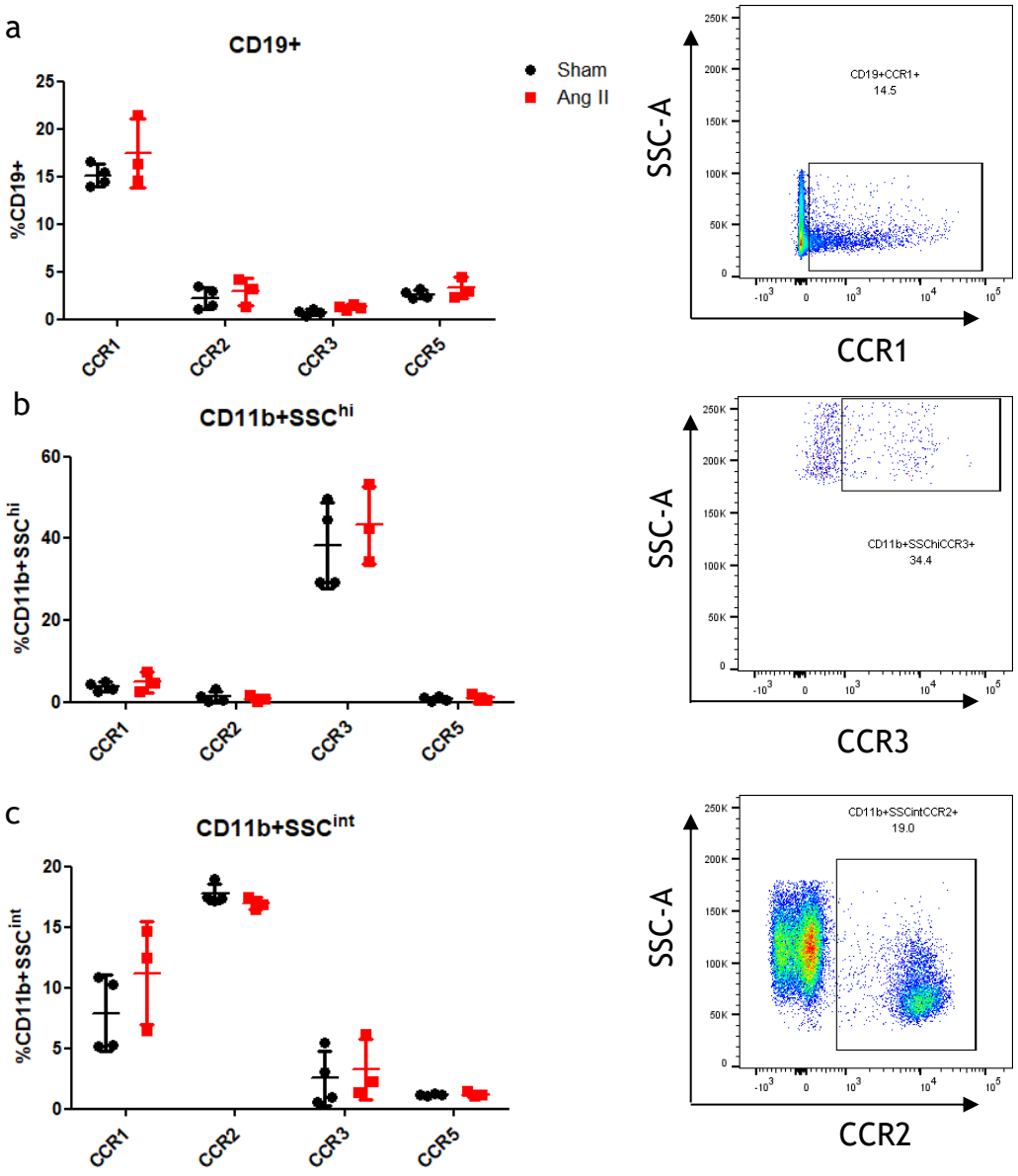


Figure 3-17. iCCR expression by leukocytes in the bone marrow of REP mice after 14 days of Ang II induced hypertension
The expression of CCR1, CCR2, CCR3 and CCR5 by CD19+ cells (a), CD11b+SSChi cells (b) and CD11b+SSCint cells (c) in the bone marrow of REP mice after sham or Ang II treatment for 14 days. Representative flow cytometry plots of CD19+ cell CCR1, CD11b+SSChi cell CCR3 and CD11b+SSCint cell CCR2 expression are shown. Data are combined from two independent experiments.

3.4.2 Blood

In sham and Ang II treated mice, blood CD19+ B cells expressed CCR1 whilst about 2 – 6 % of CD3+ T cells expressed CCR2 (Figure 3-18c, d). CD11b+SSChi cells expressed variable amounts of CCR3. This expression tended to be higher than that observed at the 7 day time point (Figure 3-18c). A small amount of CCR1, CCR2 and CCR3 could be detected on CD11b+SSCint cells (Figure 3-18d).

CD11b+SSClo cells were shown to express CCR2 and CCR5, with some cells co-expressing both receptors (Figure 3-18e, f). Furthermore, about 40 % of CD11b+SSClo cells expressed CCR5 after 14 days of treatment but only about 20 % were shown to be CCR5+ after 7 days. The expression of iCCRs by these cell subsets was not effected by Ang II treatment.

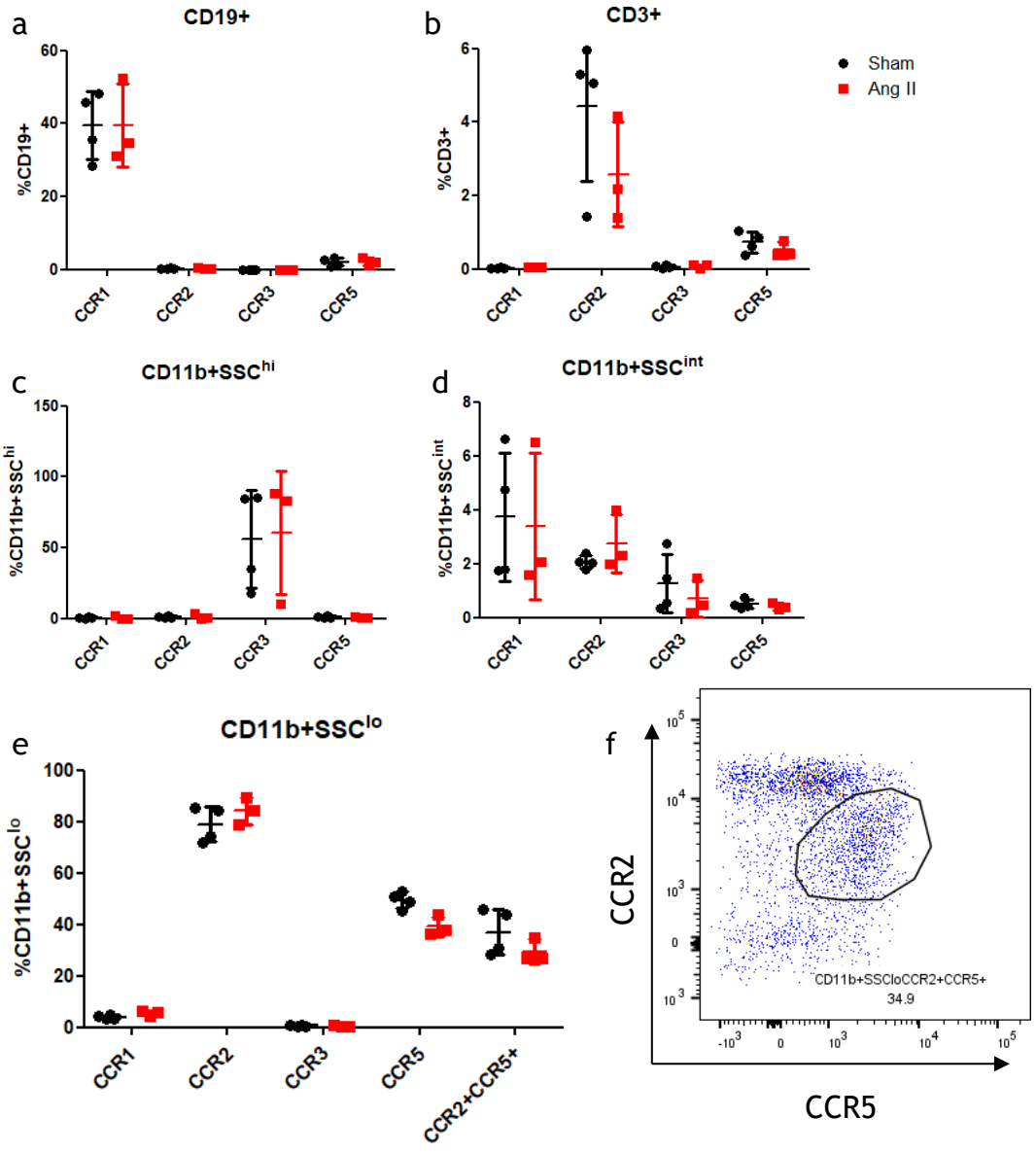


Figure 3-18. iCCR expression by leukocytes in the blood of REP mice after 14 days of Ang II induced hypertension

The expression of CCR1, CCR2, CCR3 and CCR5 by CD19+ cells (a), CD3+ cells (b) CD11b+SSChi cells (c), CD11b+SSCint cells (d) and CD11b+SSClo cells (e) in the blood of REP mice after sham or Ang II treatment for 14 days. A representative flow cytometry plot of CD11b+SSClo cell CCR2 and CCR5 co-expression is shown (f). Data are combined from two independent experiments.

3.4.3 Spleen

After 14 days of Ang II induced hypertension, spleen leukocytes showed a similar iCCR expression pattern to that observed after 7 days. About 40 – 50 % of CD19+ B cells expressed CCR1, although this was slightly higher than the expression detected after 7 days (Figure 3-19a). A small proportion of T cells expressed CCR2, CCR3 or CCR5 (Figure 3-19b). CCR3 was expressed by CD11b+SSChi cells and CD11b+SSCint cells, which may also have expressed small amounts of CCR1, CCR2 and CCR5 (Figure 3-19c, d). CD11b+SSClo cells expressed CCR2 and CCR5. Co-expression of these receptors was observed and showed that most of the CCR5+ CD11b+SSClo cells also expressed CCR2 (Figure 3-19e, f). F4/80+ cells in the spleen most highly expressed CCR3 (Figure 3-19g, h). There were no differences in iCCR expression by any of these cell types between sham and Ang II treated mice.

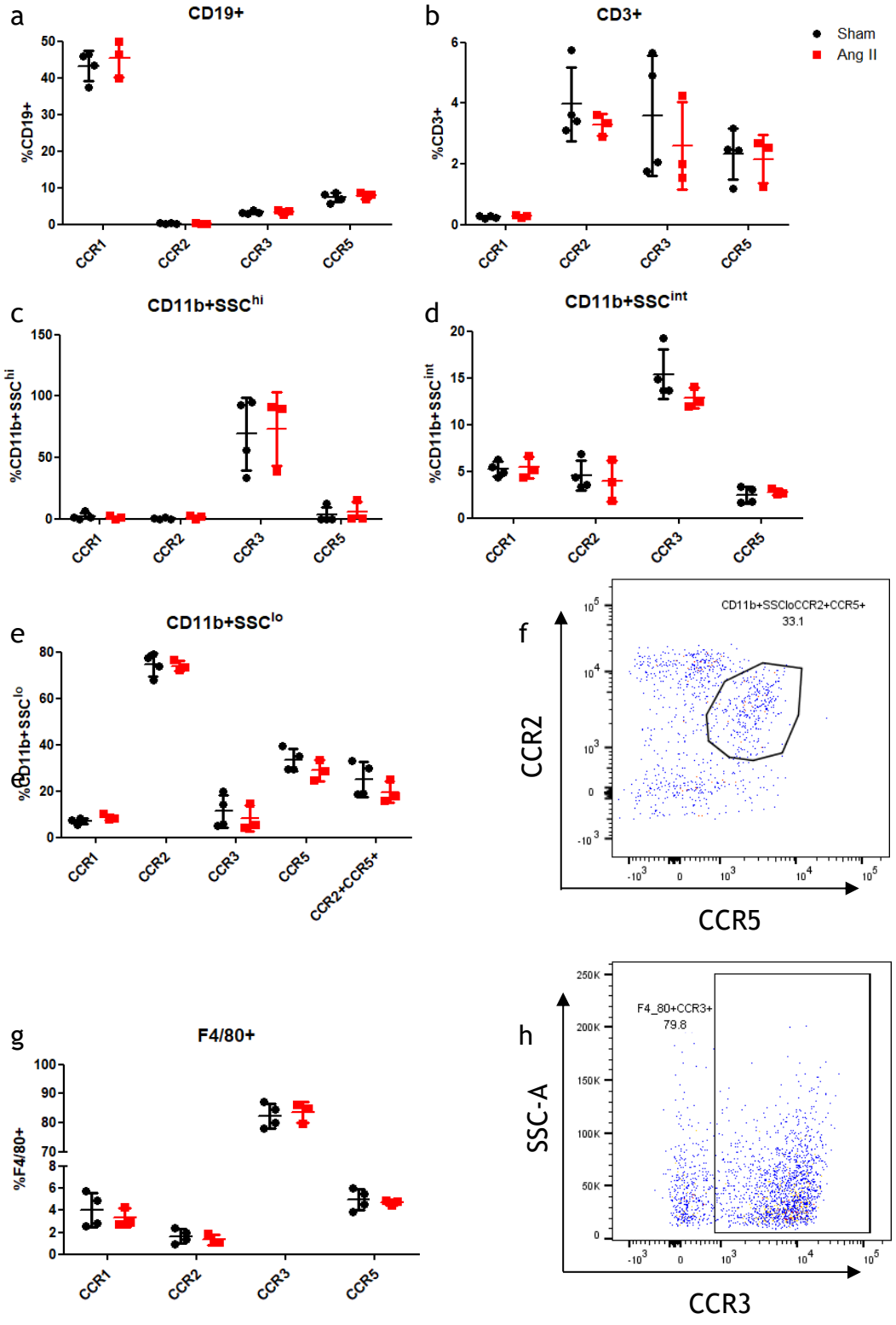


Figure 3-19. iCCR expression by leukocytes in the spleens of REP mice after 14 days of Ang II induced hypertension

The expression of CCR1, CCR2, CCR3 and CCR5 by CD19+ cells (a), CD3+ cells (b) CD11b+SSChi cells (c), CD11b+SSCint cells (d) CD11b+SSClo cells (e) and F4/80+ cells (g) in the spleen of REP mice after sham or Ang II treatment for 14 days. Representative flow cytometry plots of CD11b+SSClo cell CCR2 and CCR5 co-expression (f) and F4/80+ cell CCR3 expression (h) are shown. Data are combined from two independent experiments.

3.4.4 Kidney

Up to 40 % of kidney CD19+ B cells expressed CCR1 after 14 days of both Ang II or sham treatment (Figure 3-20a). This is higher than the amount expressing CCR1 after 7 days. CD3+ T cells expressed CCR2 and CCR5 (Figure 3-20b). CCR2 and CCR5 were also expressed by CD11b+ cells and F4/80+ cells whilst F4/80+ cells also expressed CCR1 (Figure 3-20c, d). Similar to the 7 day timepoint, most CD11b+ cells were CCR2+ or did not express any of the iCCRs and about 10 % were CCR2+CCR5+ (Figure 3-20e). Most F4/80+ cells expressed both CCR1 and CCR5 whilst 20 – 30 % only expressed CCR5 and about 20 % expressed both CCR2 and CCR5 (Figure 3-20f). None of the cell types analysed showed a difference in iCCR expression between sham and Ang II treated mice.

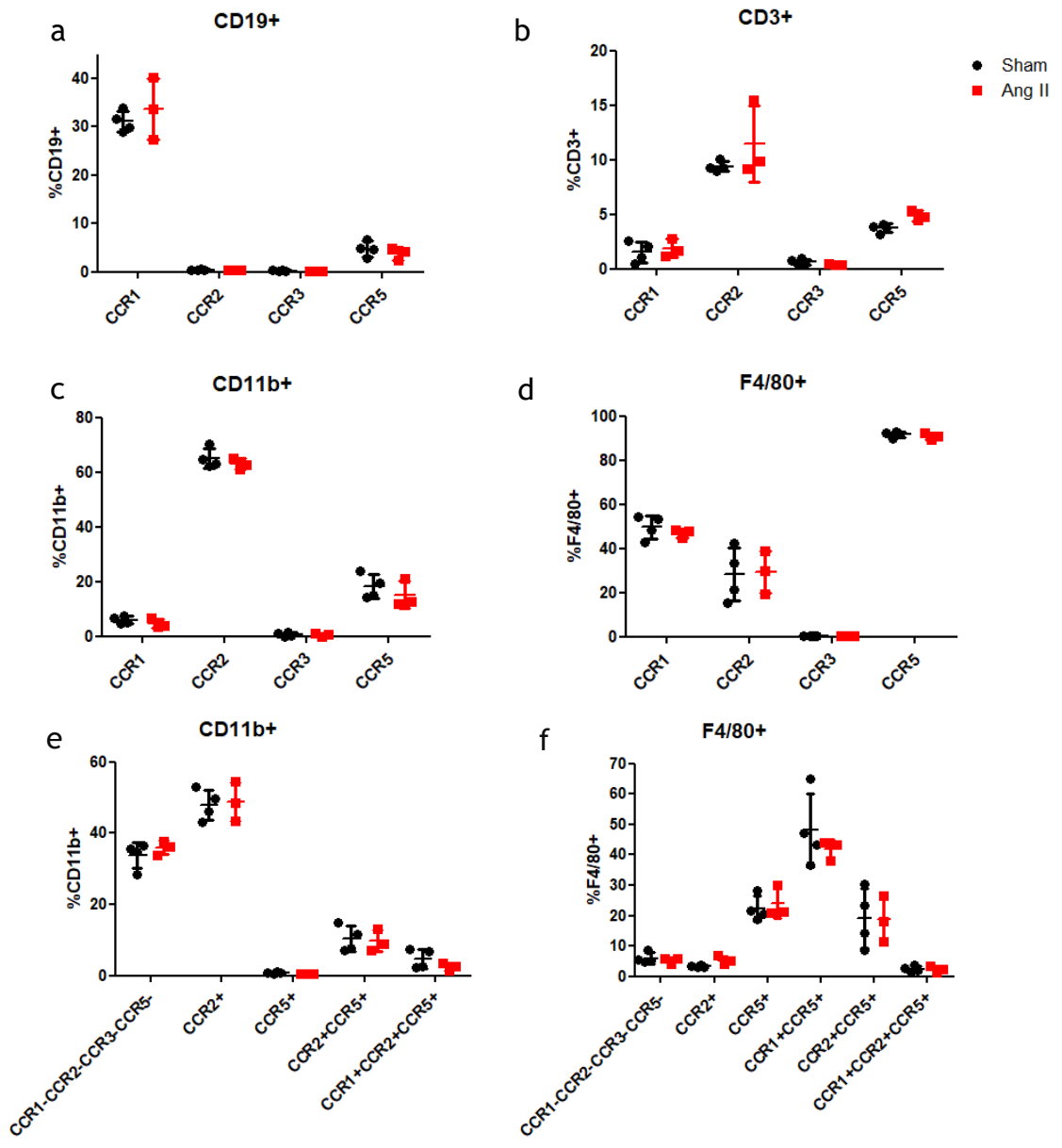


Figure 3-20. iCCR expression by leukocytes in the kidneys of REP mice after 14 days of Ang II induced hypertension
The expression of CCR1, CCR2, CCR3 and CCR5 by CD19+ cells (a), CD3+ cells (b) CD11b+ cells (c) and F4/80+ cells (d) and co-expression of CCR1, CCR2 and CCR5 by CD11b+ cells (e) and F4/80+ cells (f) in the kidneys of REP mice after sham or Ang II treatment for 14 days. Data are combined from two independent experiments.

Both CCR5 and CCR2 could be seen in the kidney when frozen tissue sections were imaged after 14 days of sham or Ang II treatment. (Figure 3-21). These may represent CD3+ T cells, CD11b+ cells or F480+ cells. CCR5 appeared to be more highly expressed than CCR2 and there was no apparent difference between sham and Ang II treatments.

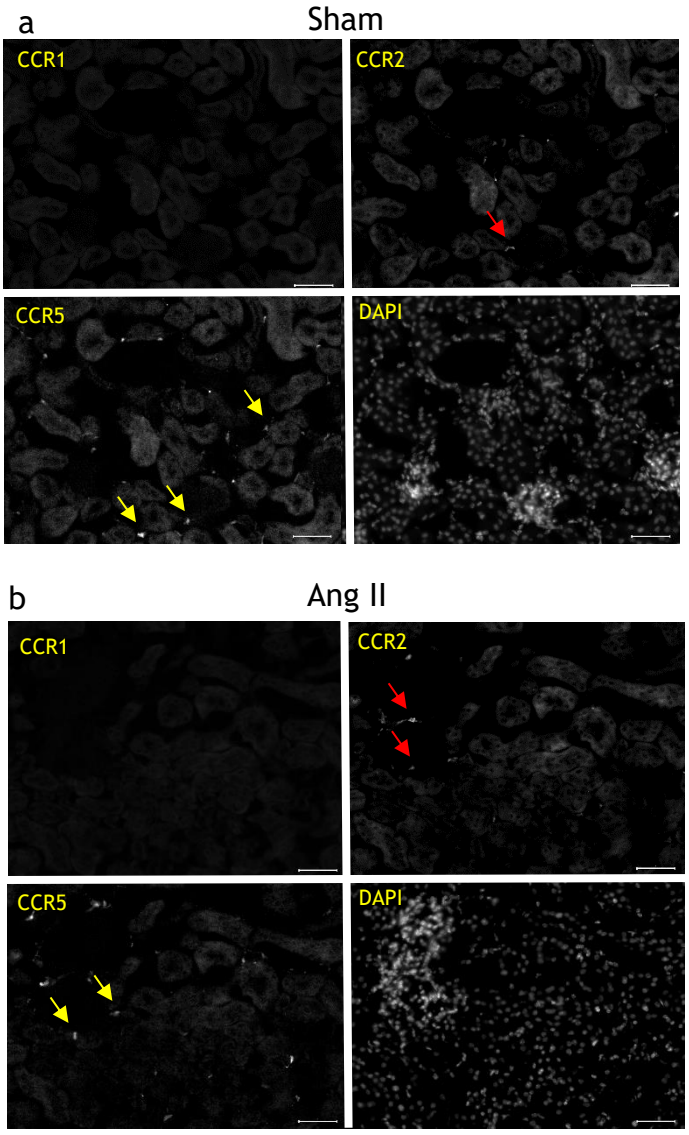


Figure 3-21. Kidney iCCR expression in REP mice after 14 days of Ang II induced hypertension
CCR1, CCR2, CCR5 and Dapi were detected in the kidneys of REP mice by fluorescent microscopy. Representative images after 14 days of sham (a) or Ang II treatment (b) are shown.

Scale bar = 50 µm. Red arrows point to some CCR2+ cells. Yellow arrows point to some CCR5+ cells.

3.4.5 Aorta and PVAT

After 14 days of Ang II induced hypertension, CCR2 and CCR5, but not CCR1, were detected in the aortic adventitia and PVAT of REP mice (Figure 3-22). These could be expressed by CD11b+ cells, which were shown to express CCR2 and CCR5 at the 7 day time point. These iCCRs were not seen in the medial or endothelial layers of the aorta and no clear difference in iCCR expression between sham and Ang II treated mice was observed.

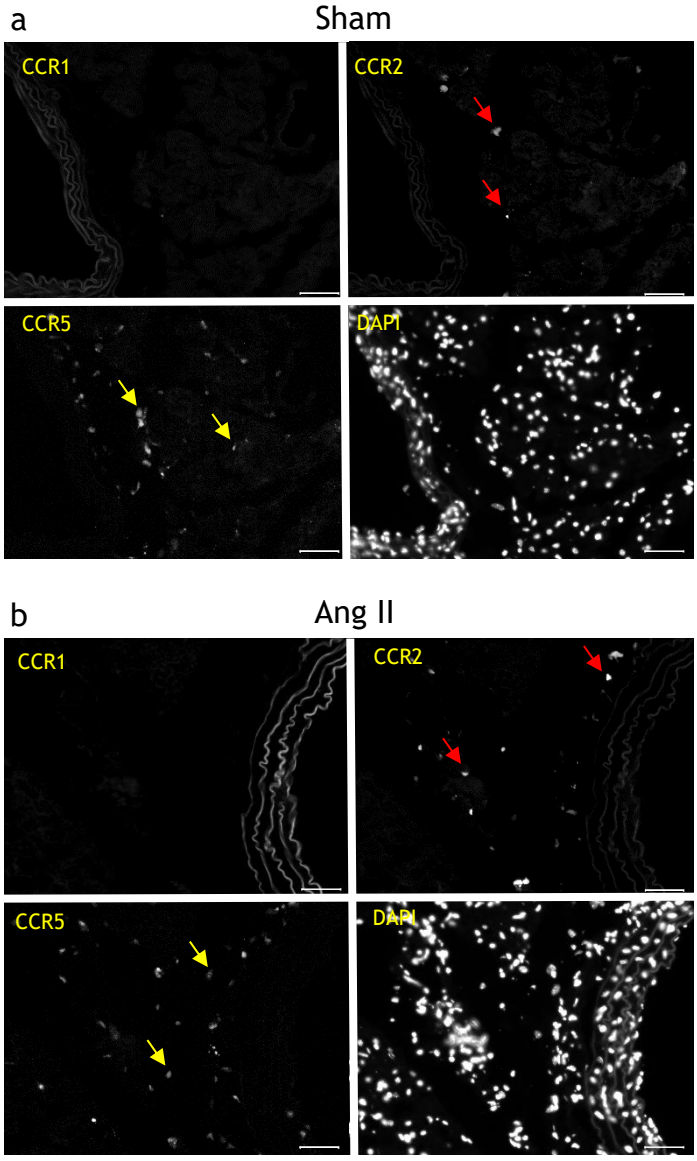


Figure 3-22. Aortic iCCR expression in REP mice after 14 days of Ang II induced hypertension

CCR1, CCR2, CCR5 and Dapi were detected in the aortas of REP mice by fluorescent microscopy. Representative images after 14 days of sham (a) or Ang II treatment (b) are shown. Scale bar = 50 µm. Red arrows point to some CCR2+ cells. Yellow arrows point to some CCR5+ cells.

3.4.6 Heart

In the hearts of sham and Ang II treated REP mice, CCR5 could clearly be detected. CCR2 was also observed in low amounts and CCR1 was not detected at all (Figure 3-23). This may indicate the presence of CD3+ cells, CD11b+ cells or F4/80+ cells in the heart. CCR2 and CCR5 expression did not appear to be affected by Ang II induced hypertension.

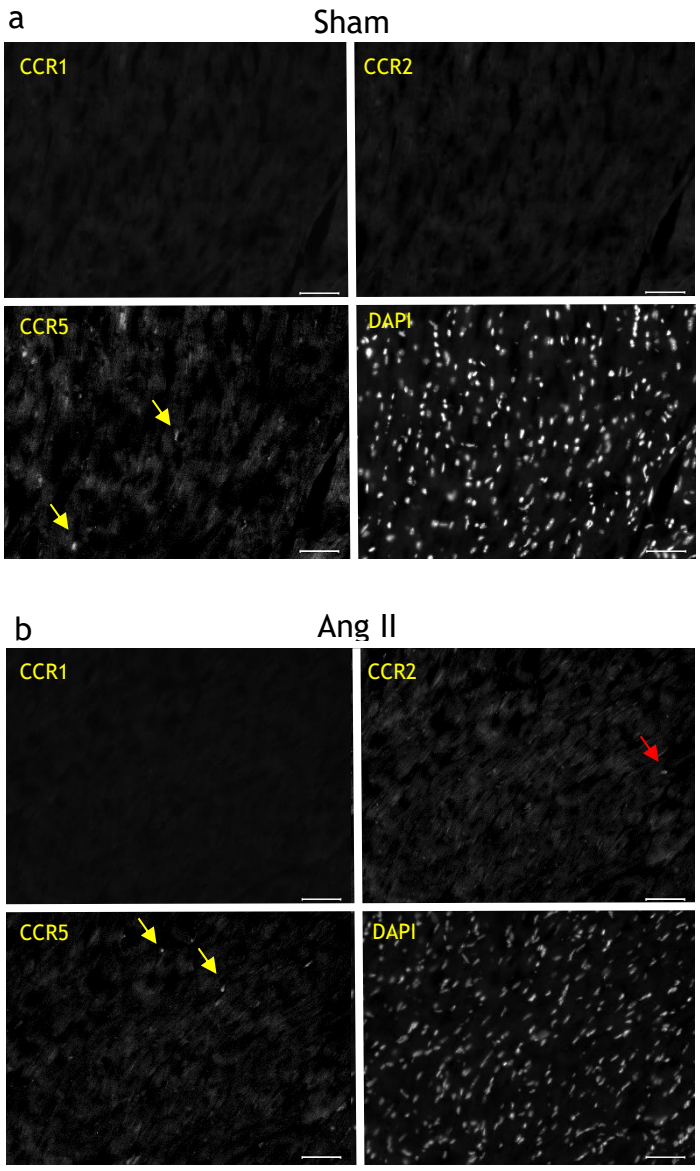


Figure 3-23. Heart iCCR expression in REP mice after 14 days of Ang II induced hypertension

CCR1, CCR2, CCR5 and Dapi were detected in the hearts of REP mice by fluorescent microscopy. Representative images after 14 days of sham (a) or Ang II treatment (b) are shown. Scale bar = 50 µm. Red arrows point to some CCR2+ cells. Yellow arrows point to some CCR5+ cells.

3.5 Use of antibodies and chemokines to determine iCCR expression by flow cytometry

To determine how flow cytometry with antibody staining compares to using REP mice, WT mice were subject to Ang II induced hypertension for 14 days and the expression of CCR2, CCR3 and CCR5 was investigated using flow cytometry. Antibodies for CCR3 and CCR5 and fluorescently labelled CCL2 to detect CCR2 (Ford et al., 2014) were used. A specific antibody for CCR1 was unavailable. The bone marrow, blood, spleen, kidney and PVAT were analysed. The gating strategies used were as described in section 3.3.

3.5.1 Bone marrow

Following 14 days of Ang II or sham treatment, CCR2, CCR3 and CCR5 were not detected on CD19+ B cells in the bone marrow (Figure 3-24a). CD11b+SSChi cells expressed CCR3 (Figure 3-24b, d) and 10 – 15% of CD11b+SSCint cells expressed CCR2 (Figure 3-24c, e). Ang II treatment did not affect iCCR expression by these cell types.

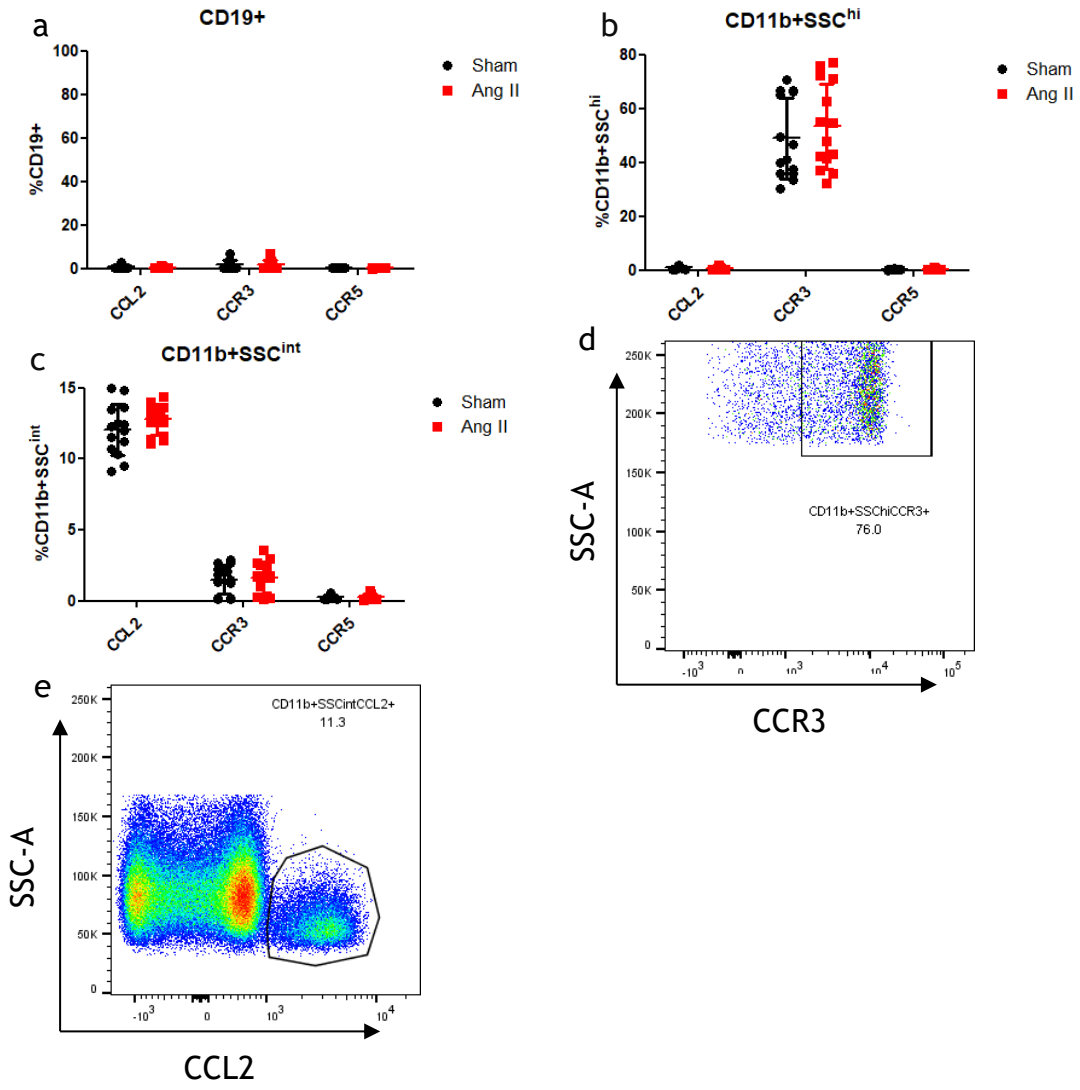


Figure 3-24. iCCR expression by leukocytes in the bone marrow of WT mice after Ang II induced hypertension
The expression of, CCR2 (using CCL2 conjugated to AF647), CCR3 and CCR5 by CD19+ cells (a), CD11b+SSChi cells (b) and CD11b+SSCint cells (c) in the bone marrow of WT mice after 14 days of sham or Ang II treatment. Representative flow cytometry plots of CD11b+SSChi cell CCR3 expression (d) and CD11b+SSClo cell CCR2 expression (e) are shown. Data are combined from three independent experiments.

3.5.2 Blood

Neither CCR2, CCR3 nor CCR5 were detected on CD19+ B cells in the blood of WT mice after sham or Ang II treatment and very little, if any, were observed on CD3+ T cells (Figure 3-25a, b). CD11b+SSChi cells highly expressed CCR3 (Figure 3-25c, d). A small proportion of CD11b+SSCint cells expressed CCR2 (Figure 3-25e) but CCR2 was most highly expressed by CD11b+SSClo cells. CCR2 expression by these cells also increased in response to Ang II (Figure 3-25f; Sham CCL2 35.1 ± 5.0 vs Ang II CCL2 43.3 ± 8.4 % CD11b+SSClo cells, $p < 0.05$, t test).

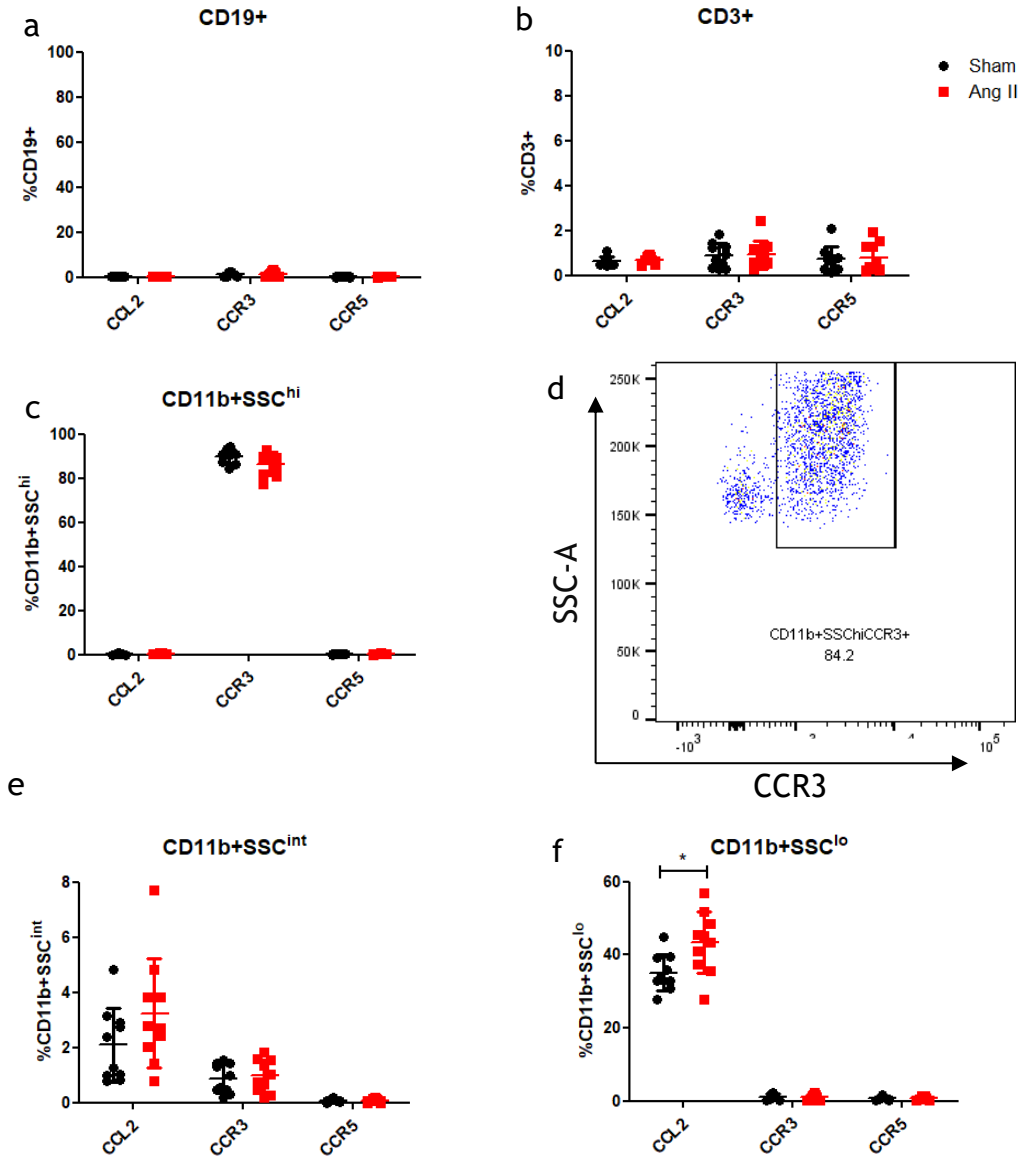


Figure 3-25. iCCR expression by leukocytes in the blood of WT mice after Ang II induced hypertension
The expression of, CCR2 (using CCL2 conjugated to AF647), CCR3 and CCR5 by CD19+ cells (a), CD3+ cells (b) CD11b+SSChi cells (c), CD11b+SSCint cells (e) and CD11b+SSClo cells (f) in the blood of WT mice after 14 days of sham or Ang II treatment. A representative flow cytometry plot of CD11b+SSChi cell CCR3 expression is shown (d). * $p < 0.05$, t test. Data are combined from two independent experiments.

3.5.3 Spleen

CCR2, CCR3 and CCR5 were not detected on spleen CD19+ B cells. Very low CCR2 and CCR3 expression was shown by CD3+ T cells in both sham and Ang II treated mice (Figure 3-26a, b). CD11b+SSChi cells only expressed CCR3 and CD11b+SSCint cells expressed CCR2, CCR3 and CCR5 (Figure 3-26c, d). Only CCR2 was expressed by CD11b+SSClo cells and CCR3 was shown on F4/80+ cells (Figure

3-26e, f). There were no differences between the mice given sham or Ang II treatments.

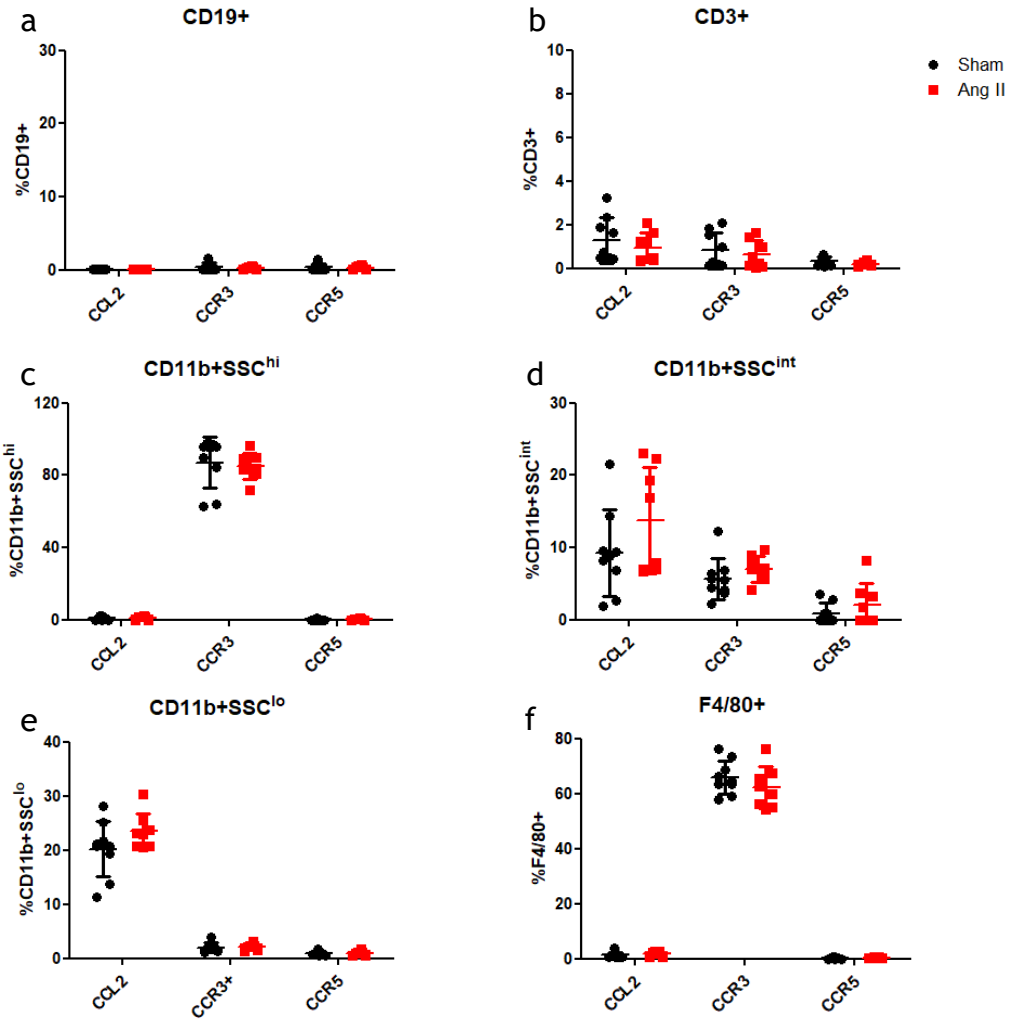


Figure 3-26. iCCR expression by leukocytes in the spleens of WT mice after Ang II induced hypertension
The expression of, CCR2 (using CCL2 conjugated to AF647), CCR3 and CCR5 by CD19+ cells (a), CD3+ cells (b) CD11b+SSChi cells (c), CD11b+SSCint cells (d), CD11b+SSClo cells (e) and F4/80+ cells (f) in the spleens of WT mice after 14 days of sham or Ang II treatment. Data are combined from two independent experiments.

3.5.4 Kidney

CCR2 and CCR5 expression by leukocytes in the kidneys was determined but CCR3 could not be measured as the antibody showed nonspecific binding when used to stain kidney cells. CD19+ B cells in sham and Ang II treated mice did not express CCR2 but may have expressed CCR5 (Figure 3-27a). Some CD3+ T cells expressed low levels of CCR2 and variable levels of CCR5 (Figure 3-27b). Both

CCR2 and CCR5 expression were detected on CD11b+ cells (Figure 3-27c) and CCR5 expression was lower in Ang II treated mice (Sham CCR5 12.4 ± 2.5 vs Ang II CCR5 7.4 ± 2.4 % CD11b+ cells, $p < 0.05$, t test). F4/80+ cells showed high levels of CCR5 expression and some CCR2 expression but there were no differences between sham and Ang II treatment (Figure 3-27d).

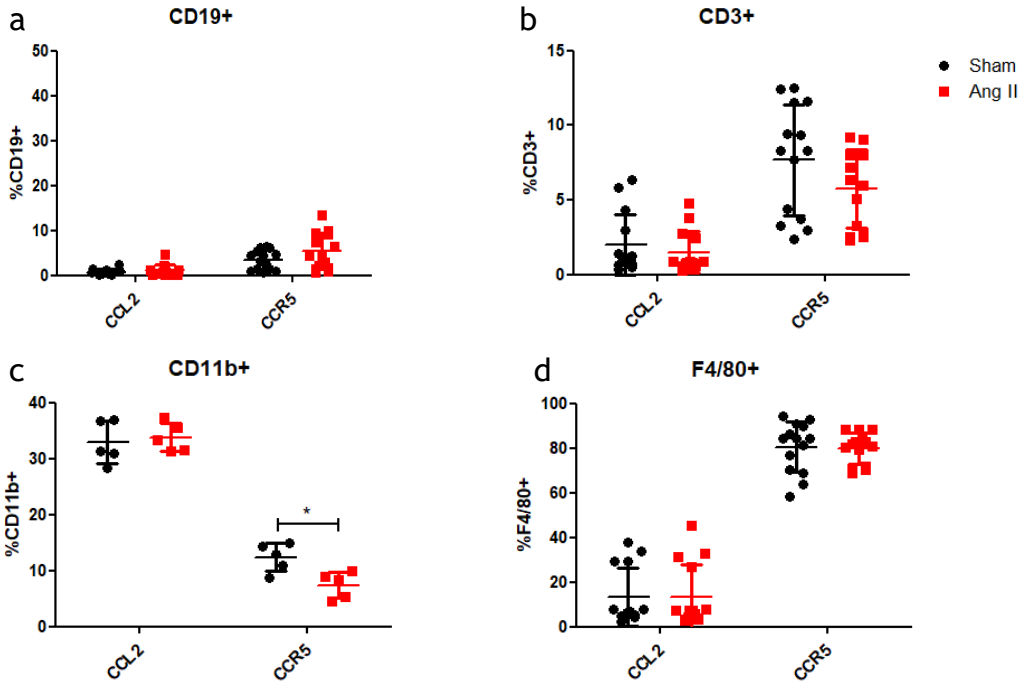


Figure 3-27. iCCR expression by leukocytes in the kidneys of WT mice after Ang II induced hypertension
The expression of, CCR2 (using CCL2 conjugated to AF647) and CCR5 by CD19+ cells (a), CD3+ cells (b), CD11b+ cells (c) and F4/80+ cells (d) in the kidneys of WT mice after 14 days of sham or Ang II treatment. * $p < 0.05$, t test. Data are combined from three independent experiments, except CD11b+ cells.

3.5.5 PVAT

CD19+ B cells and CD3+ T cells did not express CCR2 or CCR3 but may have expressed a small amount of CCR5 (Figure 3-28a, b). CD11b+ cells expressed both CCR2 and CCR5 (Figure 3-28c) and CCR5 expression was higher in the Ang II treated mice (Sham CCR5 24.7 ± 7.9 vs Ang II CCR5 39.6 ± 5.2 % CD11b+ cells, $p < 0.05$, Mann Whitney test). This was reflected in the representative flow cytometry plots that show more CCR5 staining of CD11b+ cells from Ang II treated mice (Figure 3-28e). There was also a higher number of CD11b+ cells in the mice treated with Ang II (Figure 3-28d; Sham 460 ± 200 vs Ang II 1560 ± 800

total cells/mg, $p < 0.05$, Mann Whitney test) The total number of cells was calculated per mg of tissue as the amount of PVAT isolated from each aorta was highly variable. F4/80+ cells were not detected in these samples.

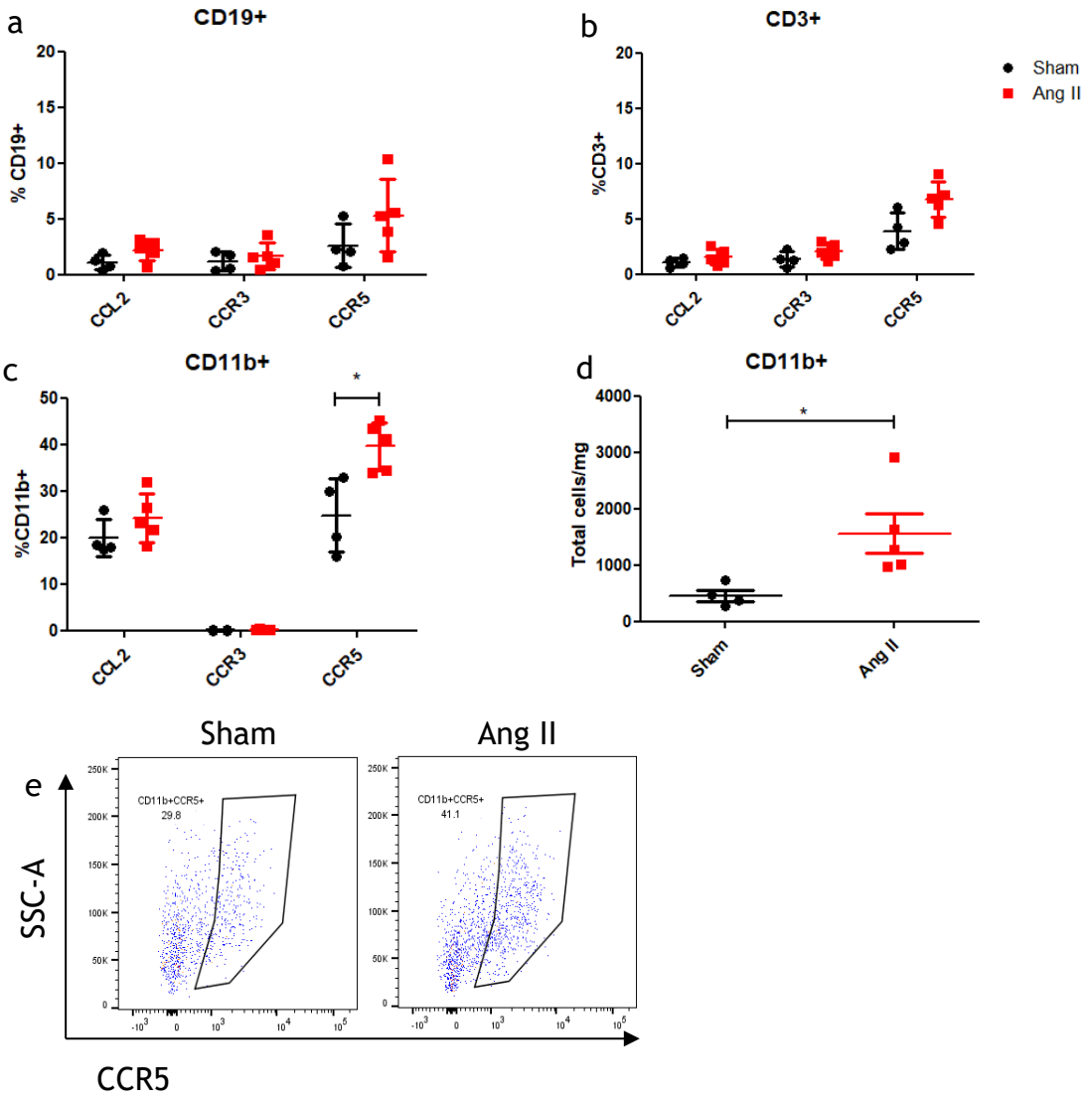


Figure 3-28. iCCR expression by leukocytes in the PVAT of WT mice after Ang II induced hypertension
The expression of, CCR2 (using CCL2 conjugated to AF647), CCR3 and CCR5 by CD19+ cells (a), CD3+ cells (b) and CD11b+cells (c) and total CD11b+ cells (d) in the PVAT of WT mice after 14 days of sham or Ang II treatment. Representative plots of CD11b+ cell CCR5 expression (e) are shown. * $p < 0.05$, Mann Whitney test.

3.6 iCCR mRNA expression

The expression of iCCRs in the kidneys, heart and aorta of WT mice subject to 14 days of Ang II induced hypertension was next determined by qPCR. In the kidney, CCR1 and CCR3 mRNA was not detected whilst there was expression of CCR2 and

CCR5 in both sham and Ang II treated mice (Figure 3-29a). There was no difference in CCR2 and CCR5 expression between the two groups. CCR3 mRNA was not detected in the hearts of WT mice whilst variable amounts of CCR1, CCR2 and CCR5 were detected in both sham and Ang II treated mice (Figure 3-29b). There was no difference in iCCR expression between the two groups.

CCR3 was not detected in the aorta, including the PVAT, of sham and Ang II treated mice. Low levels of CCR1 were detected after Ang II treatment (Figure 3-29c). Both CCR2 and CCR5 were detected and expression of these receptors increased with Ang II treatment (Sham CCR1 0.0005 ± 0.0001 vs Ang II CCR1 0.001 ± 0.0005, $p < 0.05$, t test; Sham CCR2 0.002 ± 0.0008 vs Ang II CCR2 0.004 ± 0.003, $p < 0.01$, t test; Sham CCR5 0.002 ± 0.0006 vs Ang II CCR5 0.005 ± 0.003, $p < 0.05$, t test).

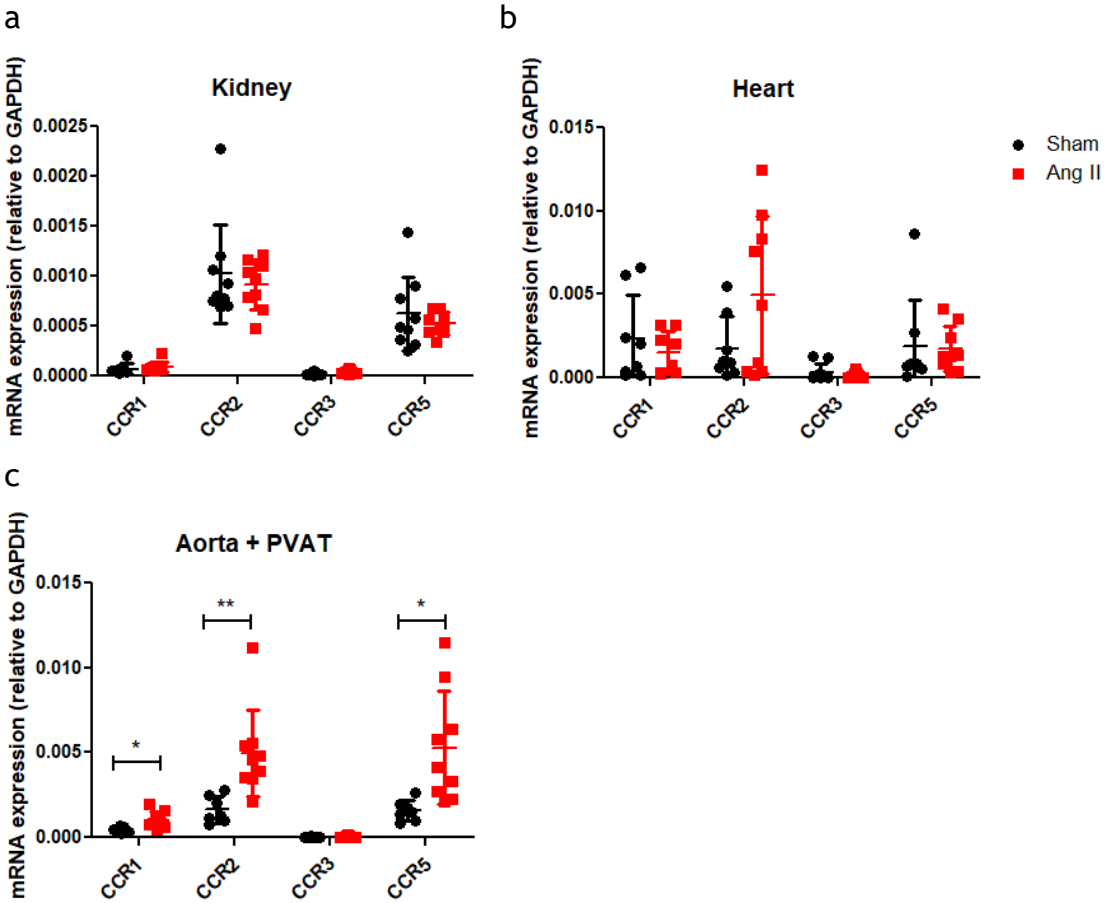


Figure 3-29. Kidney, heart and aortic iCCR mRNA expression of WT sham and Ang II treated mice

WT mice were treated with Ang II for 14 days and the expression of CCR1, CCR2, CCR3 and CCR5 in the kidneys (a), heart (b) and aorta (c) was determined by qPCR. * $p < 0.05$, ** $p < 0.01$, t test. Data are combined from two independent experiments.

3.7 Plasma chemokines and cytokines

Identifying which chemokines are upregulated in hypertension indicates which receptors are important. Therefore, the concentration of chemokines and cytokines in the plasma of WT mice after 14 days of sham or Ang II treatment was measured and are detailed in Table 3-1. Of the CC chemokines measured, CCL3, CCL7, CCL8, CCL11, CCL12, CCL19, CCL21 and CCL22 could be detected whilst CCL2, CCL4 and CCL20 were not detected. CCL5 could be measured in some samples but not all and CCL21 was lower in the hypertensive mice compared to the control mice. CXCL12 and CXCL13 were measured in the plasma but CXCL10 was not detected. CXCL13 concentration was lower in the plasma of Ang II treated mice than the sham treated mice. Of the cytokines measured, IL-1ß, IL-10 and VEGF were detected but no differences between sham and Ang II treated mice were observed. TNFα, IFNγ, IL-3, IL-6, IL-7, IL-17, IL-27 and GM-CSF could not be detected in the plasma.

Chemokine	Sham	Ang II
CCL3	1.0 ± 0.2	0.8 ± 0.2
CCL7	49.2 ± 28.6	27.3 ± 3.1
CCL8	4910 ±1691	7923 ± 1978
CCL11	969 ± 255	1086 ± 15.6
CCL12	80.7 ± 10.4	84.1 ± 4.3
CCL19	22.7 ± 19.2	12.5 ± 4.3
CCL21	1490 ± 104	1107 ± 219 *
CCL22	33.8 ± 16.0	38.2 ± 5.3
CXCL12	736 ± 274	568 ± 81.7
CXCL13	1049 ± 44.8	848 ± 131 *
IL-1ß	112 ± 6.7	110 ± 13.9
IL-10	5.4 ± 2.4	4.5 ± 1.2
VEGF	5.7 ± 1.3	4.5 ± 0.7

Table 3-1. Plasma chemokine and cytokine concentration in Ang II induced hypertension
WT mice were subject to 14 days of Ang II induced hypertension and the concentration of chemokines and cytokines (pg/ml) in the plasma was measured by a Luminex assay. * $p < 0.05$, Mann Whitney test.

3.8 Discussion

Leukocyte iCCR expression in sham and Ang II treated mice has been investigated using flow cytometry, fluorescent microscopy and qPCR. The data here show that expression of some iCCRs can be affected by Ang II and this depends on the tissue.

3.8.1 Using antibodies and fluorescent reporter mice for flow cytometry

Flow cytometry is a well-established technique used to identify leukocyte populations. However, using antibodies can lead to inaccurate results if the antibody binds to nonspecific, off-target proteins or does not effectively bind the target. The data here show various discrepancies in iCCR expression as determined using antibodies and using the REP mice (Table 3-2). For example, using fluorescently labelled CCL2, up to 40 % of kidney CD11b+ cells were found to express CCR2 whereas at least 60 % of kidney CD11b+ cells in the REP mice expressed CCR2. This was also seen for CD11b+SSClo cells in the spleen with up to 30 % of cells expressing CCR2 when detected with CCL2 compared to up to 80 % of cells expressing CCR2 in the spleens of REP mice. In the blood of REP mice, some CD11b+SSClo cells expressed CCR5 whereas the CCR5 antibody did not detect any CCR5 expression by these cells. However, this antibody did detect CCR5 on F4/80+ cells in the kidney at similar levels to that observed in the REP mice. The CCR3 antibody produced similar results to the REP mice when spleen, blood and bone marrow leukocytes were analysed but this antibody showed a lot of nonspecific staining when used to analyse kidney leukocytes. This suggests that how an antibody binds to its target depends on the tissue. This could be a result of the cell isolation protocol.

The REP mice did not respond to Ang II in the exact same way as WT mice. BP increased in the REP mice after Ang II treatment but was not as high as the WT mice. This is probably due to the variability of measurements taken using the tail cuff method and it is likely that if measured by telemetry, REP and WT mice would not have a difference in BP. Also, cardiac hypertrophy was shown in the Ang II treated WT mice but not the REP mice. This was probably due to variability of the sham treated REP mice. Carrying out a power calculation would have informed on the appropriate number of REP mice to use to show the effect of Ang II on cardiac hypertrophy and this should be considered for future experiments. Fluorescent protein expression by the REP mice reflects iCCR expression whilst antibodies are less accurate and less reliable than fluorescent reporter proteins. Therefore, the rest of this discussion will focus on data obtained from the REP mice. However differences in response to Ang II between

the WT and REP mice needs to be considered when interpreting the data from the REP mice.

Tissue	Cell type	iCCRs detected by antibodies	iCCRs detected with REP mice
Bone marrow			
	B cells	None	CCR1 CCR2 CCR5
	CD11b+SSChi	CCR3	CCR3
	CD11b+SSCint	CCR2 CCR3	CCR1 CCR2 CCR3
Blood			
	B cells	None	CCR1
	T cells	CCR2 CCR3 CCR5	CCR2 CCR5
	CD11b+SSChi	CCR3	CCR3
	CD11b+SSCint	CCR2 CCR3	CCR1 CCR2 CCR3
	CD11b+SSClo	CCR2	CCR2 CCR5
Spleen			
	B cells	None	CCR1 CCR5
	T cells	CCR2 CCR3	CCR2 CCR3 CCR5
	CD11b+SSChi	CCR3	CCR3
	CD11b+SSCint	CCR2 CCR3	CCR1 CCR2 CCR3 CCR5
	CD11b+SSClo	CCR2	CCR2 CCR5
	F4/80+	CCR3	CCR3
Kidney			
	B cells	None	CCR1 CCR5
	T cells	CCR2 CCR5	CCR2 CCR5
	CD11b+	CCR2 CCR5	CCR2 CCR5
	F4/80+	CCR2 CCR5	CCR1 CCR2 CCR5
PVAT			
	B cells	CCR5	CCR1 CCR2 CCR5
	T cells	CCR5	CCR1 CCR2 CCR3 CCR5
	CD11b+	CCR2 CCR5	CCR1 CCR2 CCR5

Table 3-2. Antibody and reporter iCCR detection on leukocytes
iCCR expression by leukocytes in the bone marrow, blood, spleen, kidney and PVAT was detected by flow cytometry using antibodies and fluorescent chemokines or REP mice. An antibody for CCR1 was not used as a specific CCR1 antibody was unavailable.

3.8.2 Leukocyte iCCR expression

B cells were shown to consistently express CCR1 in all tissues looked at here, although expression was variable between tissues. The highest expression of CCR1 by B cells was in the spleen and blood whilst the lowest was in the bone marrow. It is possible that CCR1 expression is upregulated once B cells have egressed from the bone marrow, and is involved in migration of B cells to, and localisation within, tissues. In some tissues, low levels of CCR2 or CCR5 were also detected. Few studies on B cell iCCR expression have been reported but expression of CCR1 and CCR2 by human peripheral blood B cells and CCR1, CCR2

and CCR5 by human tonsil B cells has been demonstrated by PCR or flow cytometry (Corcione et al., 2002; Kholodnyuk et al., 2017).

T cells in the kidney, spleen and blood express low levels of CCR2 and CCR5. Only up to 10 % of T cells express these receptors and expression varied between tissues. Most studies investigating iCCR expression by T cells have been carried out using human peripheral blood T cells. The data shown here are consistent with reports of CCR2 expression by 5 – 10 % of human T cells (Moon et al., 2011). However, CCR5 expression has been reported to be higher in these cells (Fukada et al., 2002; Szczepańska et al., 2015). Few studies on splenic T cell iCCR expression in mice have been reported. Kidney T cells have been shown to express CCR1 in a mouse model of renal injury, determined by RT-PCR (Anders et al., 2004). Different subsets of T cells were not analysed in this study but it is likely that Tc, Th_1, Th_2, Th_{17} and Treg cells have different iCCR expression patterns. For example, human Tregs have been shown to express CCR3 and CCR5 (Ahern et al., 2009). T cell subset iCCR expression in hypertension could be further investigated.

F4/80+ cells are a heterogenous population consisting of macrophages, eosinophils and some DC subsets (Misharin et al., 2013; Sheng et al., 2017). This study has shown that kidney F4/80+ cells express CCR1, CCR2 and CCR5 which has also recently been shown in a mouse model of renal injury (Kaneko et al., 2018). CCR2 was shown to be most highly expressed by F4/80+ cells in the renal injury model, whereas the data here suggest that CCR5 is expressed the most. This difference is possibly a result of using antibodies instead of reporter proteins or the effect of renal injury. In addition, mean fluorescence intensity (MFI) was used instead of expression as a percentage of cells so the actual proportion of F4/80+ cells expressing these receptors is not indicated. F4/80+ cells in the kidney and CD11b+F4/80+ cells in PVAT co-express CCR1, CCR2 and CCR5. This suggests that in peripheral tissues, as macrophages differentiate from CCR2+ monocytes, they upregulate CCR1 and CCR5. In the kidney 40 – 50 % of F4/80+ cells are CCR1+CCR5+. CCR1 and CCR5 may be important for localisation and activation within peripheral tissues whilst CCR2 is more important for recruitment to the tissue. However, 20 % of kidney F4/80+ cells are CCR2+CCR5+ so CCR2 may also be involved in localisation within tissues. It is unclear if iCCR

combinatorial expression is a result of redundancy or if each receptor has a unique function. It is also possible that iCCR combinatorial expression marks different macrophage lineages and functions. In the spleen, F4/80+ cells express high levels of CCR3. These cells are likely to be red pulp macrophages, cells that phagocytose red blood cells (Franken et al., 2015; Kurotaki et al., 2015).

CD11b+SSChi cells are likely to be eosinophils (Geslewitz et al., 2018; Suzukawa et al., 2008). These cells have consistently been shown here to express only CCR3 in the spleen, blood and bone marrow. Expression of CCR3 by eosinophils has been widely reported (Kong et al., 2016; Phillips et al., 2003; Ueki et al., 2013). In certain contexts, CCR3 expression by eosinophils can be altered. For example retinoic acid and IL-5 induce increased CCR3 expression (Stirling et al., 2001; Ueki et al., 2013). However, Ang II induced hypertension had no effect on eosinophil CCR3 expression which therefore is unlikely to be involved in the pathogenesis of the disease. Few studies have associated eosinophils with hypertension but eosinophils have been shown to mediate the anti-contractile properties of PVAT (Withers et al., 2017) so CCR3 and eosinophils may be important in basal BP regulation.

CD11b+SSCint cells in the spleen and blood represent a heterogeneous population of cells. Neutrophils are likely to form a high proportion of this population and monocytes, NK cells and DCs may also be included (Lakschevitz et al., 2016; Rose et al., 2012). Variable and low expression of CCR1 and CCR2 by CD11b+SSCint cells is shown in this study, likely due to the heterogeneity of cell types in the population. In the spleen, these cells also expressed CCR3 which may be due to eosinophils being included in the population. Similar to the data here, mouse blood neutrophils have been shown to express low levels of CCR1 and CCR2 (Mercer et al., 2014). CCR1 and CCR2 expression is higher in lung neutrophils than blood neutrophils so expression of these receptors may be upregulated when neutrophils infiltrate tissues (Mercer et al., 2014).

CD11b+SSClo cells in the spleen and blood constitute a population of monocytes. NK cells and DCs may also be included (Lakschevitz et al., 2016; Rose et al., 2012). These cells have high CCR2 expression and some CCR5 expression. Both of these receptors have been shown to be expressed by mouse blood monocytes (Tacke et al., 2007). Some of these cells also co-express the two iCCRs with the

majority of CCR5+ CD11b+SSClo cells also expressing CCR2. Whether CCR5 has a unique function on these cells or acts redundantly with CCR2 is unclear. It has been suggested that Ly6Clo monocytes use CCR5 to migrate to plaques in a model of atherosclerosis (Tacke et al., 2007). It is possible that these cells use CCR5 to migrate in a tissue specific manner. Monocytes were not separated into Ly6Chi and Ly6Clo populations in this study which should be carried out to further characterise monocyte iCCR expression as this could highlight differences of CCR2 and CCR5 expression between monocyte subsets. Similar to CD11b+SSClo cells, the CD11b+ cells identified in the kidneys and PVAT in this study also showed CCR2 and CCR5 expression. These cells were either CCR2+, CCR2+CCR5+ or did not express any of the iCCRs. These may represent different cell types that are CD11b+ and could be further identified with more specific cell markers.

3.8.3 Altered iCCR and chemokine expression in Ang II induced hypertension

RNA was extracted from combined aorta and PVAT tissue and CCR2 and CCR5 mRNA expression significantly increased following 14 days of Ang II treatment. This has previously been observed in DOCA-salt induced hypertension (Chan et al., 2012). An increase in CCR2+ and CCR5+ leukocytes in the aorta and PVAT may influence the mRNA expression of these receptors detected in the whole tissue. In this study, Ang II did not affect PVAT leukocyte infiltration but previously PVAT infiltration by T cells, including CCR5+ T cells, and macrophages has been reported in hypertensive mice (Guzik et al., 2007; Mikolajczyk et al., 2016). CCR5 has been implicated in monocyte migration to atherosclerotic plaques and monocyte CCR5 expression increased in a mouse model of atherosclerosis (Tacke et al., 2007). CCR5+ monocytes may accumulate in the aorta in hypertension and could be involved in the increased risk of atherosclerosis observed in the disease. It is also possible that EC and VSMC CCR2 and CCR5 expression is upregulated after Ang II treatment, although this was not seen in microscopy images.

After 7 and 14 days of Ang II induced hypertension, no differences in iCCR expression by the leukocytes analysed in the kidney were observed. CCR2 and CCR5 mRNA expression in the whole kidney also did not change in response to Ang II treatment. Contrasting to this, the DOCA-Ang II model increased CCR5

mRNA expression in the renal cortex (Krebs et al., 2012), possibly an effect only seen in longer hypertension models.

Microscopy images showed CCR5 and low CCR2 expression in the heart and kidney as well as clear CCR2 and CCR5 expression in the PVAT. There were no obvious differences between sham and Ang II treated mice. Any differences in iCCR expression in these tissues could be subtle and vary between cell types so are unlikely to be revealed in 2D microscopy images. In addition, the kidney and heart are highly autofluorescent and it is possible some positive cells will not be seen.

Human circulating monocytes have been shown to have increased CCR5 expression in hypertensive patients compared to normotensive controls as well as expression of CCR1 (Syrbe et al., 2007). This was not seen here but that may be a result of species differences. Human monocyte CCR2 expression has been shown to not differ between hypertensive and normotensive individuals (Syrbe et al., 2007) which is similar to the results in this study.

In the spleen, iCCR expression by all leukocytes analysed in this study was not affected by Ang II. The spleen is a secondary lymphoid organ that facilitates the interaction of APCs and lymphocytes. The spleen regulates the immune response so could be altered in hypertension. It has been suggested that infiltrating T cells in the kidney and aorta originate from the spleen and splenectomised mice are protected from Ang II induced BP elevation (Carnevale et al., 2014). Leukocyte iCCR expression may not be affected by Ang II in the spleen, but iCCR expression could change once leukocytes have migrated, or are migrating, to target organs such as the aorta.

The iCCR ligands CCL2, CCL3 and CCL5 have been shown to be elevated in the plasma of hypertensive patients (Parissis et al., 2002). CCL2 was not detected with the Luminex assay used in this study and CCL5 was only detected in half of the samples analysed. The concentration of these chemokines should be verified using an ELISA. Only very low levels of CCL3 were detected and Ang II had no effect on CCL3 concentration. This contrasts to observations in hypertensive patients and may be a result of species differences. The concentration of CCL21 in the plasma was lower in the Ang II treated mice compared to the control

mice. CCL21 is a homeostatic chemokine involved in the homing of T cells and DCs to secondary lymphoid organs (Förster et al., 2008) and has not previously been implicated in hypertension. Through reducing CCL21 production, it is possible that T cell and DC migration is redirected from lymphoid organs to peripheral tissues. CCL21 binds to CCR7, not any of the iCCRs, so it is unlikely that altered CCL21 expression relates to iCCR expression in Ang II induced hypertension. The iCCR ligands CCL7, CCL8 and CCL11 were detected in the plasma but Ang II had no effect on their concentration. CXCL13 was lower in the hypertensive mice. CXCL13 binds to CXCR5 and few studies have focused on these in hypertension. However, a CXCL13 gene polymorphism has been associated with hypertension (Timasheva et al., 2018).

3.9 Conclusion

In this chapter, iCCR expression by leukocytes in the kidney, PVAT, spleen, blood and bone marrow in Ang II induced hypertension has been characterised. Antibodies and mice expressing iCCR reporter proteins were used for flow cytometry and it was determined that REP mice were better for analysis of iCCR expression. No changes in iCCR expression in response to Ang II were identified for the cell types analysed but CCR2 and CCR5 mRNA expression was shown to be elevated in the aortas and PVAT of Ang II treated mice.

Altered vasculature iCCR expression could be involved in the pathogenesis of hypertension. Further studies could use the Ang II model of hypertension with $CCR5^{-/-}$ mice as CCR5 mRNA expression in the vasculature was increased in response to Ang II. The longer DOCA-Ang II model of hypertension has been used to study $CCR5^{-/-}$ mice (Krebs et al., 2012) but there are no reports of $CCR5^{-/-}$ mice with 14 days of Ang II induced hypertension. As well as this, iCCR expression at more time points could be looked at, such as after 3 and 28 days of Ang II induced hypertension. This would reveal the temporal changes of iCCR expression that occur during the disease.

Overall, this chapter highlights potential involvement of aortic CCR2 and CCR5 in hypertension but further understanding is required in order to identify how these could be therapeutically targeted to treat hypertension and end organ damage.

Chapter 4

The effect of iCCR deficiency in Ang II induced hypertension

4 The effect of iCCR deficiency in Ang II induced hypertension

4.1 Introduction

Due to the complexity of the chemokine system, identifying the exact role of chemokine receptors in various contexts has been a challenge. This has resulted in the development of only three successful drugs that target chemokine receptors. These are the CCR5 antagonist Maraviroc, the CXCR4 antagonist Plerixafor and the monoclonal antibody to CCR4 Mogamulizumab (Dorr et al., 2005; Duvic et al., 2015; Teusink et al., 2016). When studying iCCRs, it has so far only been practical to generate mice deficient for a single iCCR as the genes for each receptor are in close genomic proximity and it is not practical to generate compound receptor deficient mice by interbreeding. The full role, combinatorial or individual, of the iCCRs may not be revealed when using single receptor deficient mice as there could be compensation from the other receptors. To overcome this, mice deficient for the whole genomic cluster containing the iCCR genes (iCCR-KO) have been generated (Dyer et al., 2019).

Some of the iCCRs and their ligands have been implicated in the pathogenesis of hypertension. For example, the expression of CCR2 and CCR5 in the aorta and the expression of CCR5 in the kidney have been shown to be elevated in hypertensive animal models (Chan et al., 2012; Krebs et al., 2012; Moore et al., 2015). As well as this, hypertensive patients have elevated circulating CCL2 (Antonelli et al., 2012; Madej et al., 2005). The previous chapter revealed that after 14 days of Ang II induced hypertension, aortic CCR2 and CCR5 expression increases. A deficiency of CCR2 or CCR5 has not been shown to effect BP in hypertension. However, $CCR2^{-/-}$ mice are protected against Ang II induced vascular remodelling (Ishibashi et al., 2004; Krebs et al., 2012). The effect of CCR1 or CCR3 deficiency in hypertension has not been studied.

As only single iCCR deficient mice have been used to study hypertension, the exact role of each iCCR in the disease is not fully understood. Therefore, the aim of the experiments described in this chapter was to investigate the effect of deficiency in all iCCRs in Ang II induced hypertension. iCCR-KO mice were subject to 14 days of Ang II infusion. BP, vascular function and oxidative stress

were assessed, along with plasma chemokine concentration, inflammation of the kidneys, spleen and PVAT and Ang II induced hypertrophy. This has shown how lack of all four iCCRs affects the pathogenesis of hypertension, increasing our understanding of iCCRs in the disease.

4.2 Blood pressure

WT and iCCR-KO mice were subject to Ang II induced hypertension and BP was measured for 14 days by the tail cuff method (Figure 4-1) or by telemetry (Figure 4-2). In both WT and iCCR-KO mice, upon Ang II infusion, systolic BP started to increase 1 day after treatment and increased by about 40 mmHg by day 14 (Figure 4-2a). Diastolic BP increased by about 30 mmHg (Figure 4-2b). There was no difference in BP between the WT and iCCR-KO mice. When BP was measured in sham treated mice by the tail cuff method, the WT mice had a higher BP than the iCCR-KO mice at days 12 – 14. There was no difference in baseline BP before Ang II infusion, measured by both the tail cuff method and telemetry.

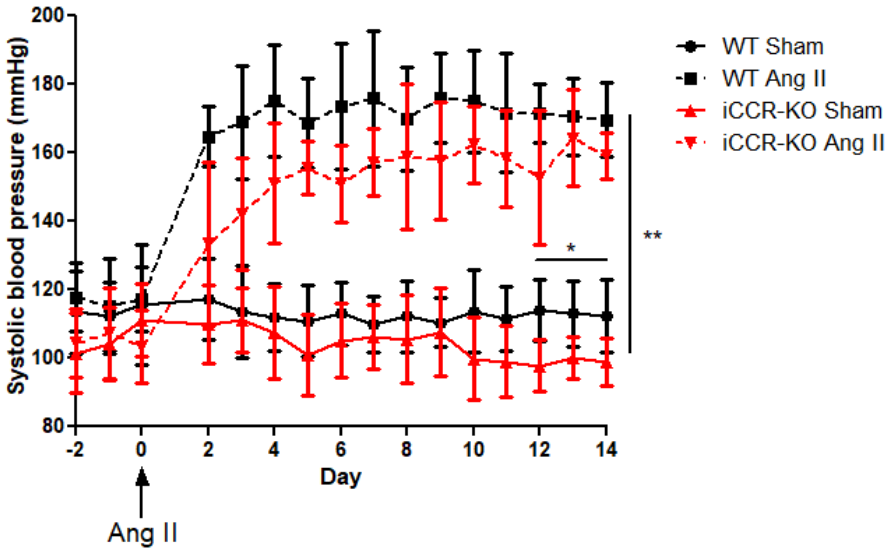


Figure 4-1. The effect of iCCR deficiency on BP measured by the tail cuff method
WT and iCCR-KO mice were subject to Ang II induced hypertension or sham treatment for 14 days and systolic BP was measured by the tail cuff method. WT sham n = 8; WT Ang II n = 8; iCCR-KO sham n = 10; iCCR-KO Ang II n = 11. Data are combined from two independent experiments. * $p < 0.05$, ** $p < 0.01$, Two-Way ANOVA with repeated measures.

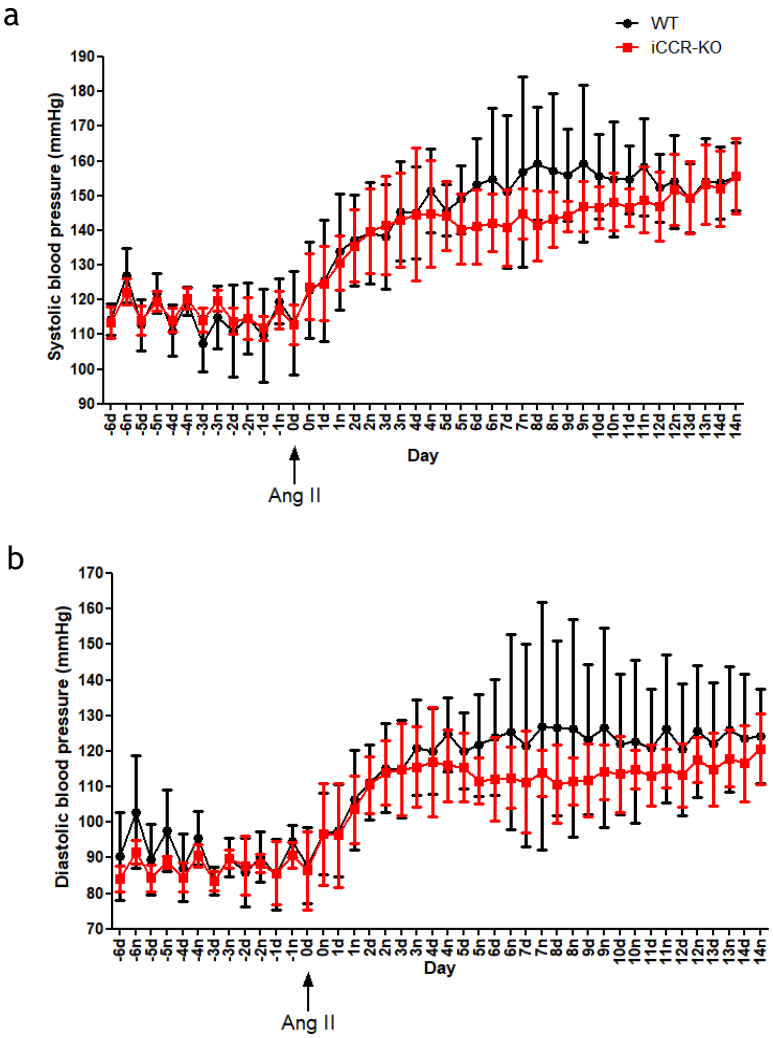


Figure 4-2. The effect of iCCR deficiency on BP measured by telemetry
Telemetry probes were implanted into WT and iCCR-KO mice and BP was measured. After recovery the mice were given Ang II for 14 days and systolic (a) and diastolic (b) BP was measured. WT n = 7; iCCR-KO n = 7. Data are combined from two independent experiments.

4.3 Vascular function and oxidative stress

ACh induces blood vessel relaxation through eNOS activation in ECs and the subsequent production of NO. NO then induces VSMC relaxation (Gewaltig and Kojda, 2002). Vascular function of the aorta and mesenteric arteries, resistance vessels that contribute to systemic BP, from sham or Ang II treated WT and iCCR-KO mice was determined by measuring the relaxation response of the vessels to ACh. This measured endothelium-dependent relaxation. Endothelium-independent relaxation was also determined by measuring the relaxation response to the NO donor SNP (Patik et al., 2016). The relaxation response to ACh was impaired in both WT and iCCR-KO aortas following Ang II infusion

compared to sham aortas. However, the iCCR-KO aortas showed a slight protection from this impairment compared to WT aortas. There was also a difference in the relaxation response between sham treated WT and iCCR-KO aortas. There was no difference in relaxation response to SNP between the WT and iCCR-KO aortas (Figure 4-3a). The mesenteric arteries from Ang II treated iCCR-KO mice showed improved relaxation to ACh compared to the mesenteric arteries from WT Ang II treated mice but no differences in the response to SNP (Figure 4-3b).

Oxidative stress in the aortas of sham and Ang II treated WT and iCCR-KO mice was assessed by measuring superoxide levels. Superoxide reacts with NO to form peroxynitrite, reducing NO bioavailability (Mordi et al., 2016). Increased vascular superoxide has been associated with impaired vascular function (Guzik et al., 2007). There were no differences in aortic superoxide between the WT and iCCR-KO mice. Upon Ang II infusion, superoxide levels were more variable than after sham treatment and tended to be higher but this was not significant (Figure 4-3c).

Overall, the aorta and mesenteric arteries of iCCR-KO mice are slightly protected from Ang II induced vascular dysfunction compared to WT mice but iCCR deficiency or Ang II treatment did not affect aortic superoxide levels.

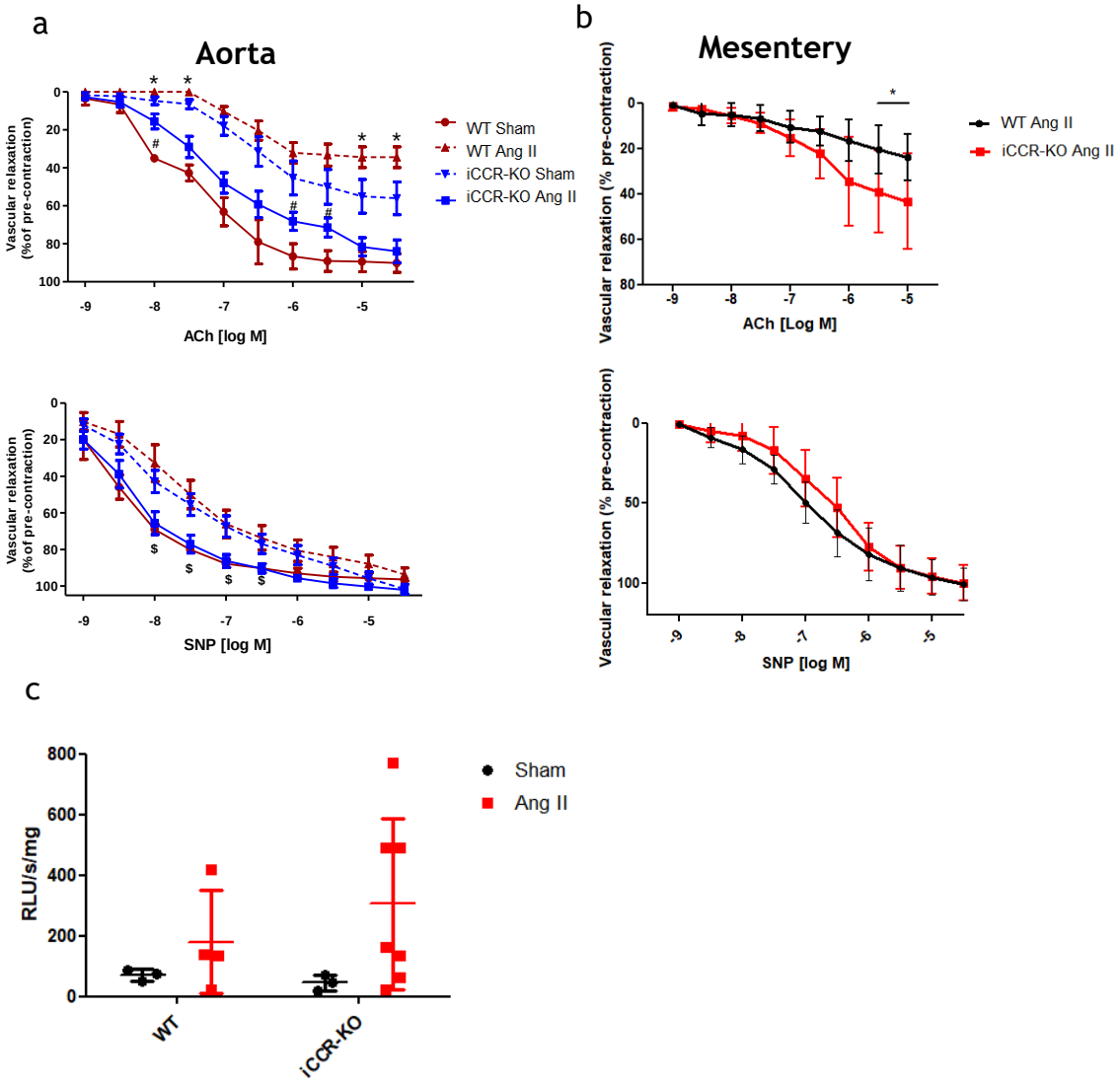


Figure 4-3. The effect of iCCR deficiency on vascular function and oxidative stress in Ang II induced hypertension
WT and iCCR-KO mice were subject to Ang II induced hypertension and vascular function was determined by measuring the relaxation response of the aorta (a) and mesenteric arteries (b) to increasing concentrations of ACh or SNP. Oxidative stress was measured by detecting superoxide in the aorta (c). Aorta vascular function: WT sham n = 3; WT Ang II n = 7; iCCR-KO sham n = 7; iCCR-KO Ang II n = 9. Data are combined from two independent experiments. Error bars = SEM. Mesentery vascular function WT Ang II n = 5; iCCR-KO Ang II n = 5. # $p < 0.05$ WT sham vs iCCR-KO sham; * $p < 0.05$ WT Ang II vs iCCR-KO Ang II; $ $p < 0.05$ iCCR-KO sham vs iCCR-KO Ang II, Two-Way ANOVA with repeated measures.

4.4 Plasma chemokines

Chemokine expression may increase in Ang II induced hypertension to enhance migration of leukocytes to tissues that regulate BP such as the kidneys and vasculature. Any changes to chemokine expression could indicate which chemokine receptors are important. Therefore, the concentration of CC chemokines was determined in the plasma of WT and iCCR-KO mice following 14

days of Ang II or sham treatment and are described in Table 4-1. Plasma CCL3 and CCL7 levels were significantly higher in the iCCR-KO mice compared to WT mice. This was observed for both sham and Ang II treated mice. In the iCCR-KO mice, Ang II treatment induced an increase in plasma CCL3 concentration. CCL5, CCL8, CCL11, CCL12, CCL19, CCL21 and CCL22 were detected in the plasma but neither iCCR deficiency nor Ang II treatment had an effect on plasma concentration. CCL2 and CCL4 were not detected. CCL20 was detected in some, but not all samples.

Chemokine	WT Sham	WT Ang II	iCCR-KO Sham	iCCR-KO Ang II
CCL3	1.4 ± 0.3	1.4 ± 0.1	98.7 ± 37 **	191 ± 81.6 ##; †
CCL5	171 ± 21.4	121 ± 48.7	315 ± 103	283 ± 116
CCL7	57.9 ±21.7	59.9 ± 49.5	748 ± 431 **	509 ± 263 ##
CCL8	7881 ± 1734	6335 ± 1313	8051 ± 3089	5245 ± 1290
CCL11	1328 ± 681	994 ± 715	1902 ± 786	1546 ± 851
CCL12	334 ± 126	254 ± 288	776 ± 483	620 ± 399
CCL19	29.2 ± 1.9	26.4 ± 8.2	31.0 ± 9.2	30.3 ± 7.0
CCL21	5520 ± 101	5330 ± 440	5543 ± 153	4274 ± 1852
CCL22	182 ± 84.7	116 ± 91.3	155 ± 62.2	122 ± 74.5

Table 4-1. Plasma chemokine concentration in Sham and Ang II treated WT and iCCR-KO mice
The concentration (pg/ml) of CC chemokines in the plasma of WT and iCCR-KO mice following 14 days of sham or Ang II treatment was measured using a Luminex assay. ** $p < 0.01$ vs WT sham; ## $p < 0.01$ vs WT Ang II; † $p < 0.05$ vs iCCR-KO Sham, Two-Way ANOVA.

4.5 Inflammation

The accumulation of leukocytes in the kidneys and vasculature has been implicated in hypertension (Guzik et al., 2007; Mikolajczyk et al., 2016; Ozawa et al., 2006). Therefore, the infiltration of CD19+ B cells, CD3+ T cells, CD4+ and CD8+ T cells and CD11b+F4/80+ cells was investigated in the kidneys and PVAT of WT and iCCR-KO mice following 14 days of sham or Ang II treatment. These cell types were also analysed in the spleen as this could indicate whether these cells migrate from, or accumulate, in the spleen in hypertension. In addition, CD11b+ monocyte and CD11c+MHC II+ DC populations were investigated in the bone marrow and blood of Ang II treated WT and iCCR-KO mice as these cells have been implicated in hypertension (Vinh et al., 2010; Wenzel et al., 2011). This also indicates how these cells migrate from the bone marrow to the blood and then to tissues. This analysis revealed some differences between WT and iCCR-KO mice. The gating strategy for kidney, PVAT and spleen analysis is detailed below (Figure 4-4).

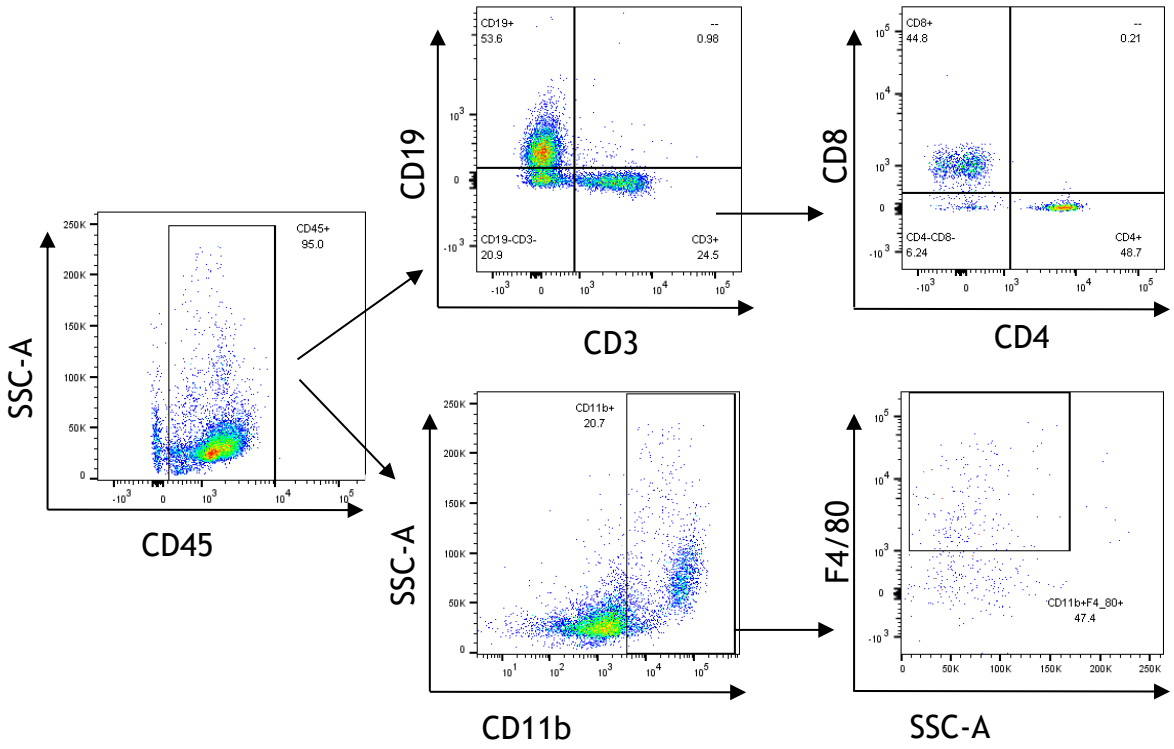


Figure 4-4. Gating strategy for the analysis of kidney, PVAT and spleen leukocytes from WT and iCCR-KO mice with Ang II induced hypertension
CD45+ cells were gated from live, single cells. CD19+ and CD3+ cells were gated from the CD45+ population then CD8+ and CD4+ cells were gated from the CD3+ population. F4/80+ cells were gated from CD11b+ cells.

4.5.1 Bone marrow

The effect of iCCR-deficiency on bone marrow CD11b+ monocyte and DC populations in Ang II induced hypertension was investigated. CD11b+Ly6C- cells, which could be neutrophils, CD11b+Ly6C+ patrolling monocytes and CD11b+Ly6C++ inflammatory monocytes (Evrard et al., 2018; Kratofil et al., 2017) were gated from CD11b+ cells, as were CD11c+MHC II+ DCs (Figure 4-5). Sham treated mice were not used as these experiments were carried out using the Ang II treated mice subject to telemetry BP measurements. iCCR-KO mice had more CD11b+Ly6C- cells (Ang II WT 1.2 ± 0.1 vs Ang II iCCR-KO 1.7 ± 0.1 % CD45+ cells, $p < 0.01$, Mann Whitney test) and more CD11b+Ly6C++ cells (Ang II WT 6.8 ± 0.5 vs Ang II iCCR-KO 8.4 ± 0.9 % CD45+ cells, $p < 0.05$, Mann Whitney test) in the bone marrow than WT mice (Figure 4-6). There was also an increase in CD11b+CD11c+MHC II+ cells in the bone marrow of iCCR-KO mice (Ang II WT 0.3 ± 0.1 vs Ang II iCCR-KO 1.0 ± 0.2 % CD45+ cells, $p < 0.01$, Mann Whitney test).

These differences are demonstrated by representative flow cytometry plots (Figure 4-6d, f).

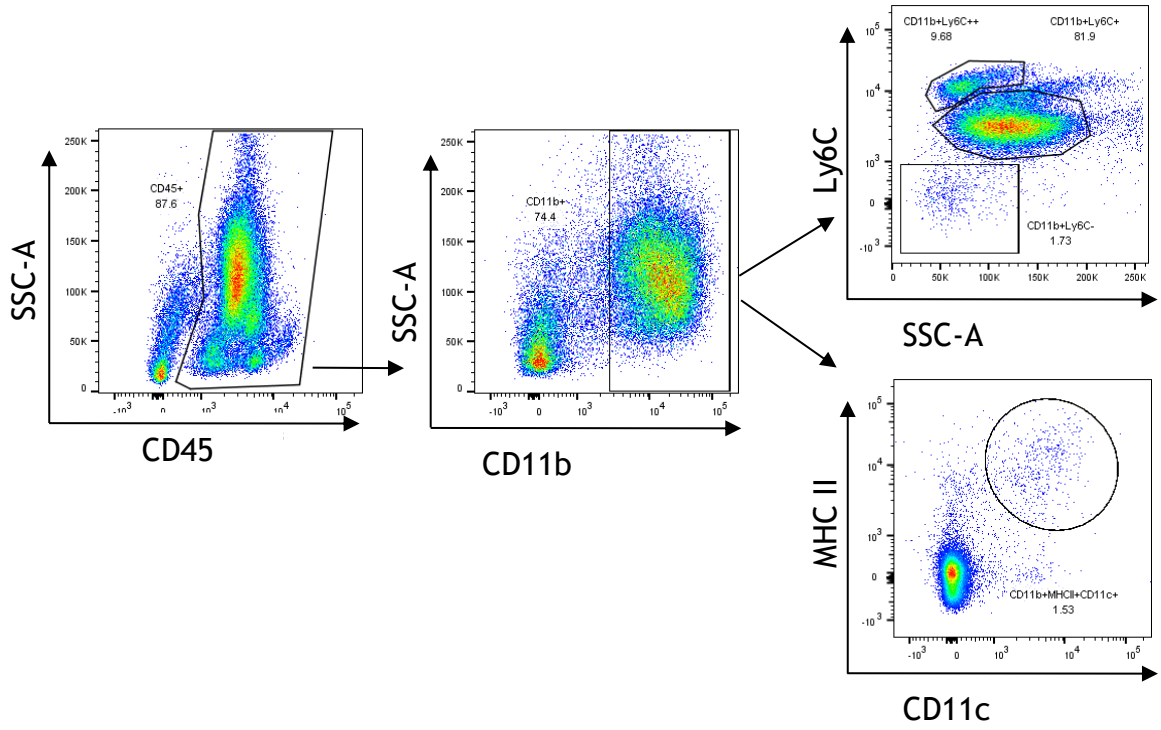


Figure 4-5. Gating strategy for the analysis of bone marrow leukocytes in 14 day Ang II treated WT and iCCR-KO mice
CD45+ cells were gated from live, single cells. CD11b+ cells were gated on CD45+ cells and gated into Ly6C-, Ly6C+, Ly6C++ and CD11c+MHC II+ cells.

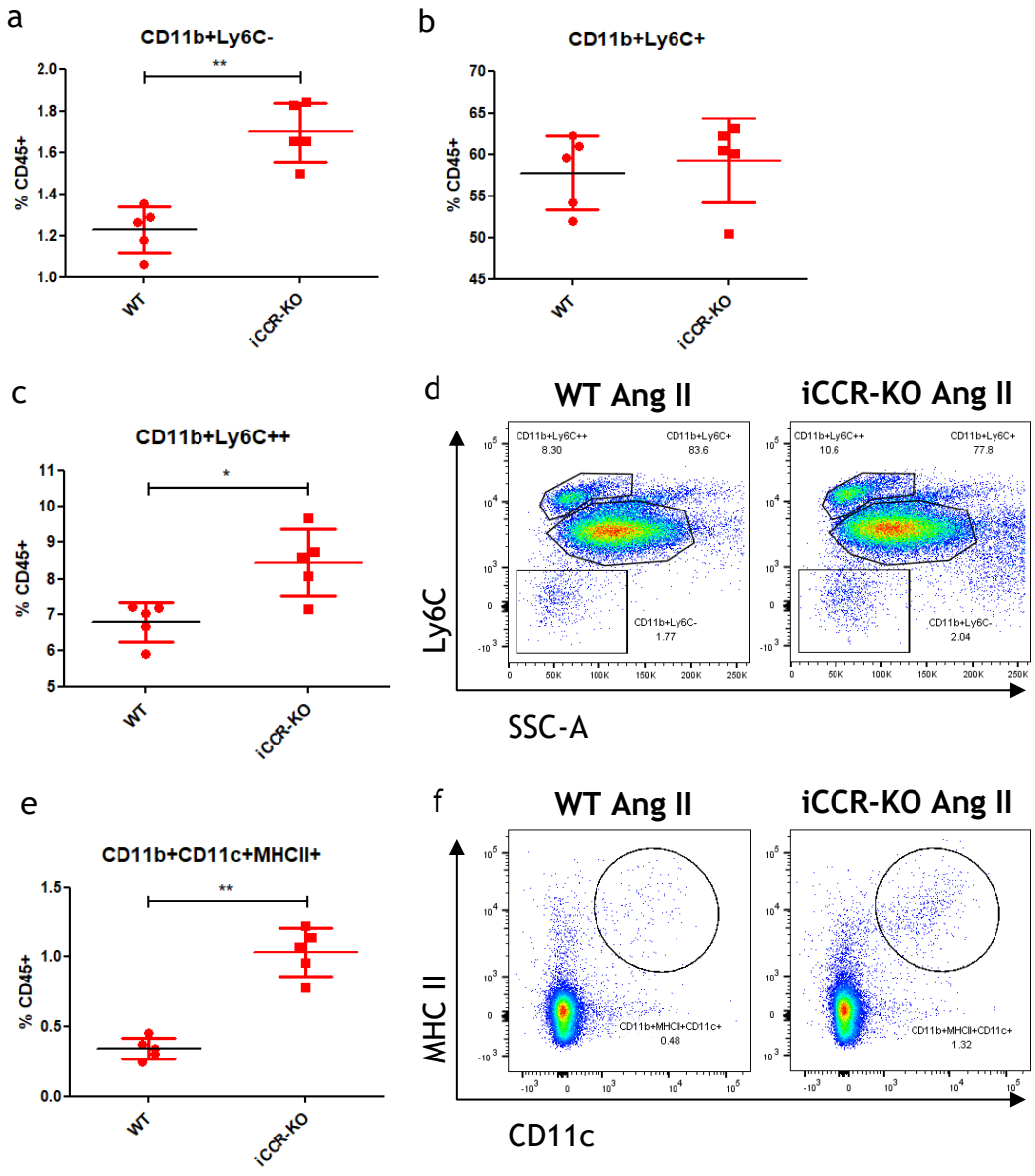


Figure 4-6. The effect of iCCR deficiency on bone marrow leukocytes in Ang II induced hypertension
WT and iCCR-KO mice were treated with Ang II for 14 days and the number of CD11b+Ly6C- cells (a), CD11b+Ly6C+ cells (b), CD11b+Ly6C++ cells (c) and CD11b+CD11c+MHC II+ cells in the bone marrow was determined. Representative flow cytometry plots of CD11b+Ly6C+ (d) and CD11c+MHC II+ (f) populations are shown. * $p < 0.05$; ** $p < 0.01$, Mann Whitney test.

4.5.2 Blood

The effect of iCCR-deficiency on blood B cell and T cell populations in Ang II induced hypertension was investigated, along with the CD11b+ monocyte and DC populations described above. CD19+ B cells and CD3+ T cells were gated from CD45+ cells. Expression of CD44, a marker of activated and memory T cells (Baaten et al., 2010; Maeshima et al., 2011), was also determined (Figure 4-7).

As with the bone marrow analysis, sham treated mice were not used for these experiments.

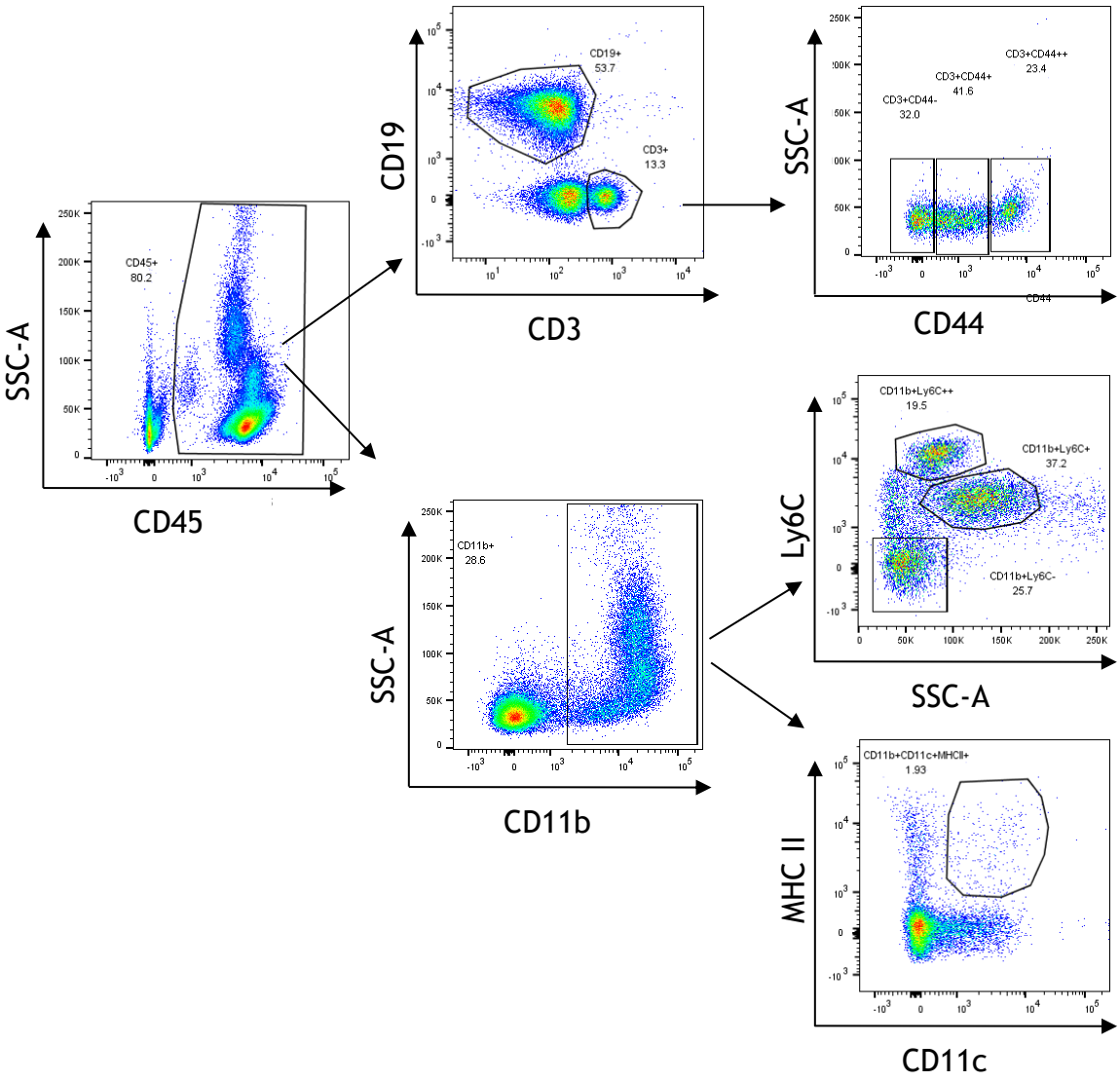


Figure 4-7. Gating strategy for the analysis of leukocytes in the blood of WT and iCCR-KO Ang II treated mice
CD45+ cells were gated from live, single cells. CD19+ and CD3+ cells were gated from the CD45+ population. The CD3+ cells were gated into CD44-, CD44+ and CD44++ populations. CD11b+ cells were gated from CD45+ cells and further gated into Ly6C-, Ly6C+, Ly6C++ and CD11c+MHC II+ cells.

Similar to the bone marrow, iCCR-KO mice had more CD11b+Ly6C- cells in the blood than WT mice (Ang II WT 7.1 ± 0.4 vs Ang II iCCR-KO 9.6 ± 1.3 % CD45+ cells, $p < 0.01$, Mann Whitney test). However, there were fewer CD11b+Ly6C++ cells in the blood of WT mice (Ang II WT 5.9 ± 0.9 vs Ang II iCCR-KO 1.5 ± 0.6 % CD45+ cells, $p < 0.01$, Mann Whitney test) which contrasts to the bone marrow (Figure 4-8). The blood of iCCR-KO mice also had fewer CD11b+CD11c+MHC II+ cells than the blood of WT mice (Ang II WT 0.5 ± 0.1 vs Ang II iCCR-KO 0.3 ± 0.1

% CD45+ cells, p < 0.05, Mann Whitney test), contradicting the observations for the bone marrow. These reductions are reflected in the representative flow cytometry plots (Figure 4-8d, f).

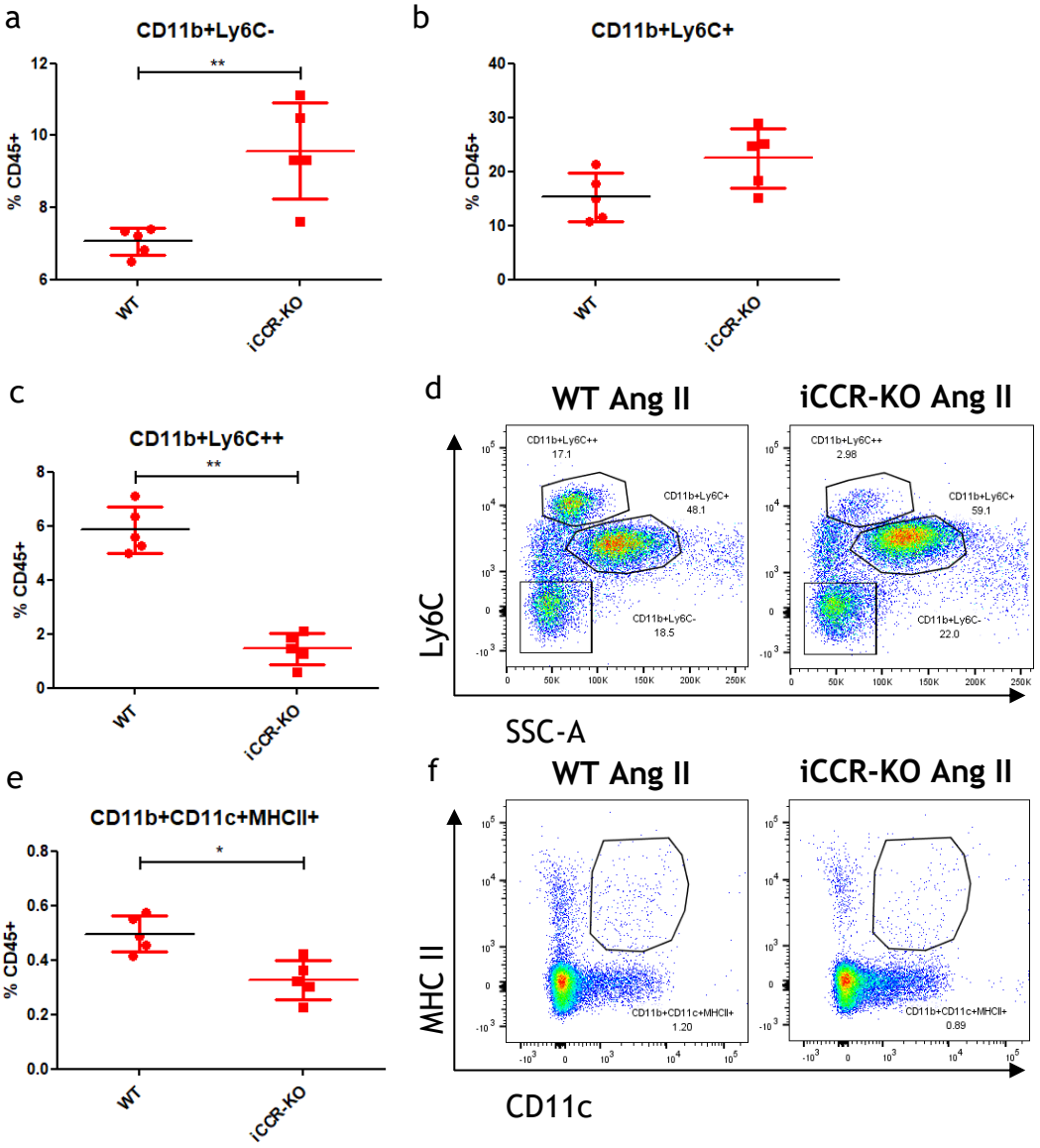


Figure 4-8. The effect of iCCR deficiency on blood leukocytes in Ang II induced hypertension
WT and iCCR-KO mice were treated with Ang II for 14 days and the number of CD11b+Ly6C- cells (a), CD11b+Ly6C+ cells (b), CD11b+Ly6C++ cells (c) and CD11b+CD11c+MHC II+ cells in the blood was determined. Representative flow cytometry plots of CD11b+Ly6C+ (d) and CD11c+MHC II+ (f) populations are shown. * p < 0.05; ** p < 0.01, Mann. Whitney test.

Blood B cells were reduced in Ang II treated iCCR-KO mice (Ang II WT 43.5 ± 8.5 vs Ang II iCCR-KO 32.4 ± 1.5 % CD45+ cells, p < 0.05, Mann Whitney test) whilst blood T cells were increased (Ang II WT 14.2 ± 1.4 vs Ang II iCCR-KO 18.7 ± 1.4 % CD45+ cells, p < 0.01, Mann Whitney test) compared to Ang II treated WT mice

(Figure 4-9a, b). CD44 expression by T cells was also assessed (Figure 4-9c). There were more CD44- T cells (Ang II WT 4.0 ± 0.4 vs Ang II iCCR-KO 5.8 ± 0.4 % CD45+ cells, $p < 0.01$, Mann Whitney test) and CD44+ T cells (Ang II WT 5.0 ± 0.7 vs Ang II iCCR-KO 7.1 ± 0.5 % CD45+ cells, $p < 0.01$, Mann Whitney test) in the blood of iCCR-KO mice than WT mice. CD44++ T cells did not differ between WT and iCCR-KO mice. These differences are demonstrated in representative flow cytometry plots (Figure 4-9d).

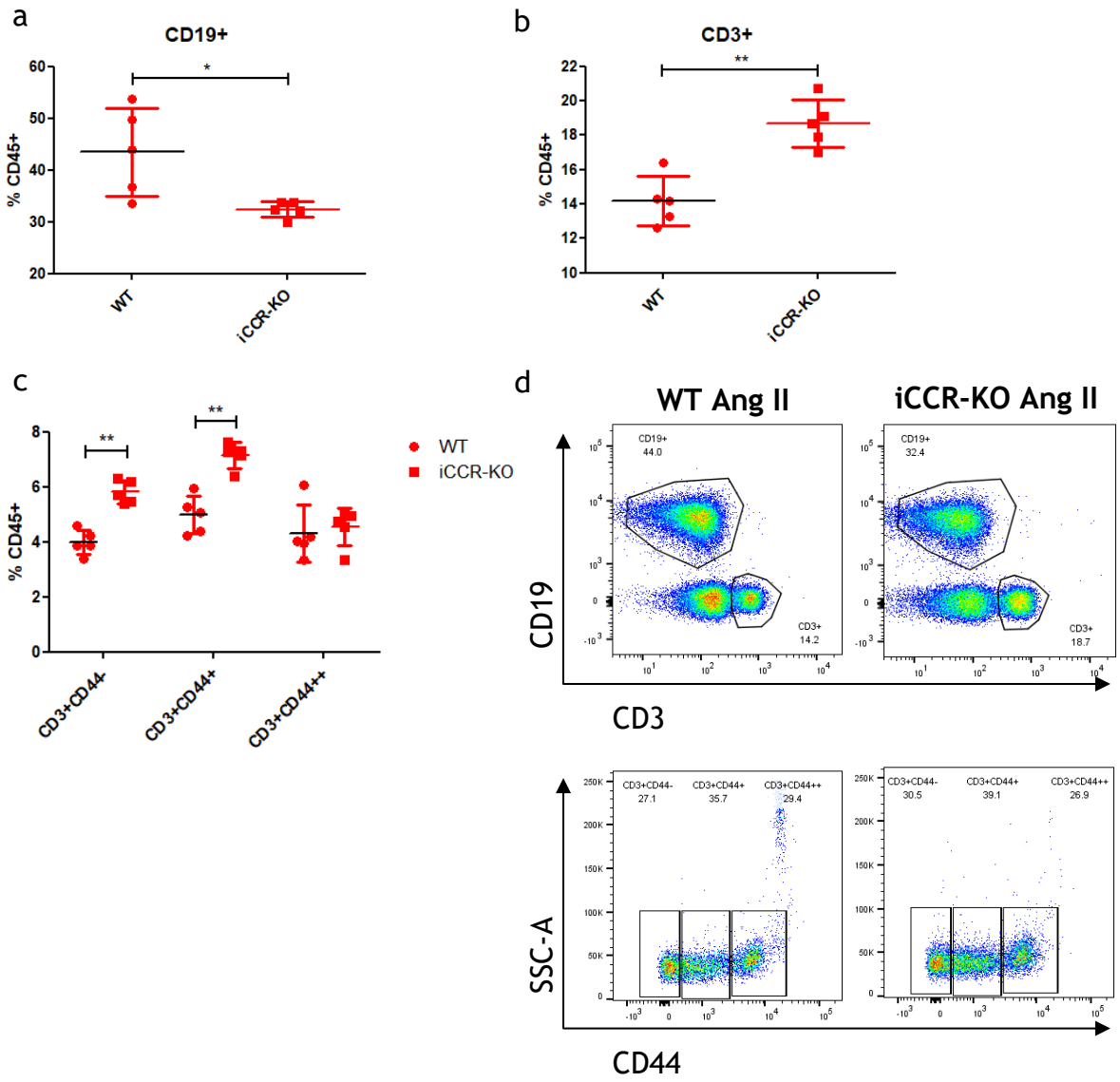


Figure 4-9. In Ang II induced hypertension, iCCR deficiency reduces the proportion of B cells in the blood whilst increasing the proportion of CD44- and CD44+ T cells
WT and iCCR-KO mice were treated with Ang II for 14 days. The proportion of circulating CD19+ cells was lower in the iCCR-KO mice (a) whilst the proportion of CD3+ cells was higher (b). CD3+CD44- and CD3+CD44+ cells were increased in iCCR-KO mice but CD3+CD44++ cells were not affected by iCCR deficiency (c). Representative flow cytometry plots of CD19+, CD3+, CD3+CD44-, CD3+CD44+ and CD3+CD44++ populations are shown (d). * $p < 0.05$, ** $p < 0.01$, Mann Whitney test.

4.5.3 Spleen

The spleens of iCCR-KO mice had fewer CD45+ cells as a proportion of live cells than WT mice after both sham and Ang II treatment (Figure 4-10a; Sham WT 92.8 ± 1.3 vs Sham iCCR-KO 84.2 ± 1.4 % live+ cells, $p < 0.05$, Two-Way ANOVA; Ang II WT 92.0 ± 2.0 vs Ang II iCCR-KO 80.3 ± 6.8 % live+ cells, $p < 0.01$, Two-Way ANOVA). This difference was not obvious from representative flow cytometry plots and was not reflected in the total number of CD45+ cells (Figure 4-10b, d). CD45+ cell infiltration was not affected by Ang II treatment. Ang II infusion also did not affect the infiltration of CD11b+F4/80+ cells, B cells and T cells in both WT and iCCR-KO mice (Figure 4-10, Figure 4-11) but iCCR deficiency did alter the infiltration of T cells in the spleen (Figure 4-11b, c). After Ang II treatment, the total number of T cells in the spleen was lower in the iCCR-KO mice (Ang II WT $1.5 \times 10^7 \pm 2.8 \times 10^6$ vs Ang II iCCR-KO $1.0 \times 10^7 \pm 2.4 \times 10^6$ total cells, $p < 0.05$, Two-Way ANOVA). There were also fewer CD8+ T cells in the Ang II treated iCCR-KO mice (Ang II WT 12.5 ± 2.4 vs Ang II iCCR-KO 8.5 ± 1.7 % CD45+ cells, $p < 0.05$, Two-Way ANOVA). These differences were not seen in sham treated mice.

Overall, iCCR deficiency reduced the proportion of CD45+ cells in the spleen. In the context of Ang II induced hypertension, iCCR deficiency reduced the total number of splenic T cells and the proportion of infiltrating CD8+ T cells.

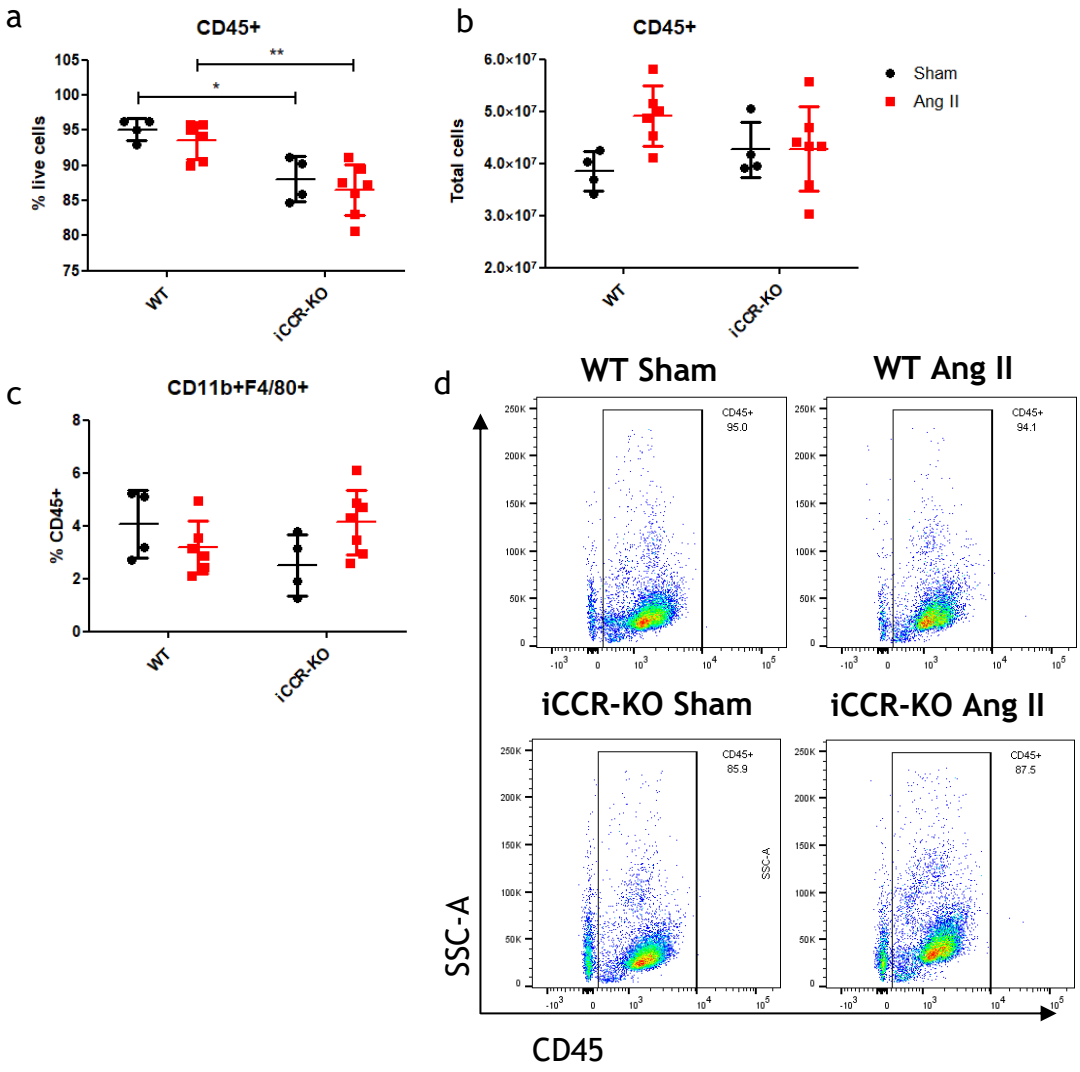


Figure 4-10. iCCR deficiency and leukocytes in the spleen in Ang II induced hypertension
The accumulation of CD45+ cells (a), total CD45+ cells (b) and CD11b+F4/80+ cells (c) in the spleens of WT and iCCR-KO mice given Ang II or sham treatment for 14 days was determined. Representative flow cytometry plots of CD45+ cells are shown (d). * $p < 0.05$, ** $p < 0.01$, Two-Way ANOVA.

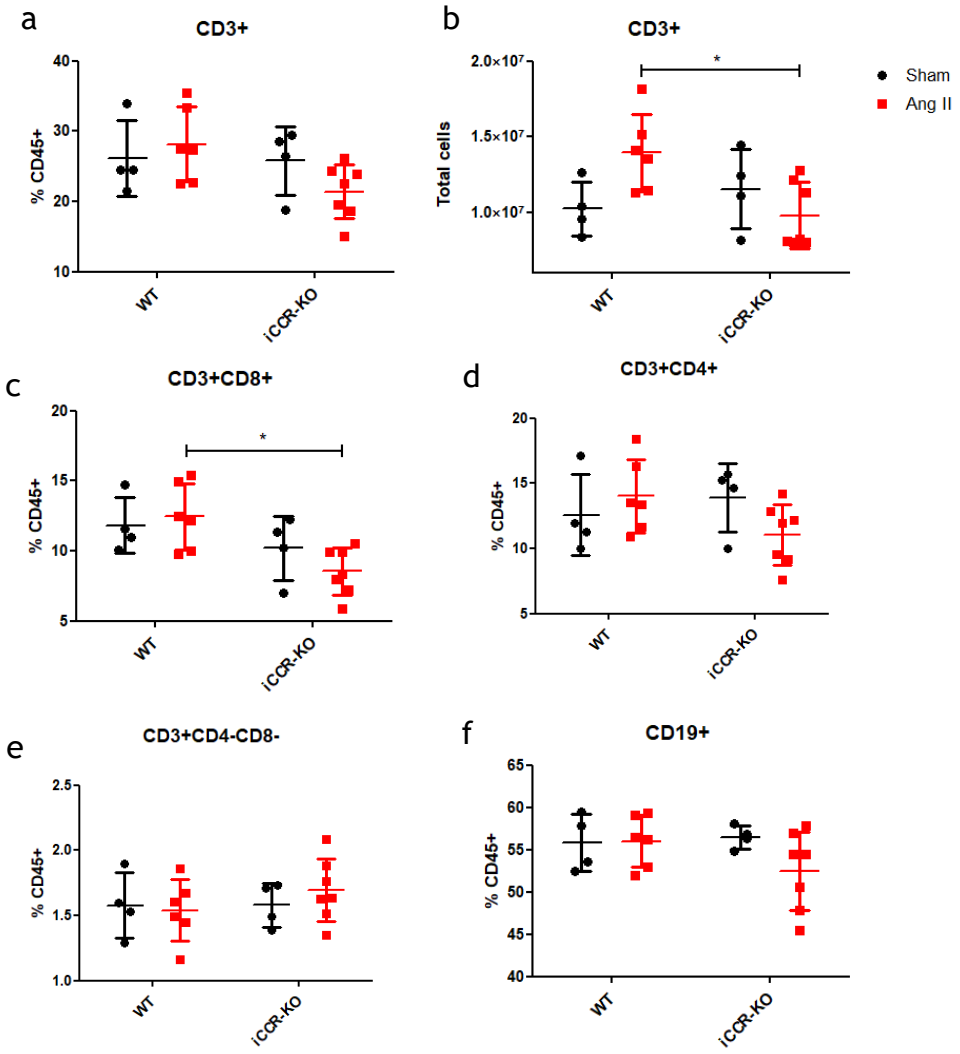


Figure 4-11. The effect of iCCR deficiency on T and B cells in the spleen in Ang II induced hypertension
The proportion of CD3+ cells (a), total CD3+ cells (b), CD8+ cells (c), CD4+ cells (d) CD4-CD8- cells (e) and CD19+ cells (f) was measured in the spleens of WT and iCCR-KO mice subject to 14 days of Ang II induced hypertension. * $p < 0.05$, Two-Way ANOVA.

4.5.4 Kidney

Infiltration of CD45+ leukocytes, B cells and CD11b+ F4/80+ cells in the kidneys was not affected by Ang II treatment or iCCR deficiency (Figure 4-12). Overall T cell infiltration was also not effected by Ang II treatment or iCCR deficiency but there was a higher infiltration of double negative CD4-CD8- T cells in the kidneys of Ang II treated iCCR-KO mice compared to Ang II treated WT mice (Ang II WT 1.7 ± 0.5 vs Ang II iCCR-KO 2.6 ± 0.5 % CD45+ cells, $p < 0.01$, Two-Way ANOVA). There were no differences in CD8+ and CD4+ T cells between all groups (Figure 4-13).

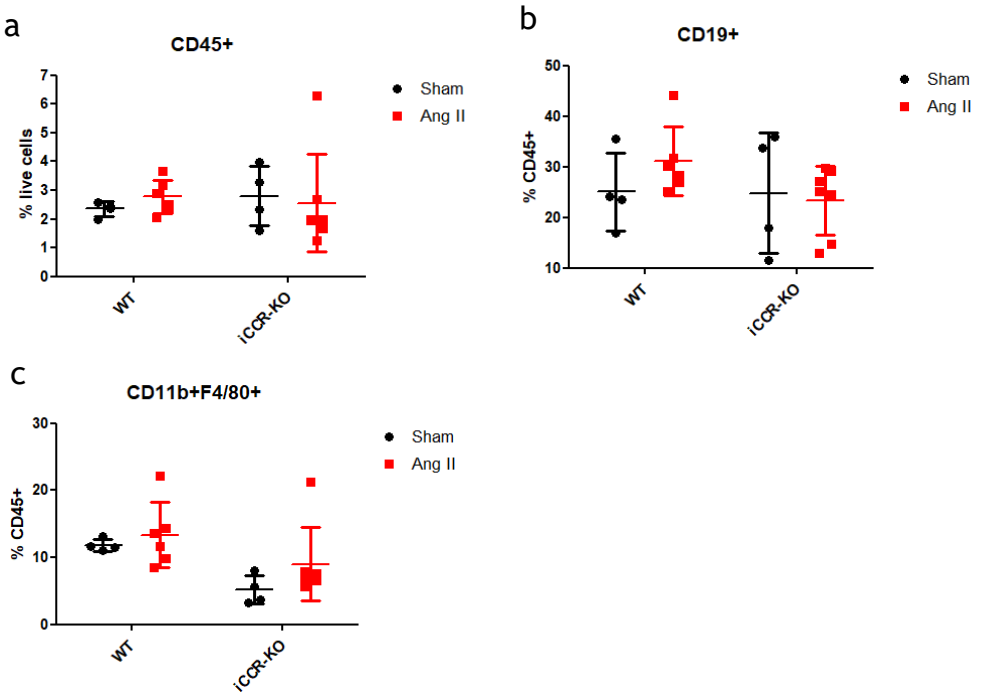


Figure 4-12. iCCR deficiency and kidney infiltration of leukocytes in Ang II induced hypertension
The infiltration of CD45+ cells (a), CD19+ cells (b) and CD11b+F4/80+ cells (c) was determined in the kidneys of WT and iCCR-KO mice following Ang II induced hypertension for 14 days.

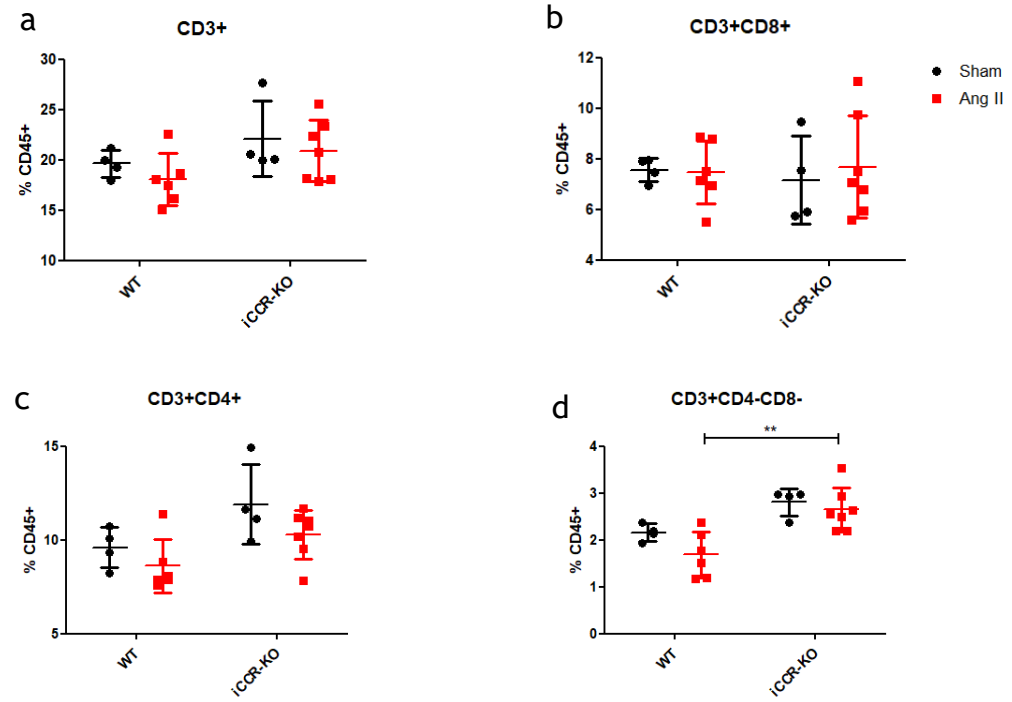


Figure 4-13. iCCR deficiency and kidney T cell infiltration in Ang II induced hypertension
The infiltration of CD3+ cells (a), CD8+ cells (b), CD4+ cells (c) and CD4-CD8- cells (d) in the kidneys of WT and iCCR-KO mice after 14 days of sham or Ang II treatment was determined. ** $p < 0.01$, Two-Way ANOVA.

4.5.5 PVAT

Infiltration of leukocytes in the PVAT was measured as a proportion of cells and total number of cells per mg of tissue. There were no differences for any cell types analysed, including B cells, T cell subsets and CD11b+F4/80+ cells, between sham and Ang II treated WT and iCCR-KO mice (Figure 4-14, Figure 4-15).

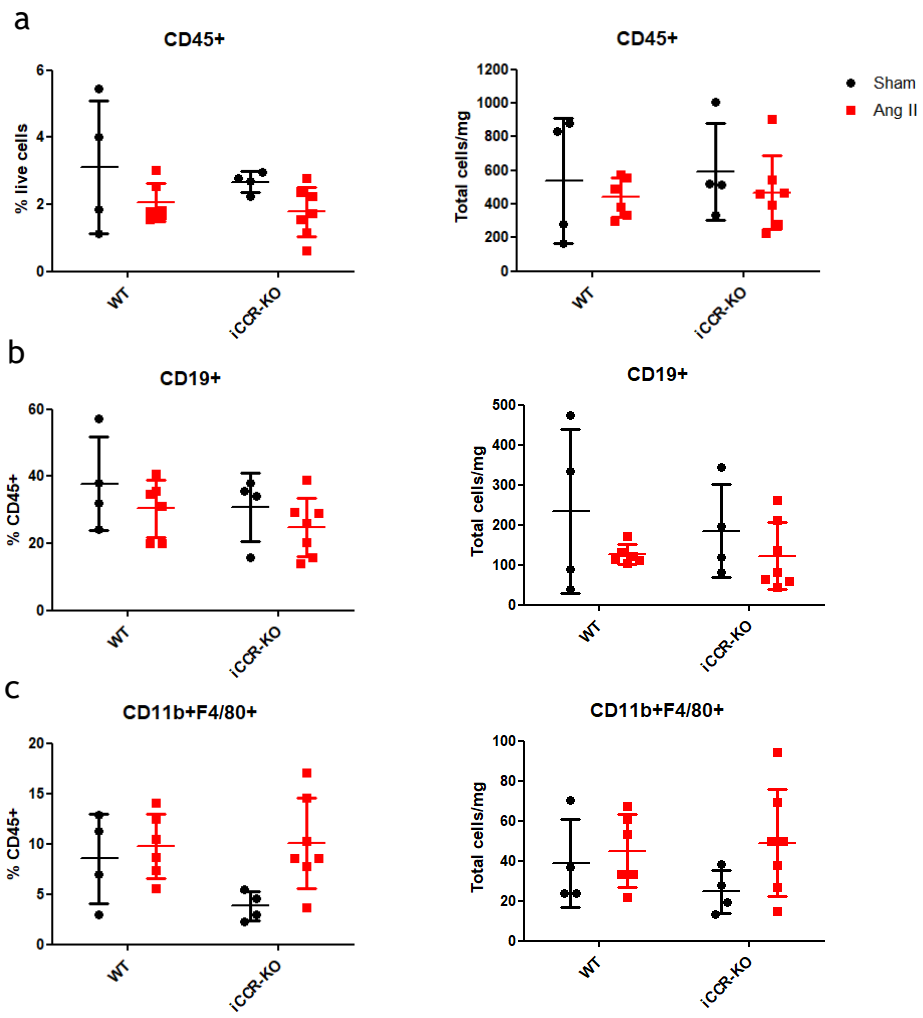


Figure 4-14. iCCR deficiency and PVAT infiltration of leukocytes in Ang II induced hypertension
The infiltration as a proportion and total number of CD45+ cells (a), CD19+ cells (b) and CD11b+F4/80+ cells (c) was determined in the PVAT of WT and iCCR-KO mice following Ang II induced hypertension for 14 days.

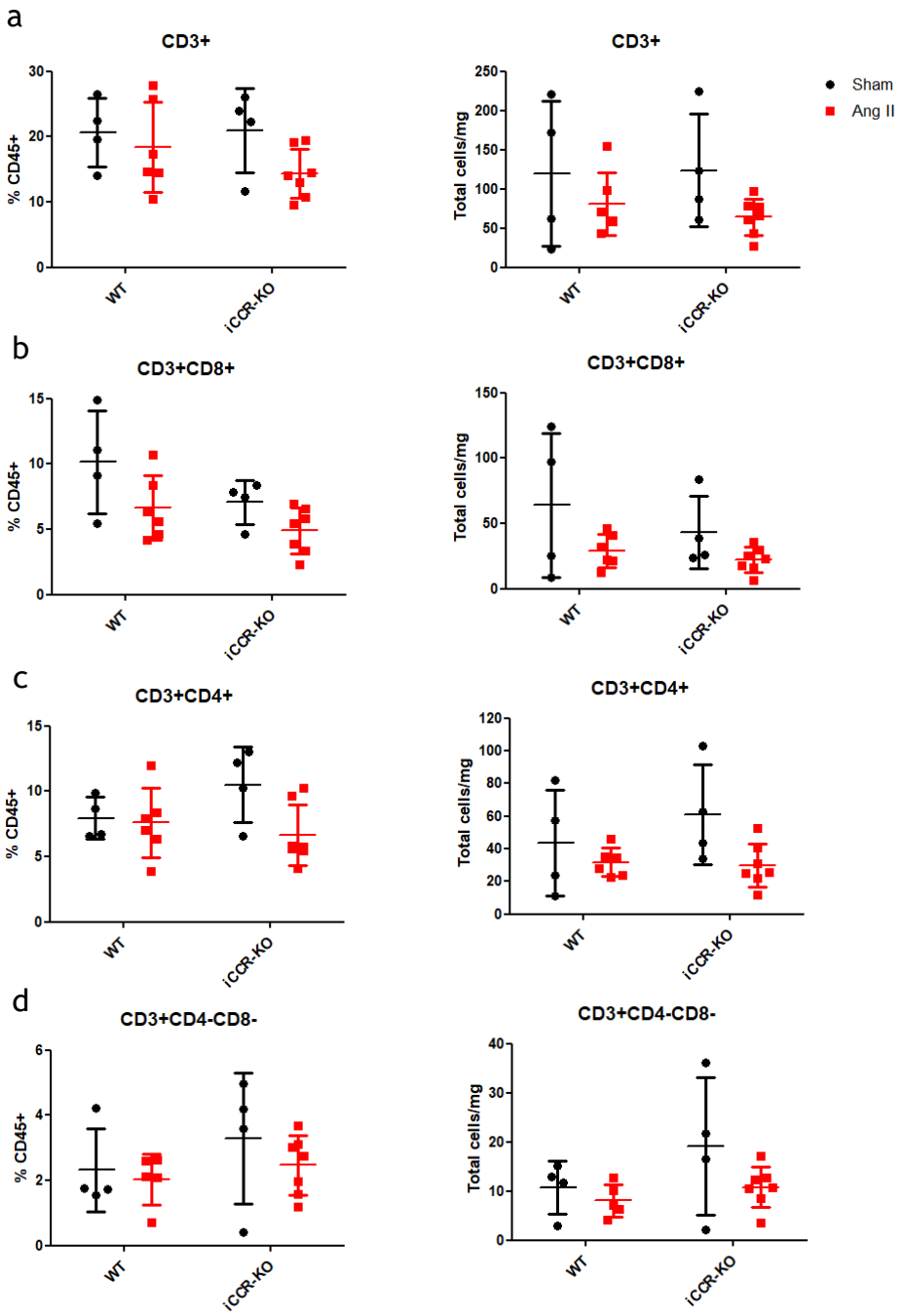


Figure 4-15. iCCR deficiency and PVAT T cell infiltration in Ang II induced hypertension
The infiltration as a proportion and total number of CD3+ cells (a), CD8+ cells (b), CD4+ cells (c) and CD4-CD8- cells (d) in the PVAT of WT and iCCR-KO mice after 14 days of sham or Ang II treatment.

4.6 Cardiac and vascular remodelling

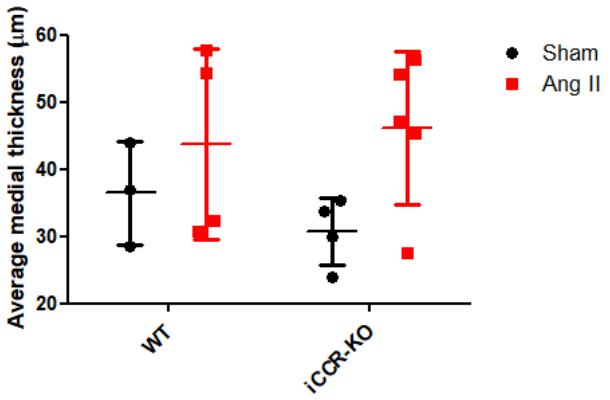
Hypertension induces the development of cardiac hypertrophy and interstitial fibrosis which can lead to heart failure (Suthahar et al., 2017). Further, Ang II can induce phenotypic switching of VSMCs from a contractile phenotype to a proliferative phenotype which leads to hypertrophy and inflammation

(Montezano et al., 2014). The effect of iCCR-deficiency on the aortic and cardiac hypertrophic effects of Ang II was investigated in WT and iCCR-KO mice after 14 days of Ang II induced hypertension.

4.6.1 Aortic hypertrophy

After 14 days of sham or Ang II treatment, the thickness of the aortic medial layer was measured for the aortas of WT and iCCR-KO mice to assess aortic hypertrophy. No differences were observed between the groups, although there appeared to be a trend for increased aortic hypertrophy in the Ang II treated mice (Figure 4-16).

a



b

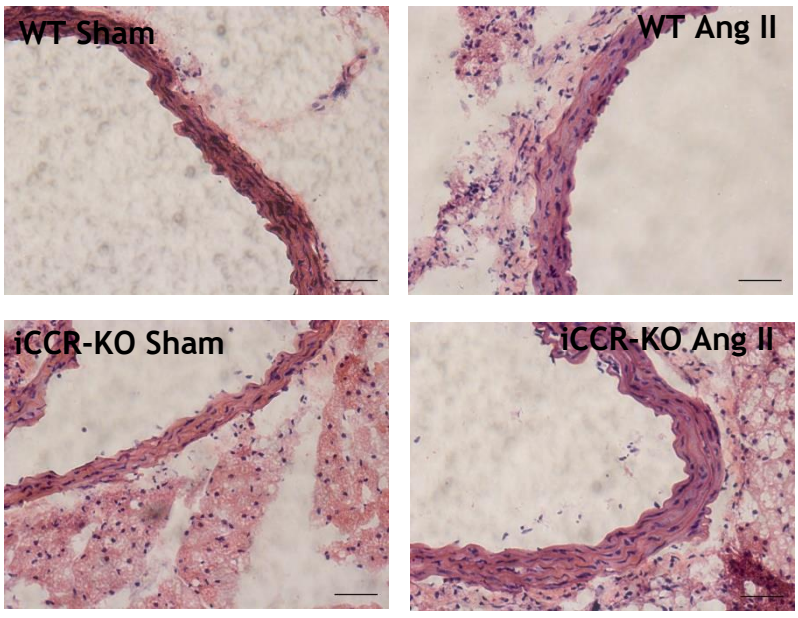


Figure 4-16. The effect of iCCR deficiency on aortic hypertrophy in Ang II induced hypertension
WT and iCCR-KO mice were subject to Ang II induced hypertension and aortic hypertrophy was assessed by measuring the width of the aortic medial layer (a) Representative H&E stained images are shown (b). Scale bar = 50 μm.

4.6.2 Cardiac hypertrophy and fibrosis

As a risk factor for heart failure, cardiac remodelling in hypertension should be treated. This was assessed in sham and Ang II treated WT and iCCR-KO mice by measuring the heart weight and looking at collagen deposition with a picrosirius red stain. When normalised to body weight, the heart weight increased in Ang II treated mice (Sham WT 5.7 ± 0.3 vs Ang II WT 7.2 ± 0.5 mg/g, $p < 0.01$, Two-Way ANOVA; Sham iCCR-KO 5.9 ± 0.8 vs Ang II iCCR-KO 7.3 ± 0.5 mg/g, $p < 0.01$, Two-Way ANOVA) and there were no differences between WT and iCCR-KO mice (Figure 4-17a). When normalised to tibia length, there was only an increase in heart weight in the iCCR-KO mice following Ang II treatment (Sham iCCR-KO 8.1 ± 1.6 vs Ang II iCCR-KO 10.4 ± 1.0 mg/mm, $p < 0.05$, Two-Way ANOVA) although there was a trend for Ang II to increase heart weight in WT mice (Figure 4-17b). Collagen deposition in the heart did not differ between the groups (Figure 4-17c, d).

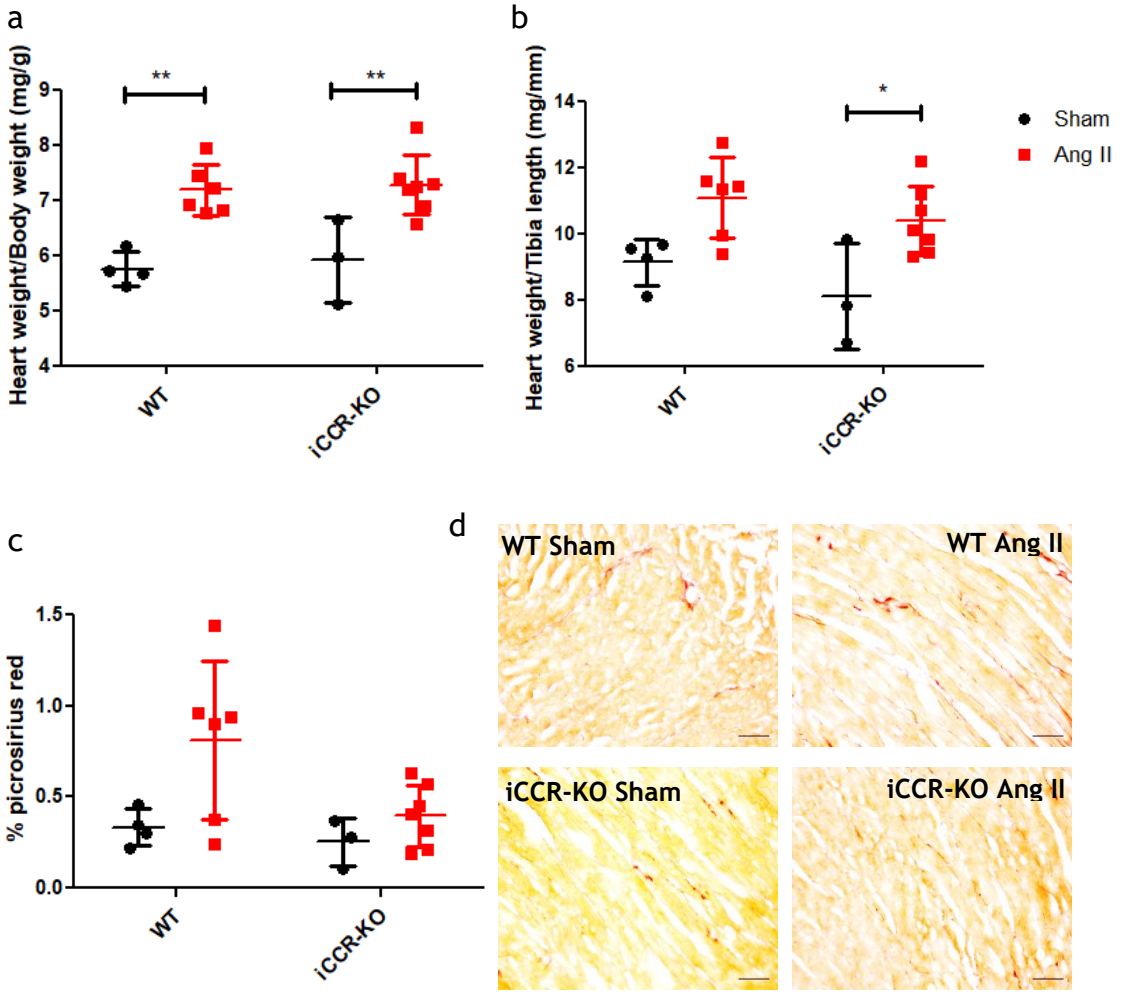


Figure 4-17. The effect of iCCR-deficiency on cardiac remodelling in Ang II induced hypertension
WT and iCCR-KO mice were subject to Ang II induced hypertension and cardiac hypertrophy was assessed by measuring the ratio of heart weight to body weight (a) and tibia length (b) and collagen deposition by picrosirius staining (c). Representative images are shown (d). Red staining indicates collagen. Scale bar = 100 µm. * $p < 0.05$, ** $p < 0.01$, Two-Way ANOVA.

4.7 Discussion

iCCR-KO mice were subject to 14 days of Ang II induced hypertension and hypertensive phenotypes were assessed. These included BP, vascular function and oxidative stress as well as inflammation and cardiac hypertrophy. This has revealed how iCCR deficiency affects hypertension, increasing our understanding of how iCCRs contribute to the disease.

4.7.1 iCCRs and BP, vascular function and oxidative stress

The BP of both WT and iCCR-KO mice increased in response to Ang II and there was no difference between the Ang II treated WT and iCCR-KO mice. Previously

it has been shown that CCR2 or CCR5 deficiency also does not affect BP in hypertension (Ishibashi et al., 2004; Krebs et al., 2012). This would suggest that the iCCRs are not involved in elevating BP in hypertension. However, treatment of mice subject to Ang II or DOCA-salt induced hypertension with the CCR2 antagonist INCB3344 reduces BP (Chan et al., 2012; Moore et al., 2015). This could suggest that CCR2, and possibly other iCCRs, contribute to BP regulation in hypertension but other mechanisms that compensate for this are established when the mice are deficient for the receptors from birth. This could be investigated using a combination of iCCR antagonists.

IL-17$^{-/-}$ mice subjected to Ang II induced hypertension undergo an initial elevation in BP that is similar to WT mice at the 14 day time point but then falls to a level lower than that of WT mice by 21 and 28 days of Ang II treatment (Madhur et al., 2010). 14 days of Ang II induced hypertension may not be long enough to see the effect of iCCR deficiency on BP and this model could be run for longer. Further, depletion of macrophages, T cells and B cells have all been associated with reduced BP in hypertensive animal models (Chan et al., 2015; Rudemiller et al., 2014; Thang et al., 2015). Factors other than iCCRs may have influenced leukocyte migration, enabling them to still regulate Ang II induced BP elevation in iCCR-KO mice.

The BP of sham treated WT mice was higher than the sham treated iCCR-KO mice at days 12 – 14 of tail cuff BP measurements. At this time point, the mice were 14 – 15 weeks old. No differences in baseline BP were seen between WT and iCCR-KO mice when measured by telemetry, when the mice were 11 – 12 weeks old. This difference in BP may be related to age and only occurs once the mice are around 14 weeks old. Measuring the BP of aged mice using the more reliable telemetry method would reveal if iCCR deficiency can protect from an increase in BP associated with aging (Parati et al., 2015).

Aortic vascular function was impaired in Ang II treated mice compared to sham treated mice, as has been shown before (Guzik et al., 2007). The iCCR-KO mice showed some protection from this impairment, although variability between mice meant that there was not a large protection. CCL5$^{-/-}$ mice have been shown to be protected from Ang II induced aortic dysfunction (Mikolajczyk et al., 2016). As CCL5 binds to CCR1 and CCR5, leukocytes may be recruited to the

aorta via these receptors and contribute to vascular dysfunction. Recruitment of NK cells to the aorta has been associated with Ang II induced vascular dysfunction (Kossmann et al., 2013) and mice with depleted monocytes are also protected from Ang II induced aortic dysfunction (Wenzel et al., 2011). Leukocytes have a role in vascular dysfunction and it is likely that iCCR deficiency is protective from Ang II induced vascular dysfunction through altered leukocyte recruitment. The infiltration of leukocytes in the PVAT was determined in this study but further analysis of the whole aorta is required.

iCCR-KO mesenteric arteries showed improved relaxation to ACh after Ang II treatment compared to Ang II treated WT vessels. Sham treated mice were not used so the extent of the effect of the Ang II was not determined, although impaired relaxation to ACh of hypertensive mesenteric arteries compared to controls has been shown (Ye et al., 2019). The improved relaxation seen in iCCR-KO mice may be due to altered leukocyte recruitment. NK cells accumulate in mesenteric arteries from L-NAME treated mice (Taherzadeh et al., 2010) but further characterisation of mesenteric artery leukocyte infiltration in hypertension is required. The response of both vessel types to SNP was not effected by iCCR deficiency, showing that iCCRs may mediate endothelium-dependent, but not -independent, relaxation.

Multiple studies have associated increased vascular superoxide with impaired vascular function and mice which are protected from Ang II induced vascular dysfunction, such as Op/Op, Rag-$1^{-/-}$ and $CCL5^{-/-}$ mice, are also protected from Ang II induced vascular superoxide elevation (De Ciuceis et al., 2005; Guzik et al., 2007; Mikolajczyk et al., 2016). No differences in aortic superoxide were shown between sham and Ang II treated WT and iCCR-KO mice. There was a trend for the Ang II treated mice to have increased aortic superoxide but the data were variable and therefore, not significant. A way to verify superoxide levels would be to determine the expression and activity of NADPH oxidases. As there was no difference in aortic superoxide between the WT and iCCR-KO mice, it is likely that superoxide level is not mediating the protection from Ang II induced vascular dysfunction in the iCCR-KO mice. NO release by mesenteric arteries has been shown to be reduced in the vessels of hypertensive rats compared to control rats (Stankevicius et al., 2002). It is possible that vessels

from hypertensive iCCR-KO mice release more NO than vessels from hypertensive WT mice and this should be further investigated.

4.7.2 iCCRs and plasma chemokines

iCCR-KO mice have a higher concentration of CCL3 and CCL7 in the plasma than WT mice and there was a trend to have increased CCL5. This was seen in both sham and Ang II treated mice. CCL3 binds to CCR1 and CCR5 whilst CCL7 binds to CCR1, CCR2, CCR3 and CCR5. Previously, iCCR-KO mice have been shown to have elevated plasma levels of CCL5, CCL7 and CCL11 (Dyer et al., 2019). This increase suggests that these chemokines have a homeostatic role and that the iCCRs scavenge these chemokines.

CCL3 concentration was higher in the plasma of Ang II treated iCCR-KO mice compared to sham treated iCCR-KO mice. This effect was not seen in the WT mice. Expression of the iCCRs may have been enough to scavenge any CCL3 that had been produced in response to Ang II and therefore no overall increase was observed. It is also possible that in WT mice, leukocytes migrate towards CCL3 and provide a feedback mechanism which switches off Ang II induced CCL3 expression. CCL3 mRNA expression has been shown to be elevated in the kidneys of hypertensive mice and to be higher in hypertensive $CCR5^{-/-}$ mice than hypertensive WT mice (Krebs et al., 2012). This suggests that CCR5 is involved in leukocyte migration towards CCL3 in the kidney. A limitation of looking at plasma chemokine concentration is that it is not possible to identify where the chemokines originated.

There is evidence of elevated CCL2 and CCL5 plasma concentration in hypertensive patients (Parissis et al., 2002). CCL2 was not detected by the Luminex assay used in this study and CCL5 concentration was not affected by Ang II. These differences could be due to differences between mice and humans. Data from the previous chapter showed that plasma CCL21 concentration slightly decreased in Ang II induced hypertension. This is not shown in this chapter and the data here show that some chemokine concentrations differ to those previously shown. This could be due to sample collection occurring at different times.

4.7.3 iCCRs and inflammation in hypertension

Ang II did not induce an increase in any of the leukocytes analysed in the kidneys of WT or iCCR-KO mice, in contrast to previous studies (Evans et al., 2017; Itani et al., 2016; Ozawa et al., 2006). This could be due to different cell isolation protocols or gating strategies and could be further investigated by IHC. CCR2 blockade with RS102895 reduces renal macrophage infiltration in hypertensive mice (Elmarakby et al., 2007; Kashyap et al., 2015) whilst CCR5 deficiency has no effect on T cell and macrophage infiltration in DOCA + Ang II induced hypertension (Krebs et al., 2012). This suggests that macrophages require CCR2, but not CCR5, to migrate to the kidney. However, as Ang II did not increase leukocyte infiltration in this study, the role of iCCRs could not be identified. iCCR deficiency resulted in an increase of kidney CD4-CD8- T cells in the hypertensive mice which was not seen in the sham treated mice. This could be due to iCCR induced changes in T cell subsets, which may only occur in hypertension. CD4-CD8- T cells have been shown to reside in the kidney and these cells are reduced in renal ischaemia reperfusion injury (Ascon et al., 2008).

T cells, macrophages and DCs have been shown to infiltrate the PVAT in Ang II induced hypertension and the expression of CCR1, CCR3 and CCR5 by T cells increases in the PVAT (Guzik et al., 2007; Mikolajczyk et al., 2016). In this study, Ang II was not shown to have this effect meaning that identifying how iCCR deficiency influences PVAT leukocyte infiltration was challenging. $CCL5^{-/-}$ and $IL\text{-}17^{-/-}$ mice are both protected from Ang II induced PVAT leukocyte infiltration (Madhur et al., 2010; Mikolajczyk et al., 2016). It is hypothesised that CCL5 recruits T cells to the PVAT via iCCRs. which then produce cytokines that mediate oxidative stress and endothelial dysfunction. If this is the case, Ang II treated iCCR-KO mice would have fewer infiltrating T cells, and possibly other leukocytes, which was not observed.

As inflammation develops in hypertension, it is likely that changes are seen in lymphoid organs. In the spleen, Ang II had no effect on the number of the leukocyte types analysed here. CD69 expression, a marker of activation, has been shown to increase by splenic T cells following Ang II treatment (Caillon et al., 2017). Activation of T cells was not investigated here but it is possible iCCR

deficiency could influence T cell activation. The monocyte population in the spleen may be reduced in rats after Ang II treatment and it has been suggested that this is due to migration from the spleen to the heart (N. P. Wang et al., 2017). Only splenic macrophages were analysed in this study and these were not affected by Ang II in WT and iCCR-KO mice. Additional markers such as Ly6C would be required to further analyse splenic monocytes.

iCCR deficiency altered certain splenic populations. The proportion of total leukocytes was reduced in the spleens of iCCR-KO mice compared to WT mice, suggesting that a small number of leukocytes require iCCRs to migrate to, or be retained in, the spleen. Hypertensive iCCR-KO mice also have a reduced total number of T cells compared to hypertensive WT mice, although this was not seen in the sham treated mice. Splenic CD8+, but not CD4+, T cells were reduced in the hypertensive iCCR-KO mice. In a model of viral infection, CD8+ T cells have been shown to increase in the spleen and this is associated with CCR2 and CCR5 expression (Nansen et al., 2000). It is possible that CD8+ T cells require these iCCRs to migrate to the spleen when challenged by a virus and, possibly, Ang II.

iCCR-KO mice had fewer Ly6C+ monocytes in the blood and more in the bone marrow, suggesting that iCCR deficiency prevents monocyte egress from the bone marrow to the blood. This has been previously shown in resting iCCR-KO mice and it is likely that this is dependent on CCR2 (Dyer et al., 2019). The same effect is seen for CD11b+ DCs but it is unknown which of the iCCRs these DCs require to migrate from the bone marrow. It is unclear if these differences are affected by Ang II and this should be repeated with sham and Ang II treated mice. Reconstituting WKY rats with the bone marrow from SHRs results in an increase in BP (Santisteban et al., 2015), suggesting that leukocytes in the bone marrow are important in hypertension. Further studies identifying leukocyte changes in the blood and bone marrow in hypertension and how these are regulated by iCCRs should be carried out.

B and T cells were also analysed in the blood of Ang II treated WT and iCCR-KO mice. The proportion of B cells decreased while T cells increased in iCCR-KO mice. Further analysis of T cell activation revealed that there were more CD44- and CD44+ T cells in the iCCR-KO mice but no difference in the CD44++ T cells.

The increase in CD44+ T cells in the iCCR-KO mice may reflect a more proliferative state. However, there are also more CD44- T cells so this may just reflect an overall increase in T cells. Ang II has been shown to increase the number of circulating CD4+ T cells expressing CD44 (Guzik et al., 2007). This should be verified by repeating the experiment with sham treated mice. A population of CD4+CD44^{hi}CD62L^{lo} T cells can produce ACh and have been suggested to deliver ACh to ECs, mediating vascular relaxation (Olofsson et al., 2016). It would be interesting to see if this population of T cells is affected by iCCR deficiency as this could contribute to BP regulation.

4.7.4 iCCRs and cardiac and vascular remodelling

Vascular hypertrophy has been demonstrated in several hypertensive animal models and hypertensive patients (Al-gburi et al., 2017; Guzik et al., 2007; Schiffrin et al., 2000). Aortic hypertrophy in sham and Ang II treated WT and iCCR-KO mice was assessed by measuring the medial thickness of the aortas. Ang II did not induce an increase in aortic medial thickness in both WT and iCCR-KO mice. As there was no increase in medial thickness, it is hard to determine if iCCR deficiency has any effect on vascular hypertrophy. However, there was a trend for aortic medial thickness to increase in the Ang II treated iCCR-KO mice compared to sham treated iCCR-KO mice, suggesting that iCCR deficiency does not influence Ang II induced vascular hypertrophy. This contrasts to CCR2$^{-/-}$ mice which are protected from Ang II induced aortic hypertrophy (Ishibashi et al., 2004). However, the protection in CCR2$^{-/-}$ mice was seen after 28 days of Ang II treatment. It is possible that it takes more than 14 days for this effect to be seen and aortic hypertrophy should be determined in the iCCR-KO mice after a longer model of Ang II induced hypertension.

Cardiac hypertrophy, as determined by measuring the ratio of heart weight to body weight, increased in both WT and iCCR-KO mice after Ang II treatment. This is also seen in hypertensive CCR5$^{-/-}$ and CCL2$^{-/-}$ mice (Krebs et al., 2012; Shen et al., 2014), suggesting that iCCRs do not affect cardiac hypertrophy in hypertension. Cardiac fibrosis was determined by measuring the deposition of collagen in the heart and there were no differences in collagen between any of the groups. However, it was expected that Ang II treatment would result in an increase in cardiac collagen as this has been shown previously (Wei et al., 2017).

A longer model may have been required to observe this. $CCL2^{-/-}$ mice are protected from hypertension induced collagen deposition in the heart (Shen et al., 2014), suggesting that CCR2 may be involved in fibrosis. If this is the case, it is likely iCCR-KO mice would be protected from Ang II induced cardiac fibrosis. Macrophages accumulate in the hearts and aortas of hypertensive mice and this is associated with remodelling (Falkenham et al., 2015; Ishibashi et al., 2004; Moore et al., 2015). Following monocyte depletion, fewer monocytes infiltrate the hearts of mice after Ang II treatment and there is reduced cardiac fibrosis (Falkenham et al., 2015). iCCR-KO mice have been shown to have reduced circulating monocytes and impaired recruitment of monocytes to sites of inflammation, similar to $CCR2^{-/-}$ mice (Dyer et al., 2019). It is hypothesised that there will be fewer macrophages recruited to the heart and aortas of Ang II treated iCCR-KO mice compared to WT mice and that this will result in reduced fibrosis but this has yet to be investigated.

4.8 Conclusion

The effect of iCCR deficiency in Ang II induced hypertension has been investigated (Table 4-2). The BP of iCCR-KO mice increased to the same extent as WT mice but the iCCR-KO mice were protected from vascular dysfunction. Vascular oxidative stress was the same in WT and iCCR-KO mice and iCCR deficiency did not affect Ang II induced inflammation in the PVAT or kidneys. However, the effect of Ang II on inflammation and oxidative stress in WT mice seen here was not as expected, or as has been demonstrated before, so the role of iCCR deficiency in Ang II induced inflammation may not be fully revealed by these experiments. iCCR-KO mice also showed cardiac hypertrophy similar to WT mice.

Overall, this study has revealed that iCCRs are involved in vascular dysfunction in Ang II induced hypertension. Leukocyte infiltration into the aorta and mesenteric arteries should be investigated to see how leukocytes may mediate vascular function. Although iCCRs do not appear to regulate BP, through protecting vascular function the risk of atherosclerosis is reduced. Therefore, iCCRs represent potential targets to improve end organ damage in hypertension. Further studies are required to identify any individual or combinatorial roles of the iCCRs in Ang II induced hypertension.

Hypertension phenotype	WT	iCCR-KO	Further experiments needed
BP	Increased	Increased	--
Vascular function	Impaired	Protected	Mesenteric sham vessels
Aortic oxidative stress	No increase detected	No increase detected	Measure NADPH oxidase activity
Plasma CCL3	No increase	Increased	--
Kidney inflammation	No increase detected	No increase detected	Repeat/IHC
PVAT inflammation	No increase detected	No increase detected	Repeat/IHC
Aortic hypertrophy	No increase detected	No increase detected	Longer model
Cardiac hypertrophy	Increased	Increased	--
Cardiac fibrosis	No increase	No increase	Longer model

Table 4-2. The effect of iCCR deficiency in Ang II induced hypertension
WT and iCCR-KO mice were subject to 14 days of Ang II induced hypertension. BP, vascular function, aortic oxidative stress, inflammation and hypertrophy were assessed and the effect of iCCR deficiency was determined.

Chapter 5

The effect of iCCR deficiency on kidney immune cells and regulators of blood pressure

5 The effect of iCCR deficiency on kidney immune cells and regulators of blood pressure

5.1 Introduction

The kidney is an important organ for the maintenance of BP through the regulation of salt excretion and fluid homeostasis. This involves reabsorption of sodium in the nephrons of the kidney and the retention of water (Soundararajan et al., 2010). Increased sodium reabsorption can result in hypertension, as is seen in certain diseases such as Liddle disease or Gordon's syndrome (Sabbadin and Armanini, 2016). Work in the previous chapter demonstrated that iCCR-KO mice could have slightly reduced baseline BP compared to WT mice, potentially in older mice. This could be due to altered kidney function. Therefore, the effect of iCCR deficiency on the kidney was investigated. iCCR-KO mice have been shown to have reduced macrophage and monocyte numbers in the spleen, skin and lung (Dyer et al., 2019) so the presence of these cells and other leukocytes in the kidney was determined along with the expression of genes involved in sodium reabsorption and BP regulation. Mice deficient in individual iCCRs were also used to determine if any differences could be a result of lacking one iCCR or multiple iCCRs. In addition, the structure of the kidneys in WT and iCCR-KO mice was investigated. This increases understanding of how iCCRs could affect BP regulation.

5.2 Kidney leukocyte infiltration

The infiltration of CD45+ cells, CD11b+F4/80+ cells, CD11b+F4/80+Ly6C- cells and CD11b+F4/80+Ly6C+ cells in the kidneys of iCCR-KO and individual iCCR deficient mice was investigated by flow cytometry, using the gating strategy below (Figure 5-1). Cells that were CD11b+ and F4/80+ were gated from CD45+ cells and these were divided based on Ly6C expression. Kidney T cells and B cells were characterised in iCCR-KO mice following the gating strategy in the previous chapter (Figure 4-4).

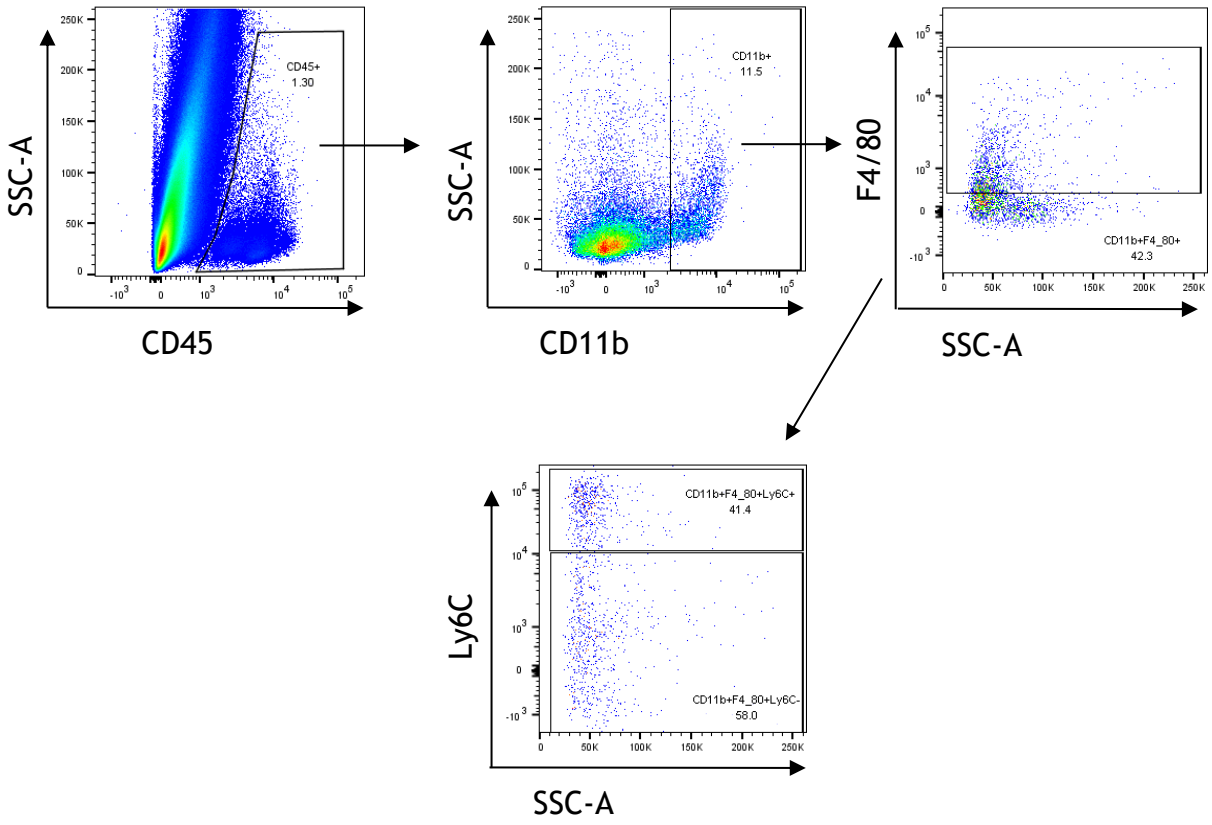


Figure 5-1. Gating strategy for the analysis of macrophages and monocytes in the kidneys of resting WT, iCCR-KO, CCR1-KO, CCR2-KO, CCR3-KO and CCR5-KO mice
CD45+ cells were gated from live, single cells. CD11b+ cells were gated from the CD45+ population and from these a population of F4/80+ cells were identified. The CD11b+F4/80+ cells were gated into CD11b+F4/80+Ly6C- and CD11b+F4/80+Ly6C+ populations.

5.2.1 Macrophages and monocytes in iCCR-KO mice

The accumulation of CD45+ cells (Figure 5-2a) in the kidneys of iCCR-KO mice did not differ from the WT mice but there was a reduction in CD11b+F4/80+ cells (Figure 5-2b; WT 5.5 ± 1.0 vs iCCR-KO 3.4 ± 0.6 % CD45+ cells, $p < 0.01$, Mann Whitney test). This was shown to be explained by a reduction in CD11b+F4/80+Ly6C+ cells (WT 1.9 ± 0.3 vs iCCR-KO 0.6 ± 0.1 % CD45+ cells, $p < 0.01$, Mann Whitney test) but not CD11b+F4/80+Ly6C- cells (Figure 5-2c, d). Representative flow cytometry plots demonstrate the reduction of CD11b+F4/80+ cells in the iCCR-KO mice (Figure 5-2e).

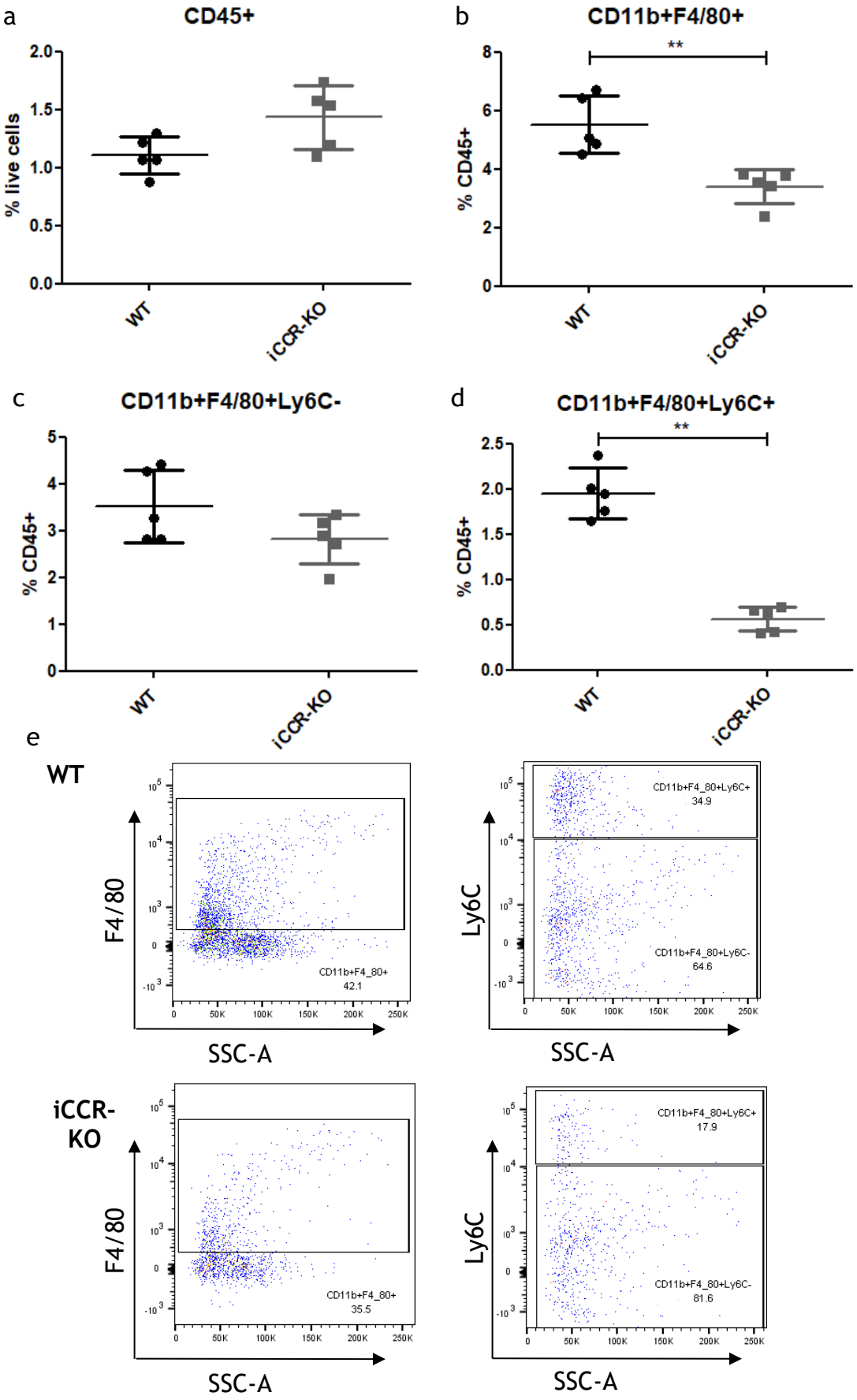

a
CD45+
% live cells
2.0
1.5
1.0
0.5
0.0
WT
iCCR-KO
b
CD11b+F4/80+
**
% CD45+
8
6
4
2
0
WT
iCCR-KO
c
CD11b+F4/80+Ly6C-
% CD45+
5
4
3
2
1
0
WT
iCCR-KO
d
CD11b+F4/80+Ly6C+
**
% CD45+
2.5
2.0
1.5
1.0
0.5
0.0
WT
iCCR-KO
e
WT
F4/80
SSC-A
CD11b+F4_80+
42.1
Ly6C
SSC-A
CD11b+F4_80+Ly6C+
34.9
CD11b+F4_80+Ly6C-
64.6
iCCR-KO
F4/80
SSC-A
CD11b+F4_80+
35.5
Ly6C
SSC-A
CD11b+F4_80+Ly6C+
17.9
CD11b+F4_80+Ly6C-
81.6

Figure 5-2. Infiltration of macrophages and monocytes in the kidneys of resting iCCR-KO mice
The infiltration of CD45+ cells (a), CD11b+F4/80+ cells (b), CD11b+F4/80+Ly6C- cells (c) and CD11b+F4/80+Ly6C+ cells (d) in the kidneys of resting iCCR-KO mice was determined by flow cytometry. Representative flow cytometry plots of CD11b+F4/80+, CD11b+F4/80Ly6C- and CD11b+F4/80+Ly6C+ populations are shown (e). ** $p < 0.01$, Mann Whitney test.

5.2.2 Macrophages and monocytes in CCR1-KO, CCR2-KO, CCR3-KO and CCR5-KO mice

To determine if the reduction in CD11b+F4/80+Ly6C+ cells in the iCCR-KO mice was a result of a deficiency of a single iCCR or multiple iCCRs, the infiltration of CD11b+F4/80+ cells, CD11b+F4/80+Ly6C- cells and CD11b+F4/80+Ly6C+ cells in the kidneys of individual iCCR deficient mice was investigated. CD45+ cells represented up to 5 % of total cells and CD11b+F4/80+ cells accounted for up to 10 % of CD45+ cells in the CCR1-KO, CCR2-KO, CCR3-KO mice and their WT controls (Figure 5-3, Figure 5-4, Figure 5-5). Analysis of the CCR5-KO mice and WT controls showed that up to about 45 % of kidney CD45+ cells were CD11b+F4/80+ (Figure 5-6). CCR1, CCR3 or CCR5 deficiency had no effect on these cell types in the kidney but CCR2 deficiency resulted in a reduction of CD11b+F4/80+ cells, CD11b+F4/80+Ly6C- cells and CD11b+F4/80+Ly6C+ cells (CD11b+F4/80+: WT 6.4 ± 1.4 vs CCR2-KO 2.8 ± 0.5 % CD45+ cells, $p < 0.01$, Mann Whitney test; CD11b+F4/80+Ly6C-: WT 4.1 ± 0.8 vs CCR2-KO 2.2 ± 0.4 % CD45+ cells, $p < 0.01$, Mann Whitney test; CD11b+F4/80+Ly6C+: WT 1.7 ± 0.5 vs CCR2-KO 0.4 ± 0.2 % CD45+ cells, $p < 0.01$, Mann Whitney test). This reduction is demonstrated by representative flow cytometry plots (Figure 5-4).

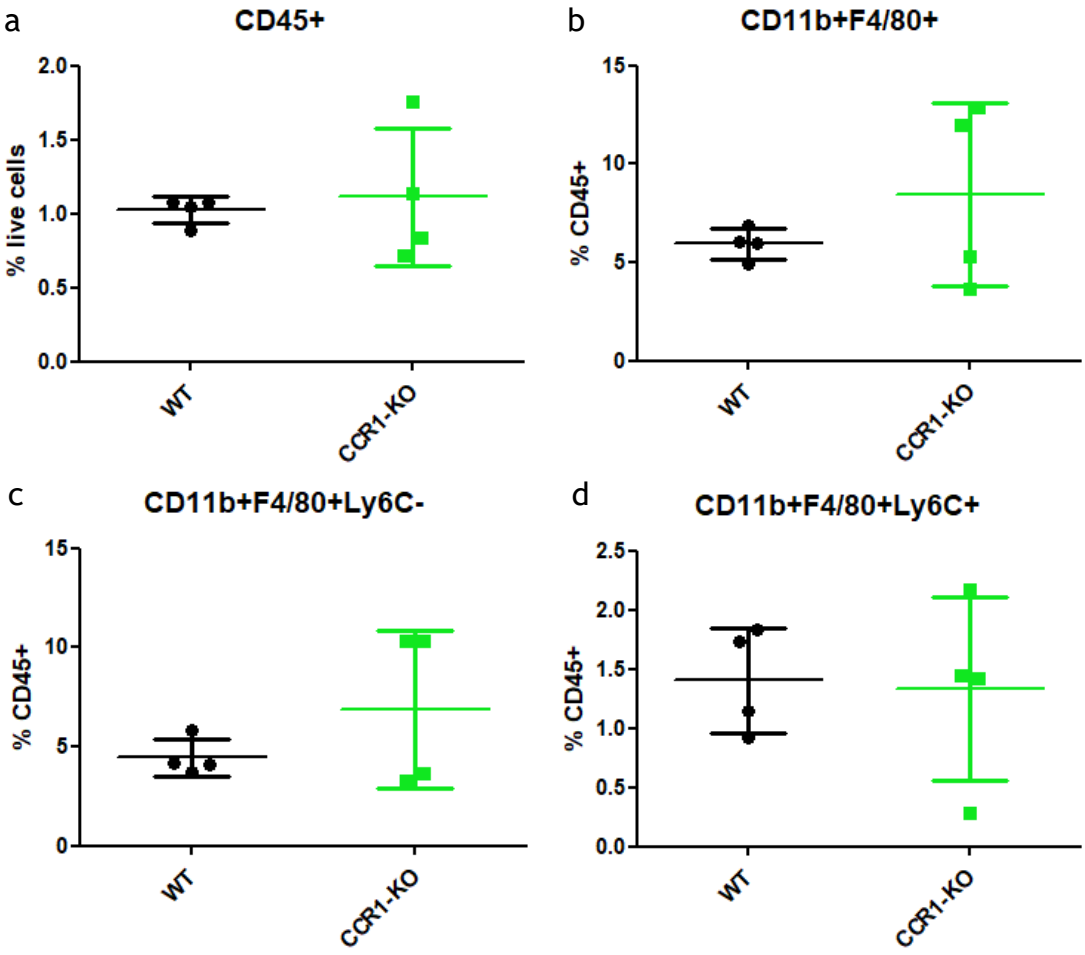


Figure 5-3. Infiltration of macrophages and monocytes in the kidneys of resting CCR1-KO mice

The infiltration of CD45+ cells (a), CD11b+F4/80+ cells (b), CD11b+F4/80+Ly6C- cells (c) and CD11b+F4/80+Ly6C+ cells (d) in the kidneys of resting CCR1-KO mice was determined by flow cytometry.

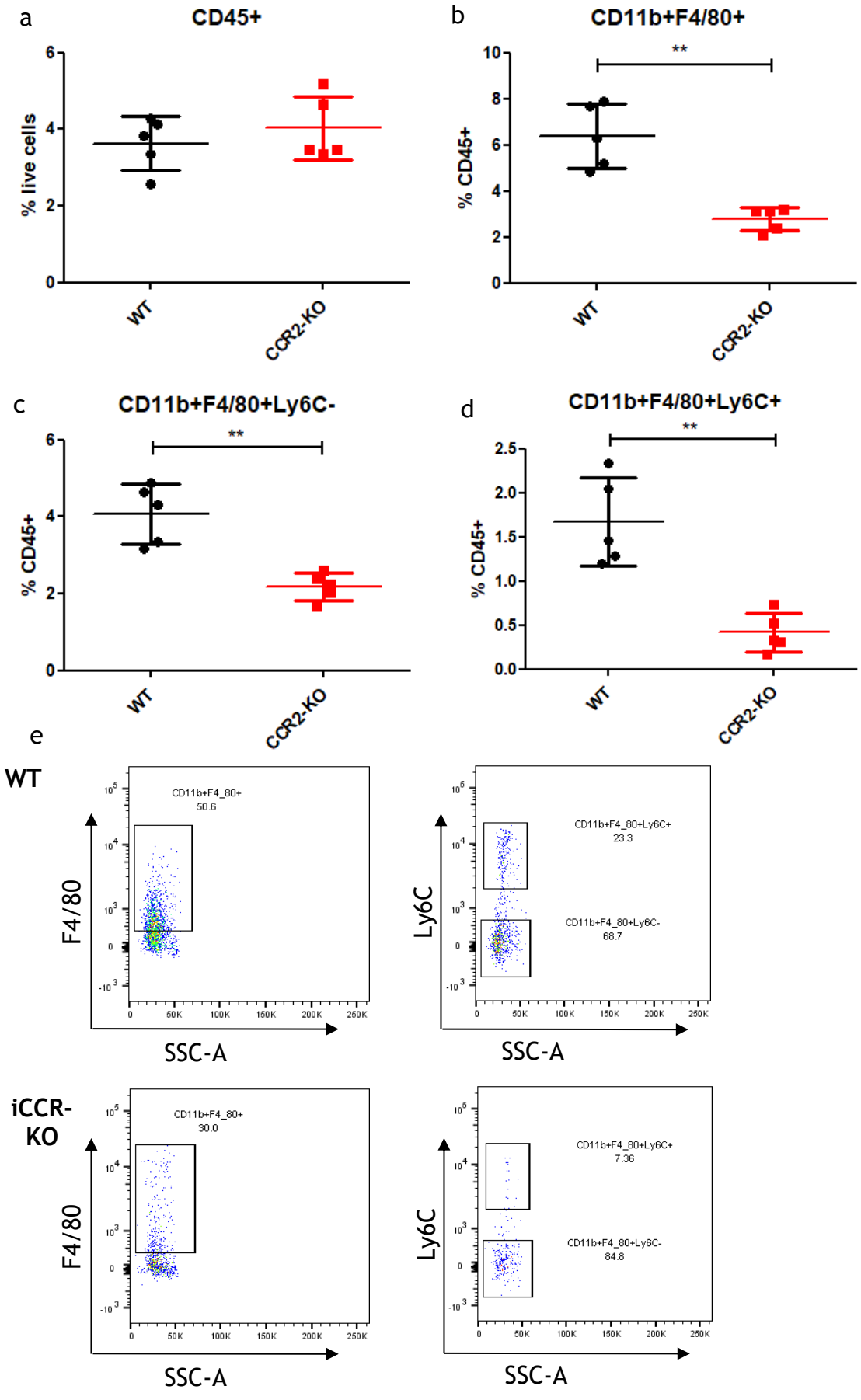

a
CD45+
% live cells
WT
CCR2-KO
b
CD11b+F4/80+
**
% CD45+
c
CD11b+F4/80+Ly6C-
d
CD11b+F4/80+Ly6C+
e
WT
iCCR-KO
CD11b+F4_80+
50.6
30.0
F4/80
SSC-A
CD11b+F4_80+Ly6C+
23.3
7.36
CD11b+F4_80+Ly6C-
68.7
84.8
Ly6C

Figure 5-4. Infiltration of macrophages and monocytes in the kidneys of resting CCR2-KO mice
The infiltration of CD45+ cells (a), CD11b+F4/80+ cells (b), CD11b+F4/80+Ly6C- cells (c) and CD11b+F4/80+Ly6C+ cells (d) in the kidneys of resting CCR2-KO mice was determined by flow cytometry. Representative flow cytometry plots of CD11b+F4/80+, CD11b+F4/80Ly6C- and CD11b+F4/80+Ly6C+ populations are shown (e). ** $p < 0.01$, Mann Whitney test.

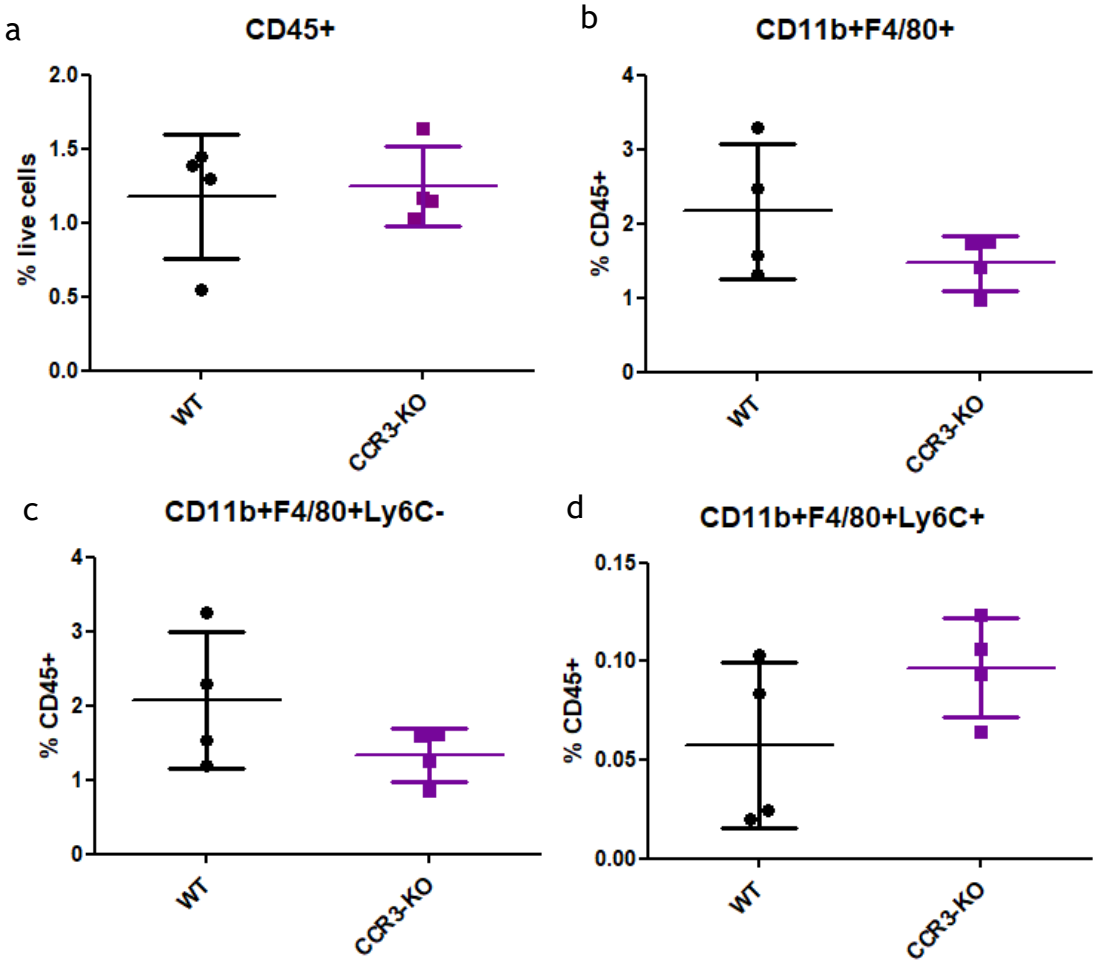


Figure 5-5. Infiltration of macrophages and monocytes in the kidneys of resting CCR3-KO mice
The infiltration of CD45+ cells (a), CD11b+F4/80+ cells (b), CD11b+F4/80+Ly6C- cells (c) and CD11b+F4/80+Ly6C+ cells (d) in the kidneys of resting CCR3-KO mice was determined by flow cytometry.

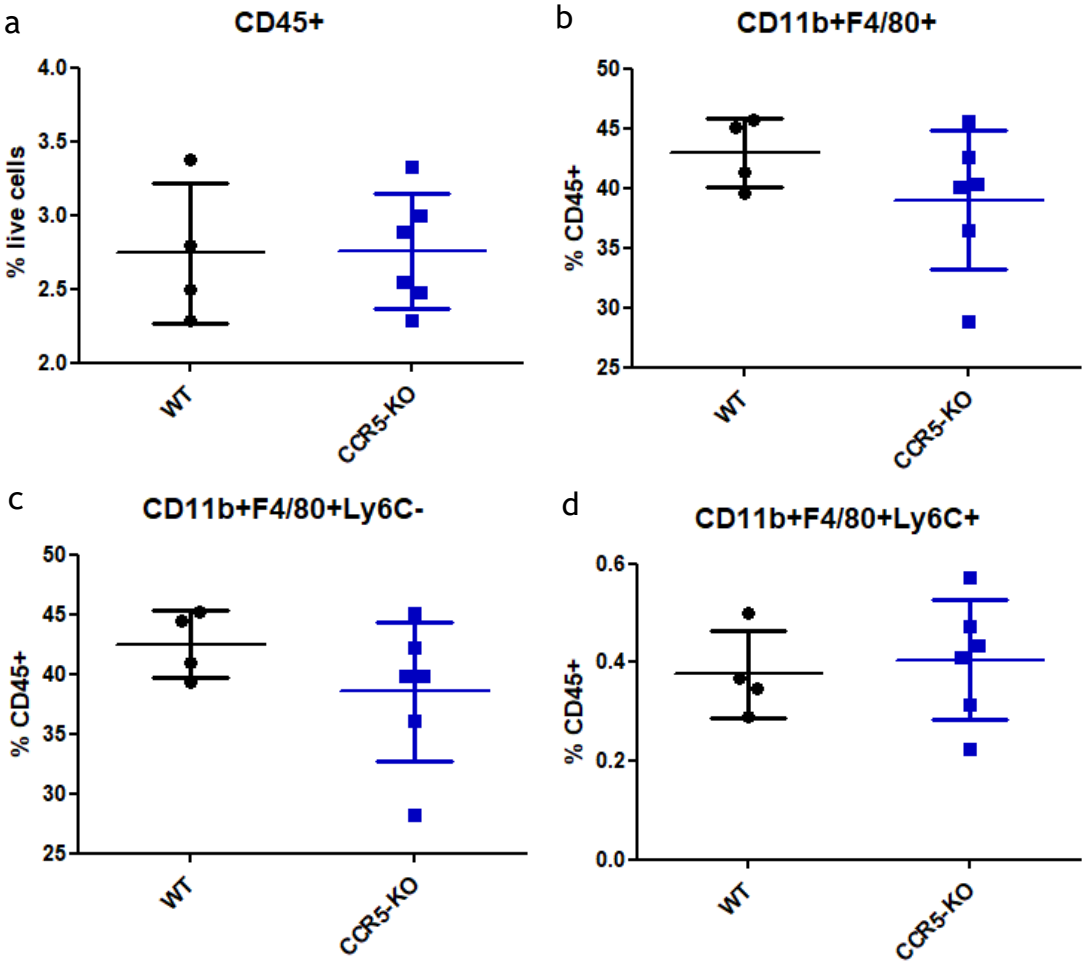


Figure 5-6. Infiltration of macrophages and monocytes in the kidneys of resting CCR5-KO mice
The infiltration of CD45+ cells (a), CD11b+F4/80+ cells (b), CD11b+F4/80+Ly6C- cells (c) and CD11b+F4/80+Ly6C+ cells (d) in the kidneys of resting CCR5-KO mice was determined by flow cytometry.

5.2.3 iCCR expression by macrophages and monocytes

REP mice were used to identify which iCCRs macrophages and monocytes express to aid the understanding of which iCCRs may be used for migration to and within the kidney (Figure 5-7). Neither CD11b+F4/80+Ly6C- cells nor CD11b+F4/80+Ly6C+ cells expressed CCR3 but they did express CCR1, CCR2 and CCR5. CD11b+F4/80+Ly6C- cells most highly expressed CCR5 whilst CCR2 was most highly expressed by CD11b+F4/80+Ly6C+ cells. Co-expression of CCR1, CCR2 and CCR5 by CD11b+F4/80+Ly6C- cells was also assessed but there were too few CD11b+F4/80+Ly6C+ cells to analyse iCCR co-expression. About 30 % of CD11b+F4/80+Ly6C- cells were CCR1+CCR5+ and 20 % were CCR5+ but none expressed just CCR1. About 5 % of these cells expressed just CCR2 and up to 15 %

expressed both CCR2 and CCR5. Further, about 30 % of these cells did not express any of the iCCRs (Figure 5-7c).

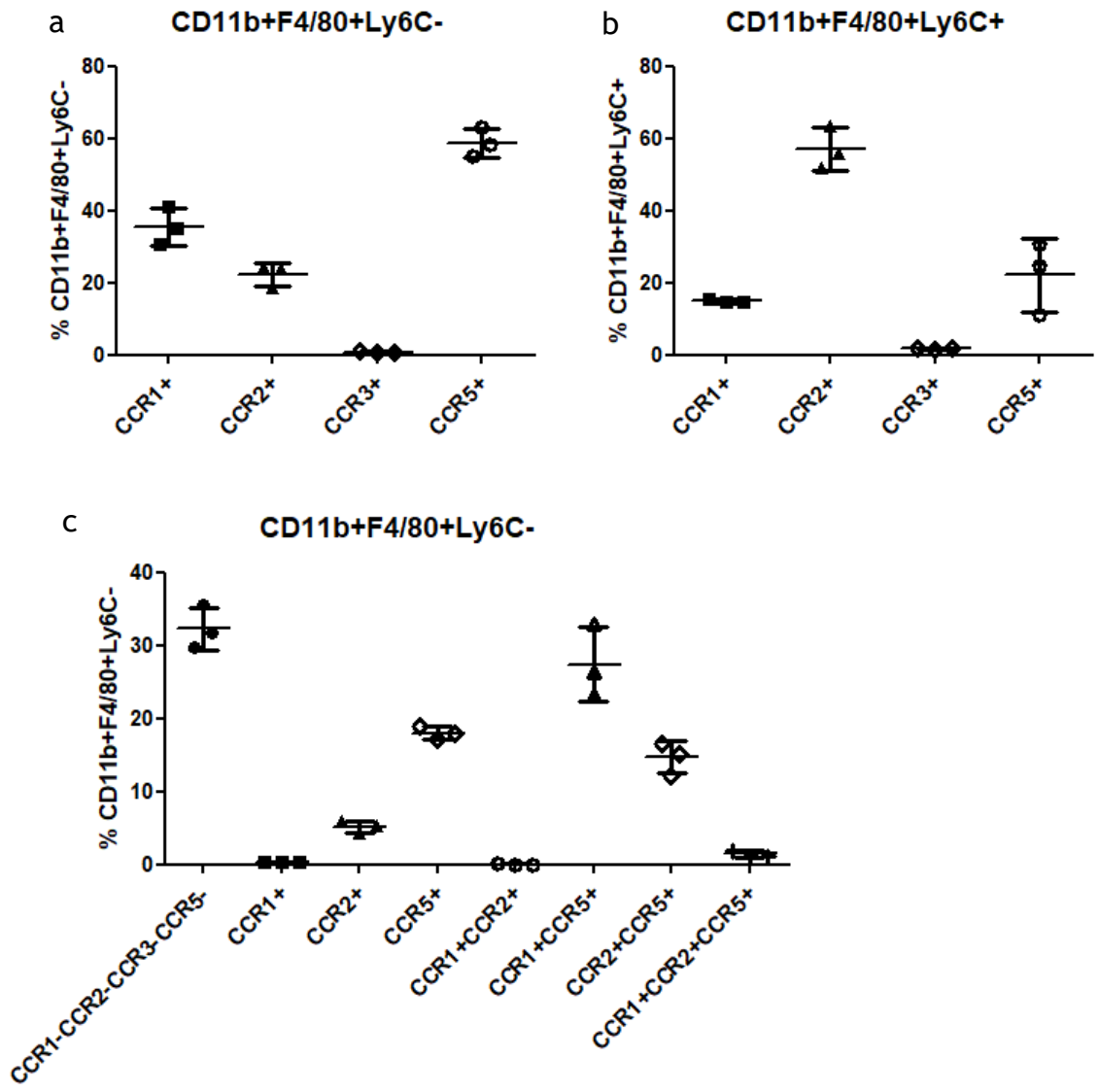


Figure 5-7. Macrophage and monocyte iCCR expression
The expression of CCR1, CCR2, CCR3 and CCR5 by CD11b+F4/80+Ly6C- cells (a) and CD11b+F4/80+Ly6C+ cells (b) was measured using REP mice. Co-expression of CCR1, CCR2 and CCR5 by CD11b+F4/80Ly6C- cells was determined (c).

5.2.4 T cells and B cells in iCCR-KO mice

Infiltrating CD19+ B cells and overall CD3+ T cells were not affected by iCCR deficiency (Figure 5-8a, e). T cells were further analysed as CD4+, CD8+ and CD4-CD8- and these were also not affected by the lack of iCCRs (Figure 5-8b, c, d).

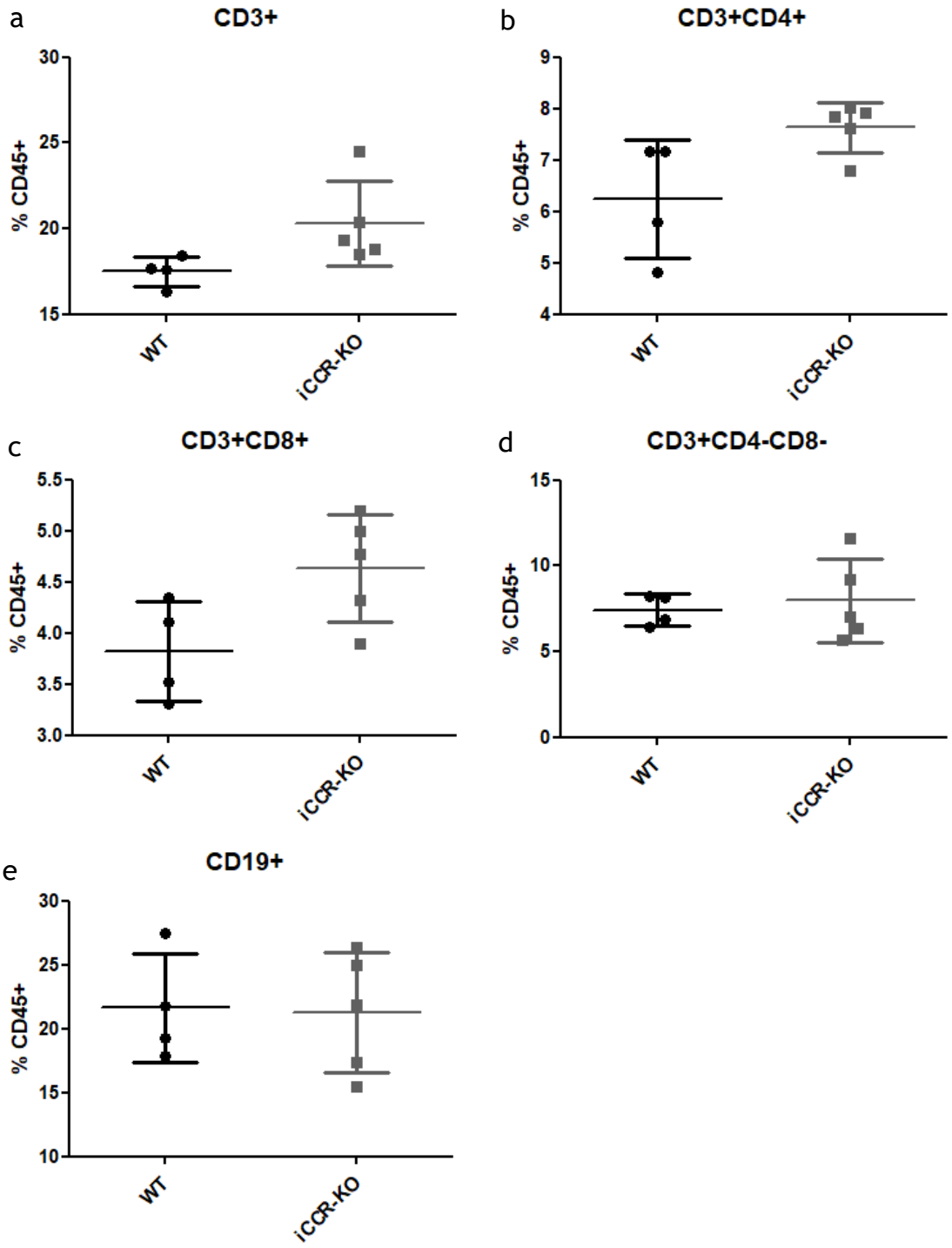


Figure 5-8. Infiltrating T cells and B cells in the kidneys or resting iCCR-KO mice
The infiltration of CD3+ cells (a), CD3+CD4+ cells (b), CD3+CD8+ cells (c), CD3+CD4-CD8- cells (d) and CD19 + cells (e) in the kidneys of resting iCCR-KO mice was determined by flow cytometry.

5.3 Kidney blood pressure regulators

As iCCR deficiency may reduce baseline BP, iCCR deficiency may affect regulators of sodium reabsorption, and therefore BP, in the kidney. The

expression of the genes for MR, serum/glucocorticoid-regulated kinase 1 (SGK1), ENaC and sodium/hydrogen exchanger 3 (NHE3) were assessed by qPCR. MR is a nuclear receptor transcription factor that binds to aldosterone and regulates transcription of genes involved in sodium reabsorption. One of these genes is SGK1. SGK1 regulates activation of ENaC, a channel that facilitates sodium reabsorption (Shibata and Fujita, 2011). NHE3 also regulates sodium reabsorption and is important in BP maintenance (Fenton et al., 2017).

5.3.1 iCCR-KO mice

The mRNA expression of MR was reduced in the iCCR-KO mice (Figure 5-9a; WT 100 ± 41 vs iCCR-KO 30 ± 14 % WT mRNA expression, $p < 0.01$, Mann Whitney test). SGK1, ENaC and NHE3 expression were not altered in the iCCR-KO mice compared to WT mice (Figure 5-9b, c, d).

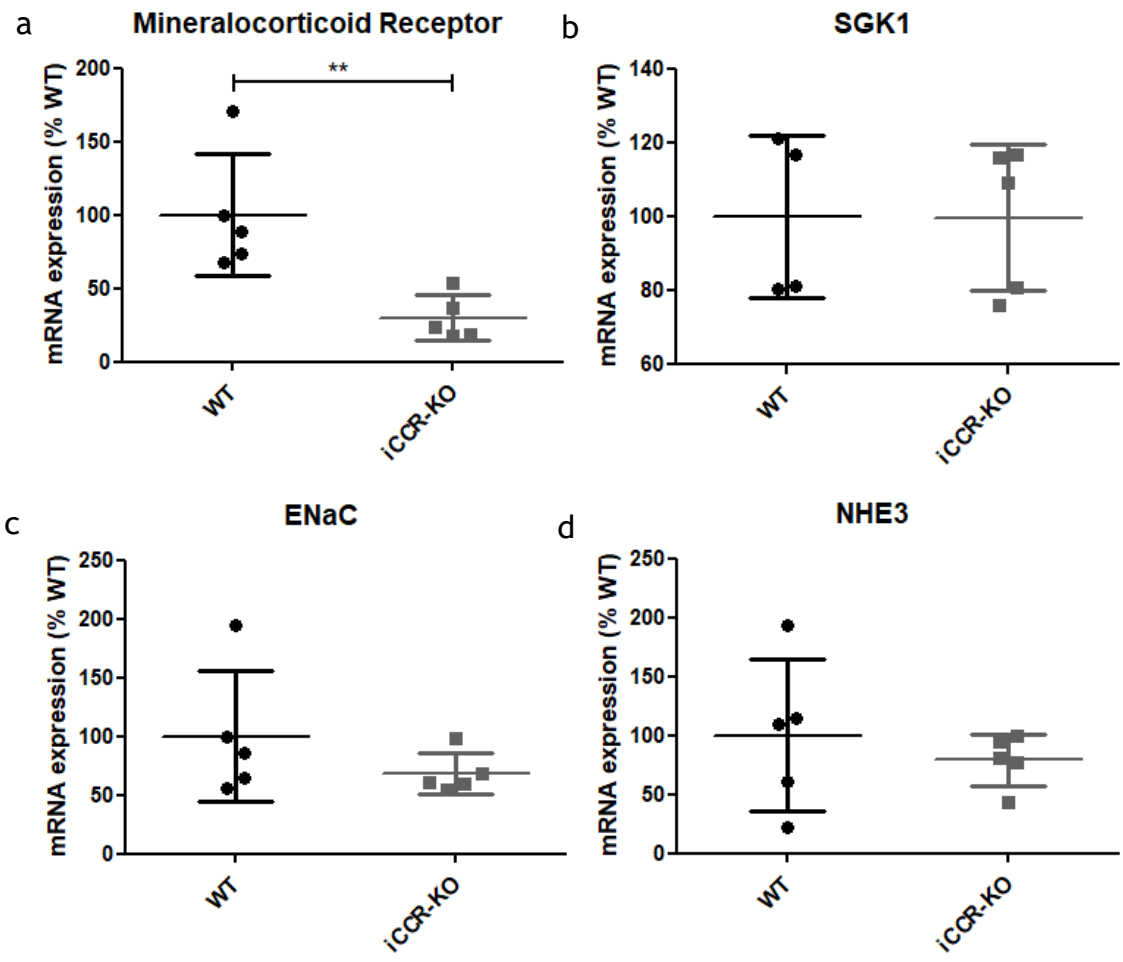


Figure 5-9. iCCR-KO kidney MR, SGK1, ENaC and NHE3 expression

The expression of MR (a), SGK1 (b), ENaC (c) and NHE3 (d) in the kidneys of resting WT and iCCR-KO mice was determined by qPCR. ** $p < 0.01$, Mann Whitney test.

5.3.2 CCR1-KO, CCR2-KO, CCR3-KO and CCR5-KO mice

To evaluate whether the reduction in MR mRNA expression in the iCCR-KO mice was a result of an individual iCCR deficiency or is specific to iCCR-KO mice, the expression of MR, SGK1, ENaC and NHE3 was determined in each of the individual iCCR deficient mice. Analysis of CCR1-KO mice showed the same MR, SGK1 and NHE3 expression as the WT mice but ENaC expression was lower in the CCR1-KO mice (Figure 5-10c; WT 100 ± 7 vs CCR1-KO 81 ± 10 % WT mRNA expression, $p < 0.05$, Mann Whitney test). CCR2 or CCR3 deficiency had no effect on the expression of MR, SGK1, ENaC and NHE3 (Figure 5-11, Figure 5-12). The expression of MR, ENaC and NHE3 in the kidneys of CCR5-KO mice was not different from the WT mice but SGK1 expression was higher in the CCR5-KO mice (Figure 5-13b; WT 100 ± 13 vs CCR5-KO 139 ± 28 % WT mRNA expression, $p < 0.05$, Mann Whitney test).

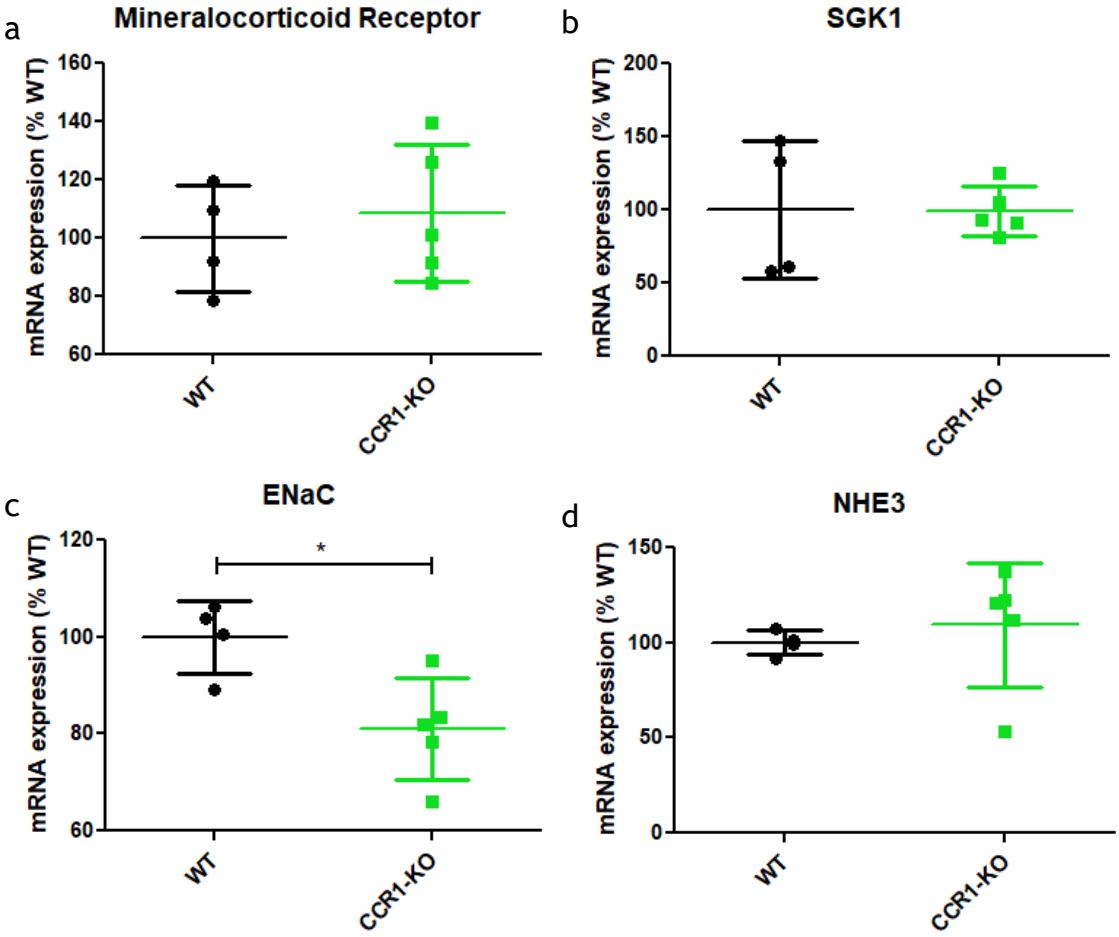


Figure 5-10. CCR1-KO kidney MR, SGK1, ENaC and NHE3 expression
The expression of MR (a), SGK1 (b), ENaC (c) and NHE3 (d) in the kidneys of resting WT and CCR1-KO mice was determined by qPCR. * $p < 0.05$, Mann Whitney test.

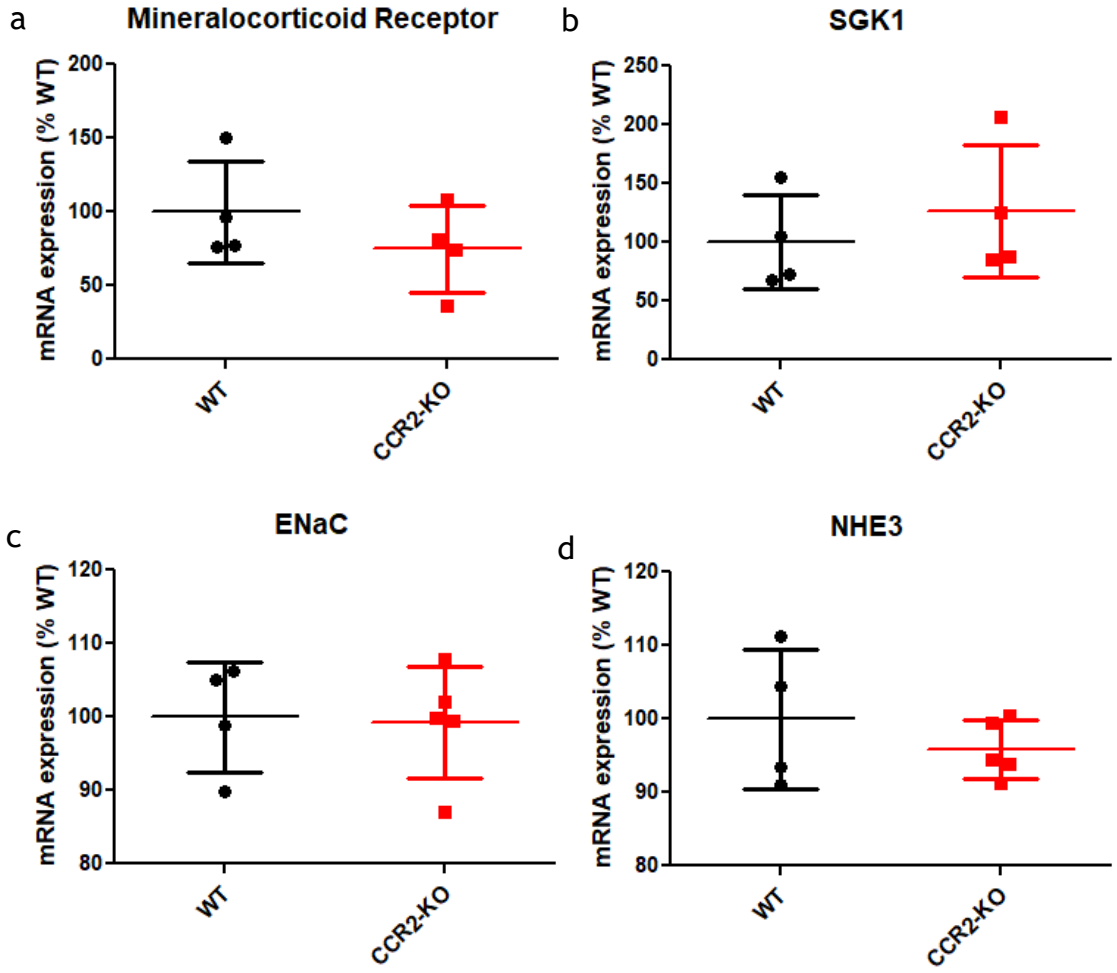


Figure 5-11. CCR2-KO kidney MR, SGK1, ENaC and NHE3 expression
The expression of MR (a), SGK1 (b), ENaC (c) and NHE3 (d) in the kidneys of resting WT and CCR2-KO mice was determined by qPCR.

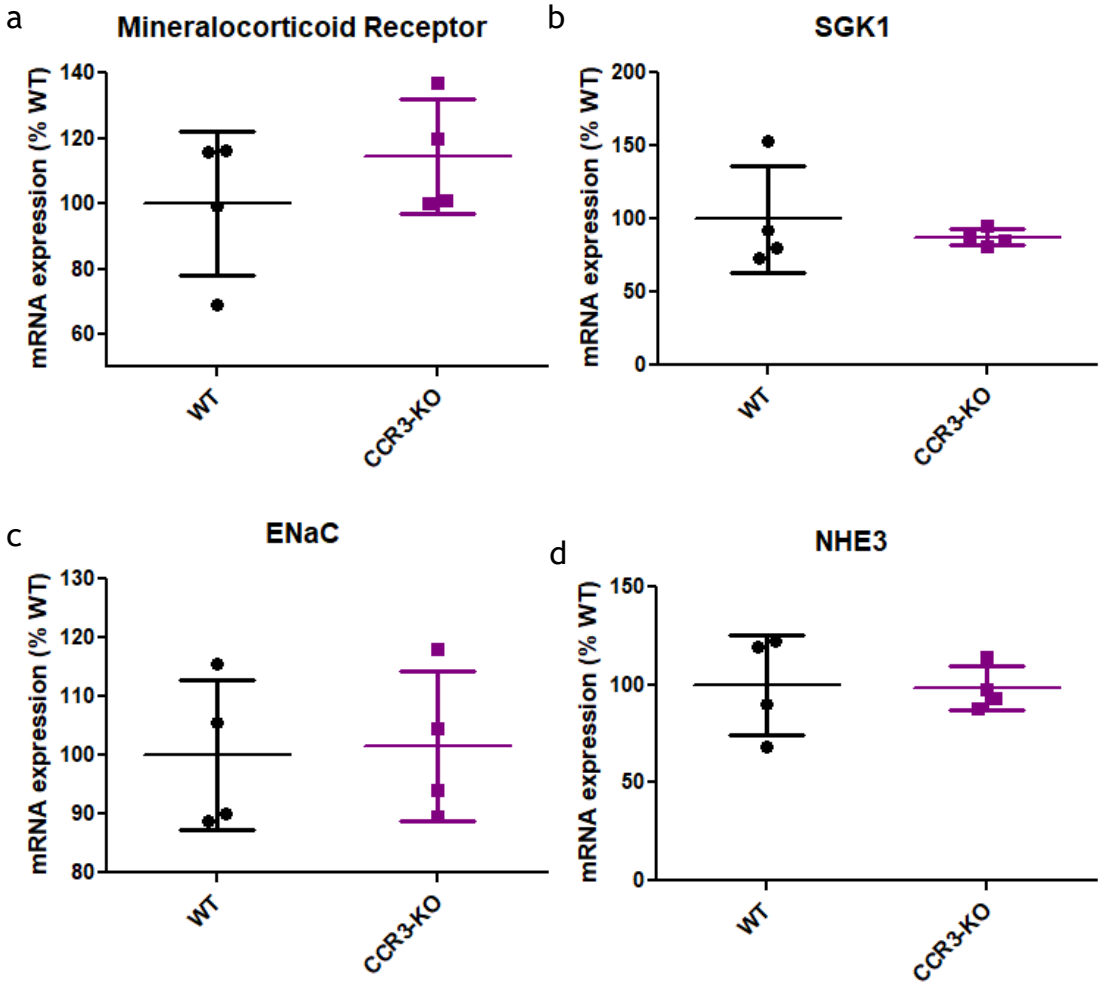


Figure 5-12. CCR3-KO kidney MR, SGK1, ENaC and NHE3 expression
The expression of MR (a), SGK1 (b), ENaC (c) and NHE3 (d) in the kidneys of resting WT and CCR3-KO mice was determined by qPCR.

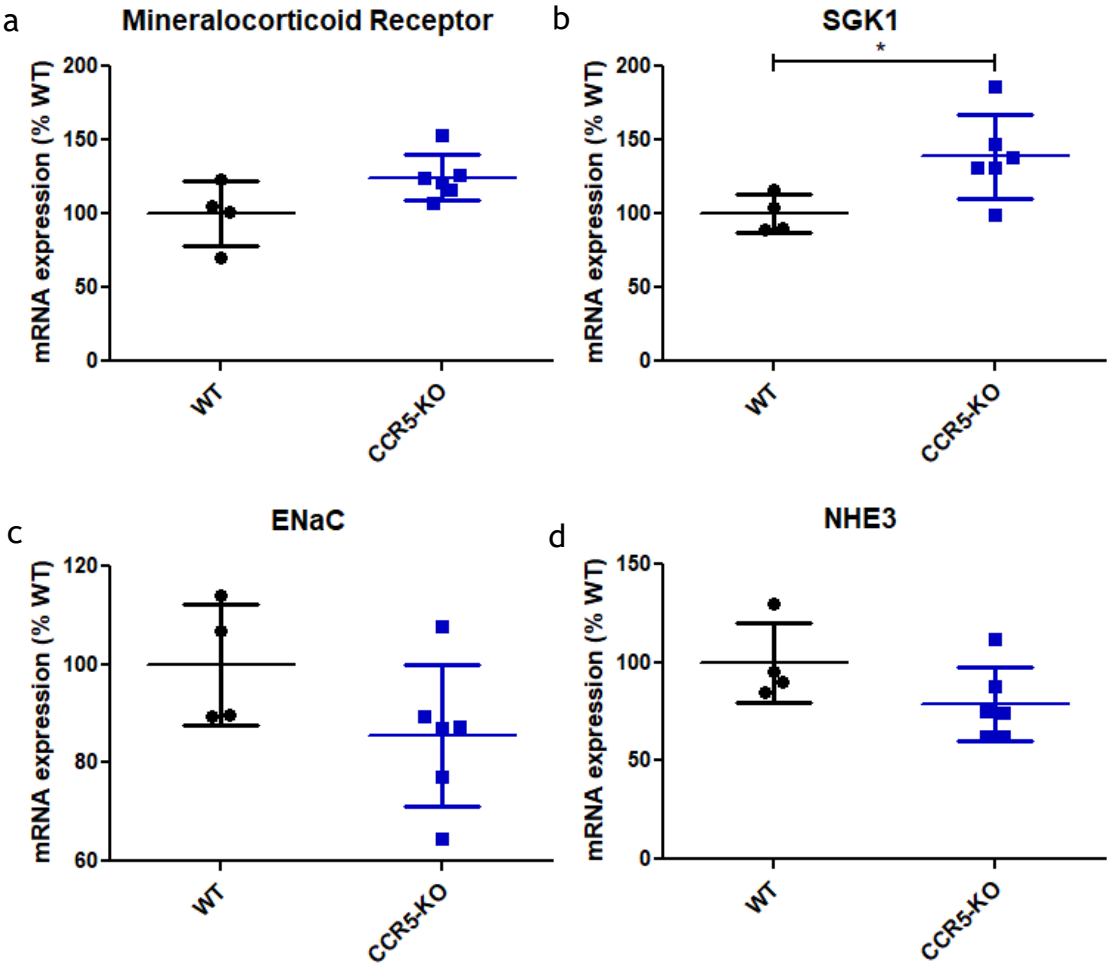


Figure 5-13. CCR5-KO kidney MR, SGK1, ENaC and NHE3 expression
The expression of MR (a), SGK1 (b), ENaC (c) and NHE3 (d) in the kidneys of resting WT and CCR5-KO mice was determined by qPCR. * $p < 0.05$, Mann Whitney test.

5.4 Immune cell MR expression

Macrophages and monocytes have been shown to express MR (Lim et al., 2007; Rickard et al., 2009). It is possible that the reduction of MR expression in the kidneys of iCCR-KO mice is a reflection of the reduction of CD11b+F4/80+Ly6C+ cells. Therefore, MR expression by immune cells, including macrophages, in WT and iCCR-KO mice was investigated.

5.4.1 Macrophage sort

F4/80+ cells were isolated from the kidneys of WT and iCCR-KO mice using magnetic columns and the MR expression by these cells was determined. Representative flow cytometry plots of the negative fraction, positive fraction and cells before the sort are shown (Figure 5-14). F4/80+ cells represented a

very small fraction of the total cells in the kidney. These cells were depleted in the negative fraction but only slightly enriched in the positive fraction. The majority of the positive fraction consisted of F4/80- cells and therefore isolating F4/80+ cells was unsuccessful and MR expression was not tested.

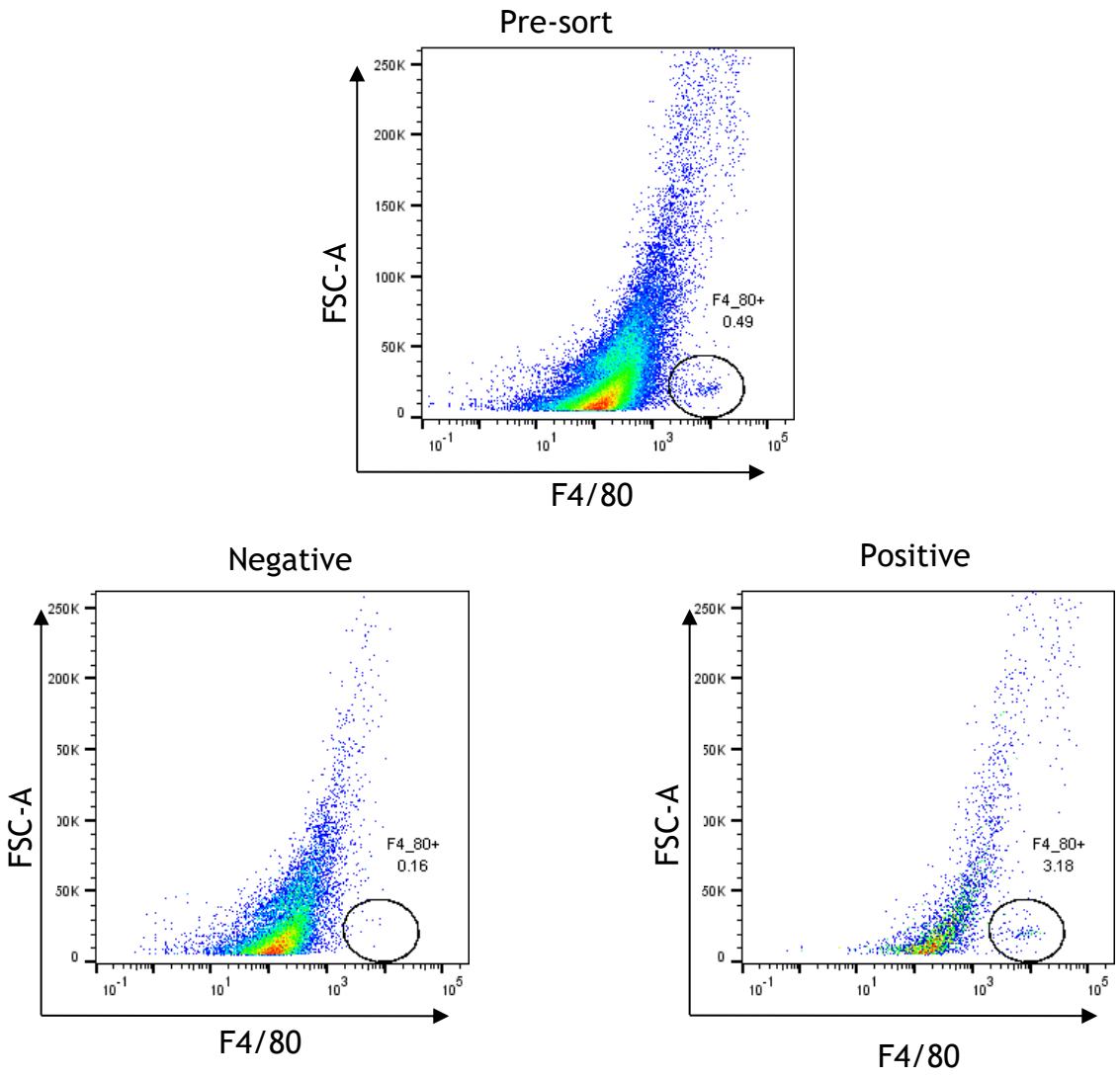


Figure 5-14. Magnetic sort of kidney F4/80+ cells
F4/80+ cells were sorted from kidney cells using magnetic columns. Representative flow cytometry plots of the cells before the sort (pre-sort) and the negative and positive fractions are shown.

5.4.2 Flow cytometry

The expression of MR by leukocytes in the kidneys of WT and iCCR-KO mice was next investigated using flow cytometry. Overall, about 4 % of kidney cells were shown to express MR in both WT and iCCR-KO mice. Representative flow cytometry plots of total kidney cells stained with the MR antibody and FMO

control are shown with a heatmap of CD45 expression. This suggests that the majority of MR+ cells are CD45+ (Figure 5-15a). 60 % of CD3+ T cells and up to 95 % of CD19+ B cells were shown to be MR+ and the majority of CD11b+F4/80+ cells were MR+ (Figure 5-15b, c, d). Slightly more CD11b+F4/80+ cells expressed MR in the iCCR-KO mice compared to WT mice (WT 93 ± 4 vs iCCR-KO 98 ± 1 % CD11b+F4/80+ cells, $p < 0.05$, Mann Whitney test). Representative flow cytometry plots of FMO controls and MR stained samples illustrate high MR staining by CD3+, CD19+ and CD11b+F4/80+ cells.

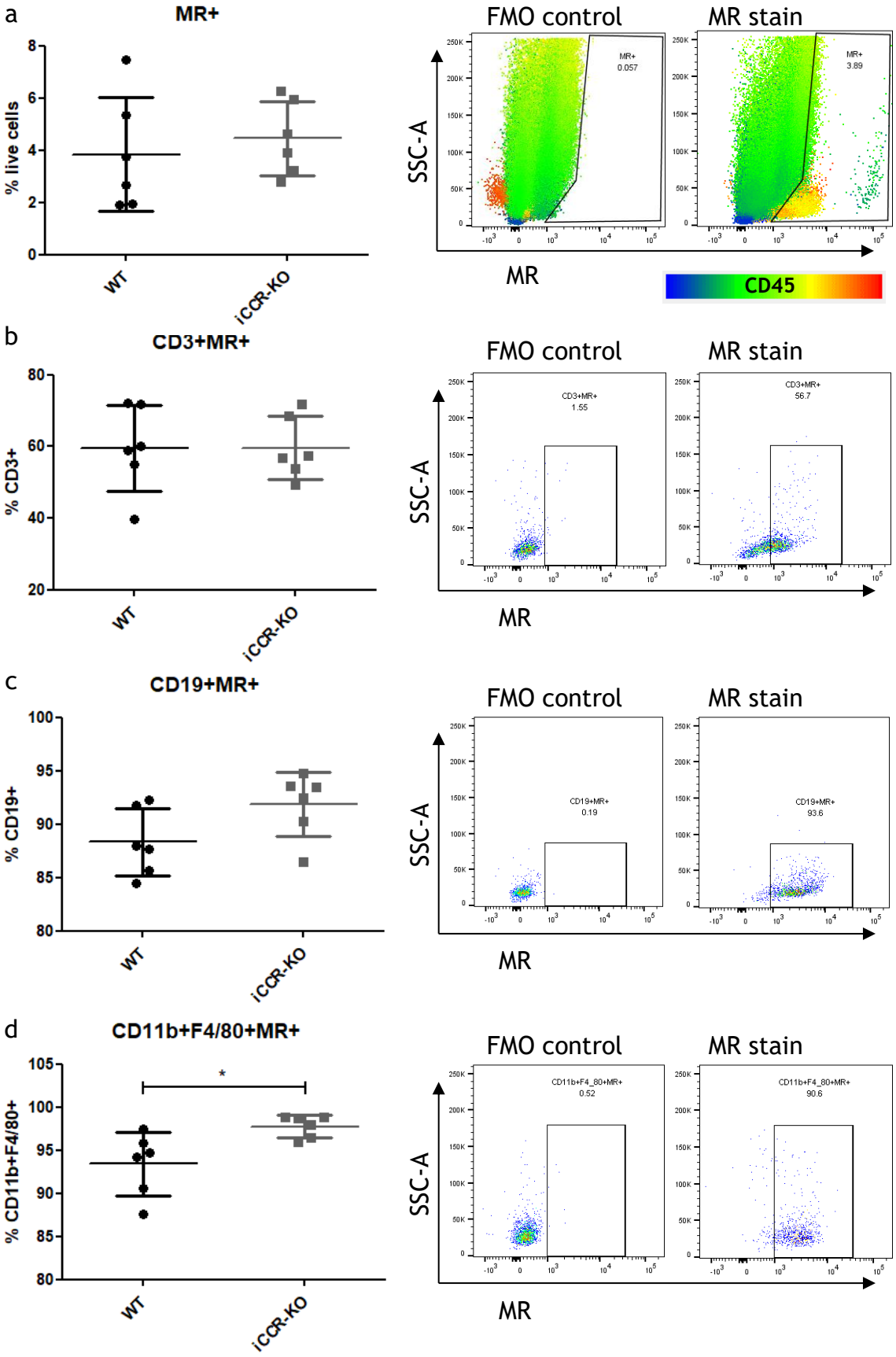


Figure 5-15. The expression MR in the kidneys of WT and iCCR-KO mice
Overall MR expression (a) and MR expression by CD3+ cells (b), CD19+ cells (c) and CD11b+F4/80+ cells (d) in the kidneys of WT and iCCR-KO mice was determined by flow

cytometry. Representative flow cytometry plots of MR staining by these cell types and an FMO control are shown. CD45 expression is presented as a heat map in a. * $p < 0.05$, Mann Whitney test.

5.4.3 Lymphoid MR expression

The high expression of MR by kidney leukocytes demonstrated by flow cytometry was unexpected and it is likely that the antibody is showing non-specific binding to these cells. To clarify this, MR expression in the spleen, thymus and kidney was determined by flow cytometry and qPCR. The spleen and thymus consist mostly of leukocytes whereas only up to 5 % of kidney cells are leukocytes. Therefore these experiments indicate whether leukocytes express MR or if the antibody binds non-specifically to them. Flow cytometry showed that the spleen had the highest MR expression, with about 4 x 10^6 MR+ cells. The thymus had 1.5 x 10^6 MR+ cells. This was much higher than the 8 x 10^4 MR+ cells in the kidney (Figure 5-16a). In contrast to this, qPCR revealed that MR expression was about 10-fold higher in the kidney compared to the spleen and thymus (Figure 5-16b). Very low levels of MR mRNA, if any, were detected in the spleen and thymus with ct values over 30.

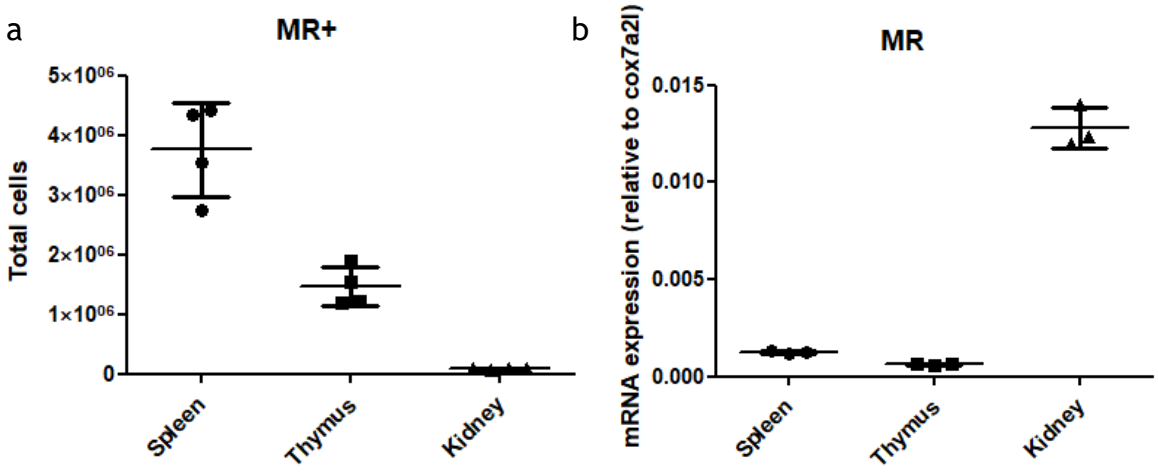


Figure 5-16. Spleen, thymus and kidney MR expression
The total expression of MR in the spleen, thymus and kidneys of WT mice was determined by flow cytometry (a) and qPCR (b).

5.5 iCCR-KO Kidney structure

Macrophages have been shown to be involved in development. They regulate angiogenesis, lymphangiogenesis and development of various organs such as the mammary glands (Fantin et al., 2010; Lee et al., 2014; Wilson et al., 2017).

Chemokines and their receptors are also involved as ACKR2 has been shown to regulate branching morphogenesis in the mammary gland and both ACKR2 and CCR2 regulate lymphangiogenesis (Lee et al., 2014; Wilson et al., 2017). Therefore, the effect of iCCR deficiency on kidney structure was investigated as this may be effected by altered kidney monocyte and macrophage populations. There appeared to be no differences in the structure of the WT and iCCR-KO kidneys, demonstrated by representative images (Figure 5-17a). The number of glomeruli were counted to indicate nephron formation and this did not differ between WT and iCCR-KO kidneys (Figure 5-17b).

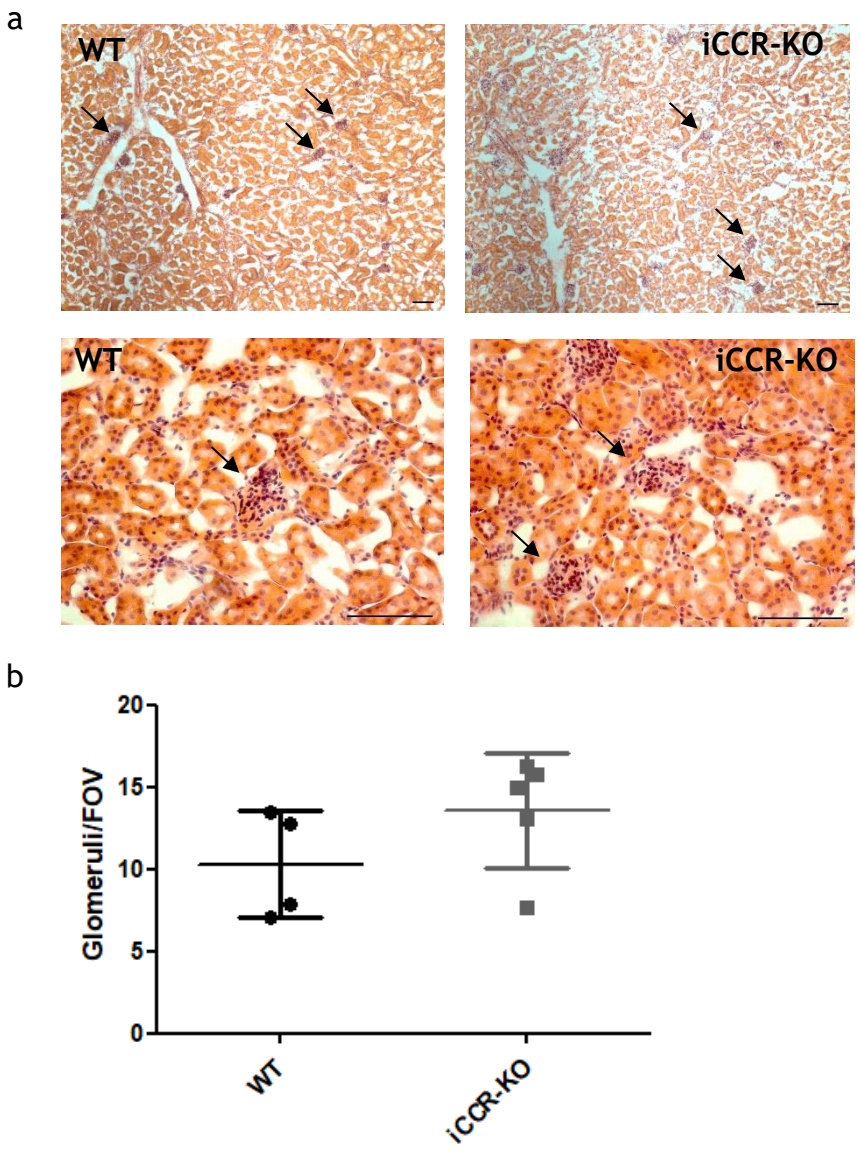


Figure 5-17. The effect of iCCR deficiency on kidney structure
The structure of kidneys from WT and iCCR-KO mice was assessed by H&E staining (a) and the average number of glomeruli per field of view (FOV) was measured (b). Arrows point to glomeruli. Scale bar = 100 µm.

5.6 Discussion

The effect of total iCCR and individual iCCR deficiency on kidney leukocytes, BP regulators and kidney structure was investigated. This has revealed how iCCRs contribute to macrophage and monocyte infiltration in the kidney and showed that MR expression is influenced by iCCRs or iCCR expressing cells.

5.6.1 Kidney monocytes and macrophages

Ly6C expression was used to identify monocyte and macrophage populations. CD11b+F4/80+Ly6C+ cells represent inflammatory monocytes whilst CD11b+F4/80+Ly6C- cells represent macrophages (Clements et al., 2016; Lin et al., 2009). iCCR deficiency resulted in fewer CD11b+F4/80+Ly6C+ cells in the kidney but CD11b+F4/80+Ly6C- cells were unaffected. CCR1, CCR3 and CCR5 deficiency had no effect on these populations whilst CCR2-KO mice had fewer CD11b+F4/80+Ly6C+ and CD11b+F4/80+Ly6C- cells. This suggests that only CCR2 is required for the migration of these cells to the kidney. The reason CD11b+F4/80+Ly6C- cells were reduced in CCR2-KO mice but not iCCR-KO mice is unclear but this has also been demonstrated in the lung (Dyer et al., 2019). It has been suggested that Ly6C^{hi} monocytes differentiate into macrophages and that these macrophages have a role in renal fibrosis (Lin et al., 2009). Whether monocyte derived macrophages and tissue resident macrophages have a role in resting kidney function has not been investigated. Cardiac macrophages have been shown to have a role in cardiac conduction (Hulsmans et al., 2017) so it is possible that kidney macrophages have functions in the kidney outside their immune and inflammatory roles. Depletion of monocytes and macrophages or CCR2 deficiency do not affect baseline BP (Huang et al., 2018; Ishibashi et al., 2004) which would suggest that the observed changes in kidney monocytes and macrophages in iCCR-KO mice are not responsible for any changes in BP compared to WT mice.

The majority of CD11b+F4/80+Ly6C- cells in the kidney express both CCR1 and CCR5 whilst CD11b+F4/80+Ly6C+ cells most highly express CCR2. This could suggest that as CD11b+F4/80+Ly6C+ cells differentiate, they downregulate CCR2 expression and upregulate CCR1 and CCR5 expression. This has been demonstrated by human monocytes *in vitro* (Kaufmann et al., 2001). The role of

CCR1 and CCR5 on kidney macrophages is unclear but these may regulate migration within the kidney or macrophage function. CCR5 may regulate macrophage polarisation as it has been suggested that CCR5 deficiency increases alternative activation of macrophages (Dehmel et al., 2010). CCR1 deficient peritoneal macrophages show increased phagocytosis and cytotoxicity, suggesting that CCR1 can regulate macrophage innate immune responses (Ness et al., 2004). At rest, CCR2 appears to regulate kidney monocyte and macrophage recruitment but other iCCRs may be involved in disease states. For example, CCR1 may regulate macrophage infiltration in renal ischaemia-reperfusion injury (Furuichi et al., 2008).

CD11b+F4/80+Ly6C- cells can be CCR2+, CCR5+, CCR1+CCR5+ or CCR2+CCR5+ and these may represent distinct subpopulations. None of these cells were just CCR1+ which could suggest a redundant role for CCR1. Some of these cells did not express any of the iCCRs and these may depend on other chemokine receptors for migration such as CX3CR1 (Geissmann et al., 2003). A population of CD11b+F4/80-Ly6C+ monocytes could reside in the kidney and these cells in iCCR-KO and individual iCCR deficient mice could be further explored, along with their iCCR expression.

5.6.2 MR, SGK1, ENaC and NHE3

It is unlikely that the reduction of CD11b+F4/80+Ly6C+ cells in the kidneys of iCCR-KO mice directly affects BP. Therefore, other regulators of BP in the kidney were investigated. These were MR, SGK1, ENaC and NHE3. NHE3 expression was not effected by individual or total iCCR deficiency. MR is a transcription factor that regulates SGK1 expression (Shibata and Fujita, 2011). MR expression was reduced in iCCR-KO mice but SGK1 expression was not effected by iCCR deficiency, suggesting that there was enough MR protein or that other transcription factors were able to regulate SGK1 expression. SGK1 expression was increased in CCR5-KO mice. How CCR5 effects SGK1 expression has not been investigated but T cell SGK1 has been shown to be important in hypertension (Norlander et al., 2017). It is possible that CCR5 regulates T cell SGK1 expression or that CCR5+ cells regulate the expression of SGK1 by other cells types such as epithelial cells. The increase of SGK1 expression in CCR5-KO mice would also be expected in the iCCR-KO mice and it is unclear why this was not observed. SGK1

inhibits ENaC degradation, leading to increased levels of ENaC and increased activity (Debonneville et al., 2001). ENaC expression was reduced in CCR1-KO mice. The effect of CCR1 deficiency on BP has not been looked at but it is possible that a reduction in ENaC would result in lower BP. ENaC is also expressed by epithelial cells in the lung, colon and bladder and expression may also be reduced in these tissues in CCR1-KO mice. ENaC expression was not reduced in the iCCR-KO mice, which would have been expected.

MR mRNA expression was reduced in the iCCR-KO mice and this effect was not seen in any of the individual iCCR deficient mice, suggesting that this is a result of loss of multiple iCCRs. Because MR can be expressed by monocytes and macrophages (Lim et al., 2007; Rickard et al., 2009), it is possible that the reduction in expression reflected the reduction in CD11b+F4/80+Ly6C+ cells. However, this may not be the case as a reduction in MR expression in CCR2-KO mice would also be expected. Nonetheless, the expression of MR by monocytes, macrophages and other leukocytes in WT and iCCR-KO mice was investigated. The data here show that B cells, T cells and macrophages express MR. There is some evidence for MR expression by T cells and macrophages. Mice with MR deficient T cells have reduced BP at baseline and after Ang II treatment and deletion of MR in macrophages protects against DOCA-salt induced hypertension (Rickard et al., 2009; Sun et al., 2017). Macrophages in iCCR-KO mice were shown to express slightly more MR than WT mice, although this difference is small and unlikely to be biologically relevant.

The antibody based data here suggest that only 4 % of kidney cells express MR and that the majority of these are CD45+ which is surprising as MR is expressed by epithelial cells in the distal convoluted tubules of the kidney (Funder, 2005; Hirasawa et al., 1997). It is therefore likely that the antibody used did not work effectively and could bind to leukocytes non-specifically. To check this, MR expression was measured in the spleen, thymus and kidney by flow cytometry and qPCR. The flow cytometry data suggested that the spleen had the most MR+ cells, followed by the thymus, and the kidney had 50-fold fewer MR+ cells than the spleen. qPCR revealed that MR expression was highest in the kidney whilst MR was almost undetectable in the spleen and thymus with ct values higher than

30. This suggests that the MR antibody used for flow cytometry was binding non-specifically to immune cells and that the data obtained using it is unreliable.

Although the expression of MR by leukocytes shown here is unreliable, it is still possible that MR expression by these cells is affected by iCCR deficiency. It is also possible that iCCR expressing leukocytes can regulate MR expression, potentially through cytokine production. The MR gene may be transcribed at the same rate in WT and iCCR-KO mice, but the mRNA could be degraded faster in the iCCR-KO mice. This could be regulated by MicroRNAs such as miR-124 and miR-135a, which have been shown to regulate MR expression. However this is likely through regulation of translation, not mRNA stability (Sõber et al., 2010). The study here was limited by the fact that only mRNA expression was measured and not protein expression. However, an antibody that could be used to measure MR expression by IHC or western blotting was not available.

Another explanation for reduced MR expression is if the iCCR-KO kidneys have a different structure to WT kidneys such as fewer nephrons and epithelial cells. This could be influenced by macrophages and may also affect BP. ACKR2 deficient mice show increased macrophage recruitment to developing mammary glands which is associated with an increase in ductal epithelial network density (Wilson et al., 2017). Macrophages have been shown to accumulate near renal tubules in the developing kidney and CSF-1 treated kidney explant cultures develop an increased number of nephrons (Rae et al., 2007). The data here indicate that the structure of the WT and iCCR-KO kidneys does not differ which suggests that kidney development is not affected by iCCR deficiency or reduced macrophage populations. However, this could be verified by studying kidney development and macrophage populations in embryonic iCCR-KO mice.

The vasculature is also important in regulating BP and how iCCR deficiency affects leukocytes and BP regulation by the vasculature should be investigated. MR has been shown to be expressed by endothelial cells and VSMCs and this is important in regulating BP (Galmiche et al., 2014; Nguyen et al., 2010). It is possible that iCCR-KO mice have reduced MR expression in the vasculature as well as the kidney. Older iCCR-KO mice tended to have a lower BP than WT mice so how iCCRs contribute to BP regulation in older mice and in age related hypertension would be interesting.

5.7 Conclusion

This study shows that the kidneys of iCCR-KO mice have fewer CD11b+F4/80+Ly6C+ cells than WT mice and that this is likely due to lack of CCR2. iCCR-KO mice also have reduced MR expression in the kidneys which may influence BP and this is specific to the iCCR-KO mice. The reason for MR expression downregulation is still unclear. As CCR2-KO mice do not show a reduction in kidney MR expression, the reduction of CD11b+F4/80+Ly6C+ cells is unlikely to be involved with the reduced MR expression. Kidney MR protein expression needs to be verified, along with which cells are expressing MR and whether reduced MR expression is responsible for any differences in BP.

Overall, this study reveals some ways that iCCR deficiency influences the kidneys. Further work on whether this influences BP needs to be done, along with studies focusing on the vasculature, but this could start to identify ways that iCCRs can be targeted to regulate BP.

Chapter 6

Discussion

6 Discussion

6.1 General discussion

The immune system is an important factor in the pathogenesis of hypertension, with the depletion of T cells, B cells, monocytes and macrophages having been shown to be protective in various hypertensive models (Chan et al., 2015; Guzik et al., 2007; Thang et al., 2015; Wenzel et al., 2011). Further, inflammation of the vasculature and kidneys develops in hypertension and this is associated with end organ damage (Moore et al., 2015; Ozawa et al., 2006).

As regulators of leukocyte migration and inflammation, chemokines and their receptors have been implicated in hypertension. Of the iCCRs, CCR2 has been most widely studied. $CCR2^{-/-}$ mice are protected from Ang II induced vascular remodelling, but not elevated BP, whilst the CCR2 antagonist INCB3344 reduces BP in Ang II induced hypertension (Ishibashi et al., 2004; Moore et al., 2015). The deficiency of CCR5 has not been shown to influence BP or kidney function and inflammation in hypertension but CCR5 expression does increase in hypertensive kidneys and aortas (Chan et al., 2012; Krebs et al., 2012). CCR1 and CCR3 have not previously been studied in hypertension.

Studying the role of chemokine receptors in hypertension, and other contexts, has been challenging due to the complexity of the chemokine system. To overcome this, iCCR-KO mice have been developed (Dyer et al., 2019) as well as REP mice which can be used to detect individual and combinatorial iCCR expression. In the experiments described here, these mice were used to investigate the role of iCCRs in hypertension. Firstly, through characterising leukocyte iCCR expression in Ang II induced hypertension and secondly, through investigating how iCCR deficiency affects Ang II induced hypertension. Finally, how iCCR deficiency may affect the regulation of BP by the kidney was investigated.

For the characterisation of leukocyte iCCR expression, B cells, T cells, monocytes and macrophages were analysed in the bone marrow, blood, spleen, kidney and PVAT by flow cytometry, using REP mice. This was carried out after 7 or 14 days of Ang II induced hypertension and there were no changes in iCCR

expression by the cell types analysed at either of the time points. However, iCCR expression in the kidney, heart and aorta (including PVAT) was also analysed by qPCR after 14 days of Ang II induced hypertension and this revealed an increase in aortic CCR2 and CCR5 expression in the Ang II treated mice. This suggests that CCR2 and CCR5 are important in regulating vascular inflammation in hypertension. Leukocytes expressing CCR2 and CCR5 may be involved in the vascular dysfunction that occurs in hypertension as iCCR-KO mice subject to Ang II induced hypertension had improved aortic and mesenteric artery function compared to Ang II treated WT mice. A similar effect is seen in $CCL5^{-/-}$ mice (Mikolajczyk et al., 2016). CCL5, and possibly other ligands for CCR5 or ligands for CCR2, could be important for the recruitment of leukocytes that impair vascular function. Vascular oxidative stress is associated with vascular dysfunction in hypertension (Guzik et al., 2007) but iCCR deficiency had no effect on aortic superoxide so it is possible that other factors were mediating the protection from vascular dysfunction.

Inflammation of the kidneys and vasculature is widely reported in hypertension (Mikolajczyk et al., 2016; Moore et al., 2015; Ozawa et al., 2006). However, the results here do not show Ang II to increase inflammation of the kidneys or PVAT. This may be due to different protocols for cell isolation or different flow cytometry gating strategies. It was hypothesised that there would be fewer inflammatory monocytes and macrophages in the kidneys and PVAT of Ang II treated iCCR-KO mice but any effect of iCCR deficiency on kidney and PVAT inflammation was difficult to identify when the overall effect of Ang II was not observed.

The BP of Ang II treated iCCR-KO mice increased to the same extent as WT mice. This, along with studies of CCR2 and CCR5 deficient mice (Ishibashi et al., 2004; Krebs et al., 2012), suggests that iCCRs do not influence blood pressure in hypertension. As a deficiency in T cells, B cells or macrophages has been reported to prevent a BP increase in hypertension, it is likely that other chemokine receptors are involved in the migration of these cells to where they regulate BP.

A difference in BP was observed between the WT and iCCR-KO sham treated mice after 12 - 14 days of BP measurements, suggesting that iCCR deficiency

results in reduced BP, an effect that might only be seen in older mice. To understand this observation, factors that regulate BP were investigated in WT and iCCR-KO mice. As a regulator of salt retention and fluid homeostasis, the experiments described here focused on the kidney. The iCCR-KO mice were shown to have fewer CD11b+F4/80+Ly6C+ cells in the kidney. Analysis of individual receptor KO mice suggested that CCR2 is the iCCR responsible for this phenotype. The iCCR-KO mice also had reduced kidney MR expression and this was not seen in any of the individual receptor KO mice. The reduction in inflammatory monocytes in the kidneys of iCCR-KO mice is unlikely to be associated with MR expression as CCR2-KO mice also had fewer monocytes in the kidney but MR expression was similar to that of WT mice.

6.2 Future work

CCR2 and CCR5 were identified as upregulated in the aortas of Ang II treated WT mice. In addition, mice deficient for the iCCRs were shown to have improved vascular function in Ang II induced hypertension compared to WT mice. It is hypothesised that CCR2 and CCR5 regulate vascular dysfunction in hypertension and this needs to be further investigated. The cells expressing these receptors in the aorta should be identified, as CCR2+ and CCR5+ leukocytes may not be recruited to the aorta in iCCR-KO mice and this could be protective against Ang II induced vascular dysfunction. Additionally, vascular function in $CCR2^{-/-}$ and $CCR5^{-/-}$ mice following Ang II induced hypertension should be determined as this may identify whether just one of these receptors is responsible for the effects seen in the iCCR-KO mice or whether both receptors are involved. This could also be carried out using CCR2 and CCR5 inhibitors. Whether these inhibitors can be protective in hypertension that has already been established would also be interesting to investigate and more clinically relevant. Although CCR1 and CCR3 were not upregulated in the hypertensive aortas, how $CCR1^{-/-}$ and $CCR3^{-/-}$ mice respond to Ang II would still be interesting to determine as this has not previously been done.

Reversible iCCR-KO mice are currently being generated. These mice are conditionally null for the iCCRs but expression of individual receptors can be switched on, in multiple combinations. CCR1-CCR2+CCR3-CCR5-, CCR1-CCR2-CCR3-CCR5+ and CCR1-CCR2+CCR3-CCR5+ mice could be subjected to Ang II

induced hypertension and vascular function assessed. This would show if mice that only express CCR2, CCR5 or CCR2 and CCR5 experience vascular dysfunction similar to WT mice, indicating how important these receptors are in regulating vascular dysfunction in hypertension.

It would also be interesting to see if iCCR-KO mice are protected from cardiac and vascular remodelling after 28 days of Ang II treatment as this was not observed after 14 days. Remodelling can be regulated by monocytes and macrophages (Falkenham et al., 2015; Moore et al., 2015). It is hypothesised that fewer monocytes would be recruited to the hearts and aortas of iCCR-KO mice and this would result in reduced pathogenic remodelling. CCR2 deficiency has already been shown to be protective against Ang II induced vascular remodelling (Ishibashi et al., 2004). WT and iCCR-KO mice could be treated with Ang II for 28 days and the infiltration of monocytes and macrophages in the aorta and heart could be determined, along with fibrosis markers such as collagen and matrix metalloproteinases (Giannandrea and Parks, 2014).

The effect of iCCR deficiency on MR expression was unexpected. How the lack of iCCRs affects other tissues and how they function would be interesting to investigate. For understanding BP regulation, characterising how iCCR deficiency affects the vasculature needs to be carried out. This would involve determining the infiltration of monocytes and macrophages in the vasculature. Assessing vascular function in resting WT and iCCR-KO mice would also be interesting as a slight difference between sham treated WT and iCCR-KO mice was identified. The effect of PVAT on vascular function in these mice should also be investigated by carrying out wire myography with and without PVAT as the experiments described here only focused on vessels after the removal of the PVAT.

6.3 Final conclusion

Overall, the work described here has increased understanding of how iCCRs influence hypertension and BP regulation. Characterising iCCR expression in Ang II induced hypertension has shown that Ang II has few effects on iCCR expression. However, aortic CCR2 and CCR5 mRNA expression increased in response to Ang II. iCCR deficiency was shown to be protective against Ang II induced vascular

dysfunction, but had no effect on BP. iCCR deficiency was also shown to reduce the infiltration of monocytes in the kidney and reduce kidney MR expression. Further experiments are required to understand if this influences BP.

These experiments have identified iCCRs as possible targets to improve vascular function in hypertension which could reduce the risk of complications of hypertension such as atherosclerosis, MI and stroke.

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