

The Role of ACKR2 in Inflammatory Pathologies

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Abstract

Chemokines are a highly conserved family of chemoattractant cytokines that are key to the movement of cells around the body under both inflammatory and homeostatic conditions. Chemokines bind to seven transmembrane G protein coupled receptors that signal and induce cell movement upon ligand binding. As well as the 'classical' chemokine receptors, there also exists a family of atypical chemokine receptors that do not induce a canonical signalling response upon ligand binding.

These atypical chemokine receptors (ACKR) have been shown to modify the chemokine response through processes such as the scavenging of inflammatory chemokines. One such receptor with this scavenging function is ACKR2 which has been shown to bind and internalise all of the inflammatory CC chemokines. The functional repertoire of ACKR2 continues to be expanded and it is now thought to have a role in inflammation, lymphatic drainage and lymphatic vessel development. It has been shown that the absence of this receptor results in impaired resolution of inflammation and, as a result, increased inflammatory pathologies *in vivo*. In models of skin inflammation a lack of ACKR2 has been shown to result in increased pathology and impaired inflammatory resolution.

Multiple models of cutaneous inflammation, including excisional wound healing and chemically induced damage, were used to further investigate the role of ACKR2 in this context. Work on wound healing suggested that although ACKR2 appears to play no role in wound closure it does have a role in the formation of scar tissue in an excisional wound. Our data suggest that ACKR2 has a role in collagen deposition in developing and maturing scars. We also found that ACKR2 had a protective role in chemically-induced models of skin inflammation.

We then looked at the role of ACKR2 in ocular inflammation. The main work performed in this section involved the use of the experimental autoimmune uveitis (EAU) model. Here we found that ACKR2 had a protective effect resulting in reduced pathology and infiltration of inflammatory leukocytes. This work also suggested, using *in vitro* analysis, that a human retinal pigmented epithelial cell line expresses functional ACKR2 protein and that our findings may be relevant to human disease.

Finally we looked at the role of ACKR2 in the inflammatory autoimmune disease rheumatoid arthritis (RA). By taking samples of peripheral blood from RA patients we assessed the transcript levels of *Ackr2* and correlated them with clinical measurements. Our findings suggested that, in patients with 'well-controlled' RA, there was an increase in the transcription of *Ackr2* in peripheral blood leukocytes. Additional work using *in vitro* methods suggest that the hypoxic nature of the rheumatoid joint, and some of the drugs used to treat the disease, may increase the transcription of *Ackr2*.

Overall the findings in this work suggest novel roles for ACKR2 in the skin and the eye. They also shed light on further environmental factors that may alter the local expression of ACKR2 in the rheumatoid joint. Taken together this work suggests that ACKR2 may have great therapeutic potential and, furthermore, this potential may be relevant to a wider range of tissues than previously thought.

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List of Accompanying Material

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'All our dreams can come true, if we have the courage to pursue them'

Walt Disney

Author's declaration

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

Signature

Printed name: Kenneth James Pallas

Abbreviations

ACKR- Atypical Chemokine Receptor
 ACPA- Anti-citrullinated peptide antibody
 ADCC- Antibody-dependent cell-mediated cytotoxicity
 AIDS- Acquired immunodeficiency syndrome
 APC- Antigen presenting cells
 BcR- B cell receptor
 BEC- Blood endothelial cell
 CD- Cluster of differentiation
 cDNA- Complimentary DNA
 CFA- Complete Freund's adjuvant
 CLA- Cutaneous lymphocyte antigen
 CLP- Common lymphoid progenitors
 CMP- Common myeloid progenitors
 CNS- Central nervous system
 COPD- Chronic obstructive pulmonary disease
 CRP- C-reactive protein
 CTLA-4- Cytotoxic T lymphocyte-associated antigen4
 CCL2- CC-chemokine ligand 2
 CXCL8- CXC-chemokine ligand 8
 CCR- CC-chemokine receptor
 CXCR- CXC-chemokine receptor
 DAMP- Damage-associated molecular patterns
 DARC- Duffy antigen receptor for chemokines
 DAS-Disease activity score
 DC- Dendritic Cells
 DMARD- Disease-modifying anti-rheumatic drug
 DNA- Deoxyribonucleic acid
 DNFB- 2,4- dinitrofluorobenzene
 EAU- Experimental Autoimmune Uveitis
 EDTA- Ethylenediaminetetraacetic acid
 EGF- Endothelial growth factor
 ESR- Erythrocyte sedimentation rate
 FCS- Foetal calf serum
 FGF- Fibroblast growth factor
 FLS- Fibroblast-like synoviocytes
 GAG- Glycosaminoglycan
 GDP- Guanosine diphosphate
 GMP- Granulocyte monocyte precursor
 GPCR- G-protein coupled receptors
 GTP- Guanosine triphosphate
 H&E- Haematoxylin and eosin
 HEK- Human embryonic kidney
 HEV- High-endothelial venules
 HIV- Human immunodeficiency virus
 HPA- Hypothalamo-pituitary-adrenocortical

HRP- Horseradish peroxidase
HSC- Haematopoietic stem cells
HSP- Heat-shock protein
ICAM- Inter-cellular adhesion molecule
IFN- Interferon
IgE- Immunoglobulin E
IgG- Immunoglobulin G
IL- Interleukin
IRBP- Interphotoreceptor retinoid binding protein
KO- Knockout
LEC- Lymphatic endothelial cells
LPS- Lipopolysaccharide
MC- Mast cell
MEP- Myeloid erythrocyte precursors
MHC- Major histocompatibility complex
MPER- Mammalian protein extraction reagent
MTX- Methotrexate
NBF- Neutral buffered formalin
NET- Neutrophilic extracellular traps
NK Cells- Natural killer cells
OCT- Optimal cutting tissue
PAF- Platelet activation factor
PAMP- Pathogen associated molecular patterns
PBL- Peripheral blood leukocyte
PBS- Phosphate buffered-saline
PBST- Phosphate buffered saline with 0.05% Tween 20
pDC- Plasmacytoid dendritic cells
PRR- Pattern recognition receptors
RA- Rheumatoid arthritis
RF- Rheumatoid factor
RNA- Ribonucleic acid
TACE- TNF- α converting enzyme
TcR- T cell receptor
TGF- Transforming growth factor
T_H1- T helper 1 cell
TLR- Toll-Like Receptor
TNF- Tumour Necrosis Factor
TPA- 12-O-tetradecanoylphorbol-13-acetate
WT- Wild-type

Chapter 1- Introduction

Chapter 1 Introduction

Given the nature of the work in this thesis, this section will provide an overview of inflammation and chemokine biology. Introduction sections in individual results chapters will provide greater detail on the background relevant to that work.

1.1 Inflammation

Inflammation is the body's response process to tissue damage and/or infection. It involves the accumulation of fluid and cells at the site of trauma. This process is driven by the immune system, a network of cells that serves to protect the body by attacking anything that is considered to be 'non-self'. It is important, during this process, to effectively and efficiently resolve the issue at hand whilst keeping 'collateral damage' to a minimum. When this system fails chronic inflammation can ensue and result in many years of continual damage. There are four classical signs of inflammation each of which are described in Latin. These are calor (heat), tumor (swelling), dolor (pain) and rubor (redness) as described by Celsus (circa 30 B.C.) in *De Medicina* (Donaldson, 2014). Loss of function was added as a fifth sign later on.

1.1.1 The Innate Immune Response

The immune response is the activation of the immune system following an insult such as physical damage, bacterial, viral or fungal infection. The first lines of defence are the barriers formed by epithelial tissues such as the skin and the mucosa of the gut. These tissues provide physical barriers that keep pathogens out of the body. The importance of these tissues can be seen when there is a breach in them during wounding. It is often following a wound that an infection can occur as pathogens may enter opportunistically. If these barriers are breached then the immune system responds to kill the pathogen.

The innate immune system develops prenatally and so is functional from birth (Pagenkemper and Diemert, 2014). One of the main functions of the innate immune system is to be a rapid responder to trauma/infection. Upon recognition of damage, or an invading pathogen, the innate immune system mounts an

immediate response that works to neutralise the infectious agent. The rapid recognition of 'non-self' is of utmost importance for the cells of the innate immune system. One of the ways in which the cells of the innate immune system are able to determine self from non-self is through pattern recognition receptors (PRR) (Sellge and Kufer, 2015). One of the main groups of PRRs are the Toll-like receptor (TLR) family which were originally discovered in *Drosophila melanogaster* (Hashimoto, Hudson and Anderson, 1988). These receptors are able to sense pathogen-associated molecular patterns (PAMPs) that tend to be molecules that are absolutely essential for the survival of pathogens and cannot be evolved away. In bacteria, and fungi, constituent parts of the cell wall make up these PAMPs whereas in viruses it tends to be viral DNA/RNA that is recognised (Sellge and Kufer, 2015). Activation of TLRs result in the downstream activation of the inflammatory transcription factor nuclear factor- kappa B (NF- κ B) (Sellge and Kufer, 2015).

There are 11 members of the TLR family in mammals and the targets of these molecules have been well characterised. TLR1 forms heterodimers with TLR2 to sense triacylated lipopeptides (Takeuchi et al., 2002), whilst TLR2 on its own can recognise peptidoglycans and lipoteichoic acid from Gram positive bacteria (Schwandner et al., 1999). TLR2 can also combine with TLR6, and the heterodimer recognises diacylated bacterial lipoproteins (Takeuchi et al., 2001). TLR3 is the cognate receptor for double stranded viral RNA that is common in retroviruses (Alexopoulou et al., 2001). TLR4 recognises lipopolysaccharide (LPS) from Gram-negative bacteria (Termeer et al., 2002). LPS is a key constituent of the cell wall of these bacteria. TLR5 is the receptor for flagellin which is a key protein expressed on bacterial flagella and is involved in the motility of these pathogens (Hayashi et al., 2001). TLRs 7 and 8 recognise single stranded viral RNA (Heil et al., 2004, Lund et al., 2004). Both viral and bacterial genomes express a higher percentage of CpG regions than mammalian DNA and TLR9 is responsible for recognising these motifs (Hemmi et al., 2000). The exact function/ligand of TLR10 is, as yet, unknown. TLR9 is highly expressed in lymphoid tissues such as the spleen, lymph node and tonsil (Chuang and Ulevitch, 2001). Mouse TLR11 has been shown to be able to recognise *Toxoplasma gondii* and uropathic *Escherichia coli* (Mathur et al., 2012). Although

humans have the TLR11 gene they do not seem to express the protein due to premature stop codons in the RNA sequence (Ishii et al., 2008).

TLRs are able to recognise pathogenic signals that are present both within and outwith the cell but there are also further mechanisms to detect foreign invaders once they have infected host cells. One of the main contributors to the recognition of intracellular PAMPs are the Nucleotide Oligomerisation Domain (NOD)-like receptors (NLRs) (Fritz et al., 2006). There are two members of the NOD family, NOD-1 and NOD-2, and each has been shown to activate the NF- κ B pathway. NOD-1 recognises D-glutamyl-meso- diaminopimelic acid (iE-DAP) and NOD-2 is activated by muramyl dipeptide (MDP). Activation of NODs has also been shown to induce apoptosis in host cells in a caspase-1 dependent manner (Warren et al., 2008).

As well as PAMPs, additional activators of the innate immune system are endogenous damage-associated molecular patterns (DAMPs). These are molecules such as heat-shock proteins that are released by damaged or stressed cells and induce an immune response similar to that triggered by pathogens (Gallucci and Matzinger, 2001).

1.1.1.1 Cells of the Immune Response

All of the cells that make up blood, including red blood cells and the leukocytes of the immune system are derived from pluripotent haematopoietic stem cells (HSCs) and originate in the bone marrow (Janeway, 2001). Many of these cells also mature at this site.

HSCs give rise to progenitor cells that are more restricted in the type of cells that they can produce. HSCs can give rise to either lymphoid or myeloid progenitor cells. Lymphoid progenitors are the precursor cells of lymphocytes and natural killer (NK) cells. The myeloid precursor cells give rise to granulocytes (neutrophils, eosinophils, basophils and monocytes), macrophages, mast cells and myeloid-dendritic cells (DC). This lineage is also responsible for the generation of red blood cells and platelets. Figure 1.1 provides an overview of this system.

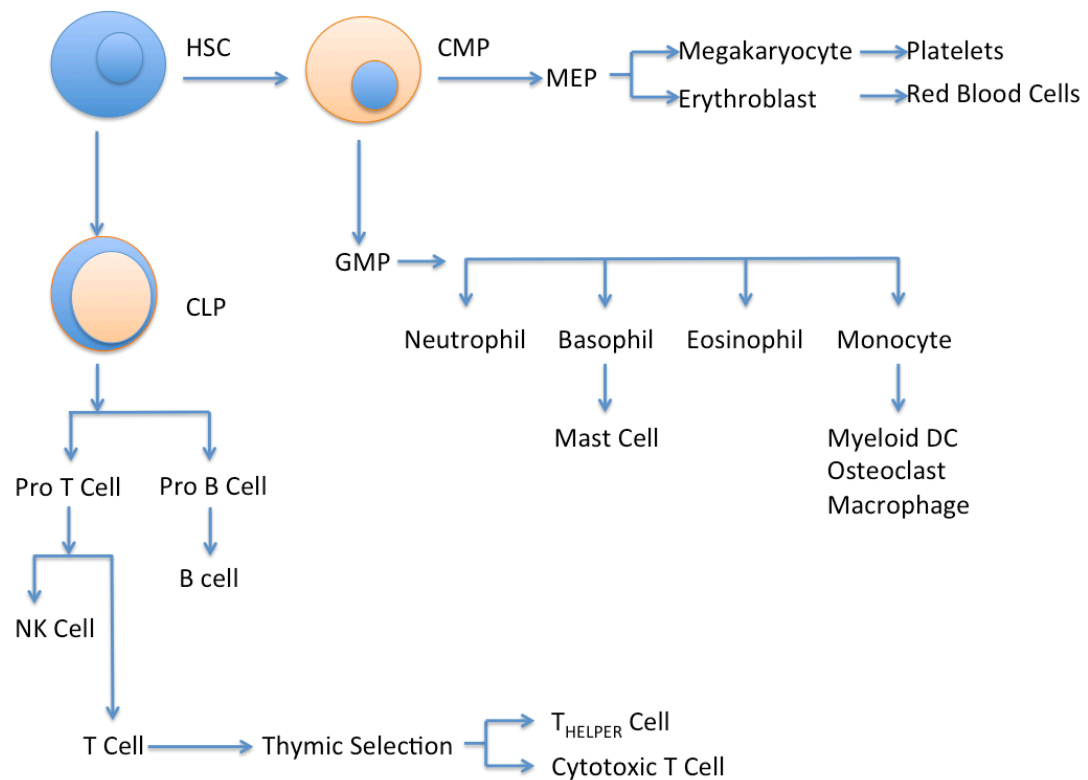


Figure 1.1- The haematopoietic system- Pluripotent haematopoietic stem cells (HSC) can become either common myeloid progenitors (CMP) or common lymphoid progenitors (CLP). CMPs can then become myeloid erythrocyte precursors (MEP) or granulocyte monocyte precursor (GMP) and both then become the end stage effector cells shown in the figure above.

As previously mentioned, infiltration of leukocytes into tissue is one of the classical features of inflammation. Macrophages are key ‘sentinel’ cells that patrol tissues under physiological conditions in order to check for any invaders (Davies and Taylor, 2015). They are often the first immune cells to recognise a pathogen and react to it. The activation and accumulation of immune cells is initiated via the release of inflammatory mediators from ‘stressed’ cells. The aforementioned macrophages, local epithelial and endothelial cells, release molecules including histamine, leukotrienes, prostaglandins, cytokines and chemokines (Metz and Maurer, 2009). These molecules activate and induce the infiltration of more cells to aid in the response.

Cells such as, in order of activation, tissue-resident macrophages, mast cells, neutrophils, monocytes/recruited macrophages, then adaptive immune cells like T-helper cells, cytotoxic T cells, and B cells all have roles to play in the immune response (Metz and Maurer, 2009). The activation and arrival of these cells tends to accelerate and amplify the recruitment of more cells by further secretion of

cytokines and chemokines. More details will be given on these cell types and molecules in subsequent sections.

1.1.1.2 Mast Cells (MC)

These are large, long-lived cells that tend to be found in connective tissue and at perivascular sites (Mekori and Metcalfe, 2000) and can enter cell cycle and proliferate *in situ* (Galli, Nakae and Tsai, 2005). Upon activation they release the pre-formed contents of granules, that are stored in the cell cytoplasm, containing histamine, lipid mediators and cytokines (Voehringer, 2013). The granules also contain tumour necrosis factor (TNF)- α which is a classic inflammatory cytokine that aids in the recruitment of more innate immune cells to potentiate the inflammatory response (Suto et al., 2006).

MCs are key cells in the initiation of the inflammatory response mainly due to their anatomical proximity to the vasculature (Mekori and Metcalfe, 2000). This means that MC-derived vasoactive mediators are able to work quickly and effectively (van Nieuw Amerongen et al., 1998). It has been shown that upon exposure of endothelial cells to leukotriene B₄, histamine or TNF- α (all contained within MC granules) P-selectin is expressed on these cells (Walsh et al., 1991, Kubes and Kanwar, 1994). The functional significance of these molecules will be discussed in more detail in section 1.1.1.3.

It is worth noting that MCs are able to limit, and quell, the immune response (Voehringer, 2013). These cells can produce, and release, the anti-inflammatory cytokine interleukin (IL)-10 (Grimbaldeston et al., 2007). It has been shown that MC are involved in immune suppression of the skin upon exposure to ultraviolet (UV) light via this IL-10 production (Chacón-Salinas et al., 2011). On the other hand it has been shown that MC can aid the adaptive immune response against parasitic infections such as nematodes by expressing both major histocompatibility complex (MHC) I and II to present antigen to T cells (Suurmond et al., 2013). They have also been shown to promote immunoglobulin (Ig) E production from B cells (Hong et al., 2014). As a result of these findings it has become apparent that these cells play an important role in promoting inflammation (Egozi et al., 2003). These cells also secrete chemokines such as

CC chemokine ligand (CCL) 2 and, as a result, contribute to inflammatory cell accumulation at sites of activation (Selander et al., 2009).

1.1.1.3 Neutrophils

Neutrophils are the first leukocytes to be recruited to sites of damage or infection during the inflammatory response (Nourshargh and Alon, 2014). Under the influence of CXC chemokine ligand (CXCL8) these cells leave the blood vessels and extravasate into tissue. Neutrophils are easily identifiable cells by histological staining and are described as having a ‘multi-lobed’ nucleus.

These cells are multi-faceted in function and have a varied armoury to attack pathogens (Scapini and Cassatella, 2014). Neutrophils have the ability to phagocytose foreign bodies in a process that sees the cell literally envelope the pathogen. Once this has occurred the organism is degraded in lysosomes within the cell. Neutrophils also have the ability to degranulate at sites of danger in a process that involves the cell releasing cytoplasmic granules that are toxic to the invading pathogen. One of the main granule-types contained in neutrophils are the azurophilic granules. These granules contain microbicidal factors such as Cathepsin G, neutrophil elastase, defensins and myeloperoxidase (Faurschou and Borregaard, 2003).

Finally, the most recently discovered addition to the offensive arsenal of the neutrophil is the release of neutrophilic extracellular traps (NETs). These NETs are extracellular fibres made predominantly of DNA that can bind and kill pathogens (Brinkmann et al., 2004). The formation of NETs is regulated by neutrophil elastase and myeloperoxidase, two key mediators of inflammation in these cells (Papayannopoulos et al., 2010). It has been shown that NETs are able to bind and kill both bacterial and fungal pathogens (Urban et al., 2006) but may have a role in the pathology of the autoimmune condition systemic lupus erythematosus (Hakkim et al., 2010).

Neutrophils are the most abundant white cell in the human body making up around 40-75% of white blood cells in the adult and pus is comprised of dead neutrophils (Janeway, 2001). Aberrant activation of neutrophils can cause

extensive tissue damage in pathologies such as rheumatoid arthritis and chronic obstructive pulmonary disease (COPD) (Bazzoni et al., 2010). It has also been shown that they can exist in pro-inflammatory and anti-inflammatory forms (Tsuda et al., 2004).

During infection/inflammation, changes in the endothelial cells in the locality of the trauma give neutrophils the ability to roll slowly along the vessel walls then extravasate. The local tissue releases the chemokine CXCL8 in high amounts rapidly upon trauma (Roupé et al., 2010). CXCL8 is stored within the Weibel-Palade bodies of endothelial cells for rapid release (Wolff et al., 1998). Rolling is achieved via the interactions of adhesion molecules like P- and E-selectin, on the luminal surface of the endothelial cell, that bind to the sialyl-Lewis x carbohydrate on the neutrophil surface- this protein is expressed by many other leukocytes (Zou et al., 2005). Neutrophils also recognise L-selectin (Andrian et al., 1992). To complement, and enhance, this binding, the integrin LFA (lymphocyte function-associated antigen)-1 (made up of β : α chains- CD18:CD11a respectively) expressed on the neutrophil binds the adhesion molecule ICAM (inter-cellular adhesion molecule)-1 (CD54) on the endothelial cells. At this point, the cell arrests and this is achieved via interactions between CXCL8 and CXC chemokine receptor (CXCR) 1 (Nourshargh and Alon, 2014). The cell can then leave the vessel under the influence of CXCL8 as shown in figure 1.2.

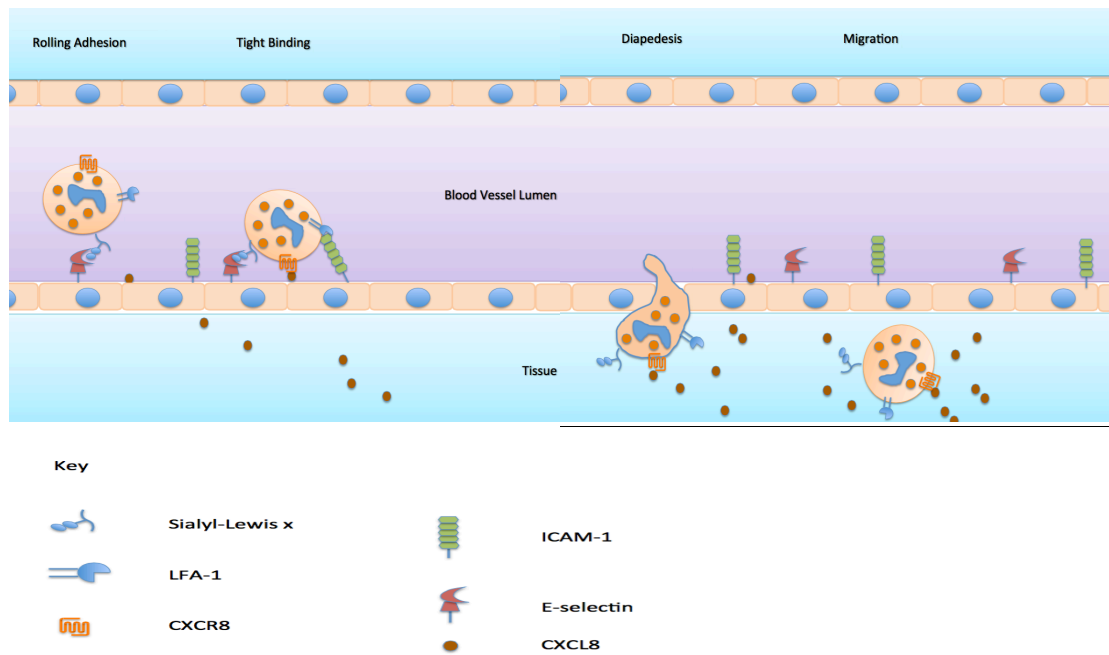


Figure 1.2- Extravasation of neutrophils- The figure above shows a simplification of the four stages involved in the exit of neutrophils from the bloodstream to sites of trauma under the signal of the chemokine CXCL8. By binding selectins and adhesion molecules on the endothelial cell the leukocyte is arrested and then under the influence of the chemokine signal the cell can leave the blood and enter the tissue.

1.1.1.4 Monocytes

Although the monocytes of the mouse are different to those of man, many parallels can be drawn regarding the roles that they play in both species. Monocytes are blood-borne macrophage precursors and make up between 10-15% of white cells in healthy humans. Human blood contains at least three sub-types of monocytes and all of them express the LPS co-receptor, CD14. Classical monocytes are $CD14^{HI}CD16^{-}$ and are inflammatory cells that tend to give rise to inflammatory macrophages upon arrival in tissue (Yona et al., 2013). The ‘non-classical’- $CD14^{LO}CD16^{HI}$ monocyte subtype tend to be less inflammatory and are thought to be precursors to more pro-fibrotic and anti-inflammatory macrophages (Yona et al., 2013). Finally the $CD14^{HI}CD16^{LO}$, so-called intermediate monocyte subtype, has been shown to give rise to monocyte-derived dendritic cells (Ziegler-Heitbrock et al., 2010).

One of the main functions of monocytes is to aid in the generation and replenishment of macrophages and dendritic cells under both physiological and inflammatory conditions. Many of the monocytes in the body are stored within

the red pulp of the spleen where they can easily access the peripheral blood vasculature when needed (Swirski et al., 2009). Monocytes are recruited to sites of inflammation through their expression of a variety of chemokine receptors including CCR1, CCR2, CCR5, CCR6 and CX₃CR1 (Anon., 2011a).

1.1.1.5 Macrophages

The name ‘macrophage’ comes from the ancient Greek words makros (large) and phagein (to eat). Ilya Ilyich Metchnikov originally discovered these cells in 1882 when he was studying starfish larvae. One of the main functions of resting macrophages is that of sentinels (Davies et al., 2013). These cells patrol tissues and constantly sample their surroundings in order to ensure a rapid detection of pathogens (Sellge and Kufer, 2015). Pathogen destruction is achieved using a receptor-independent mechanism known as macropinocytosis (Janeway, 2001). There is also emerging evidence that, like neutrophils, macrophages may be able to use extracellular traps to neutralise pathogens (Boe et al., 2015).

Upon recognition of a pathogen, macrophages change state and become activated (Sellge and Kufer, 2015). Macrophages have a variety of molecules on their surface to detect any non-self proteins. Other than the previously mentioned TLRs, macrophages also express the mannose-receptor that recognises carbohydrates found on bacteria and another LPS receptor known as CD14 as well as many others. The classical inflammatory, or M1, type macrophage is key at early stages of the immune response in providing an anti-microbial defence against any pathogens that may have entered the host (Murray and Wynn, 2011). These cells secrete proteins such as IL-6, and TNF α (Daley et al., 2010) when activated and therefore potentiate the inflammatory response.

Alternatively activated (Stein et al., 1992), or M2, macrophages have a fundamental role in promoting tissue repair and fibrosis. These cells are an anti-inflammatory subset of the classic macrophage. They express a very different cell surface receptor profile, including the mannose receptor CD206 that is absent on M1 cells. Key mediators released from these cells are IL-10 and transforming growth factor-beta (TGF β)-1, both potent anti-inflammatory

agents. Over-expression of TGF β is thought to be a major cause of hypertrophic scars (van der Veer et al., 2009).

The idea of alternative activation was first proposed by Siamon Gordon's group and the M1/M2 paradigm was then formulated by Mantovani and colleagues (Mantovani et al., 2004). In a review published in 2014, Gordon sought to reassess this paradigm and suggested that this concept has been over-interpreted and needs further details added to it (Martinez and Gordon, 2014). Another recent development in macrophage biology was the identification of the origin of tissue-resident macrophages which showed that they are developmentally distinct from their recruited counterparts (Schulz et al., 2012).

1.1.1.6 Dendritic Cells (DC)

Dendritic cells (DC) were originally discovered by Steinman and Cohn in mouse spleen and were named due to their appearance that distinguished them from macrophages (Steinman and Cohn, 1973). DCs are antigen-presenting cells (APCs) and are therefore key cells involved in the development of adaptive immune responses. Macrophages (Yamashita and Shevach, 1978), neutrophils (Potter and Harding, 2001), mast cells (Stelekati et al., 2009) and basophils (Sokol et al., 2009) have all been shown to have varying degrees of antigen presentation capability. However DCs represent the most professional of the APCs (Svensson, Stockinger and Wick, 1997). Following phagocytosis the pathogen is processed and molecular derivatives are presented on the surface of the cell on MHC II molecules. These cells change activation state and migrate through the draining lymphatics to the local draining lymph node under the CCR7/CCL19 axis (more details of this in section 1.3.2), where they interact with T cells via MHCII molecules (Sugamura, Ishii and Weinberg, 2004, Schumann et al., 2010). The interaction between T cells and DCs will be described in more detail in section 1.2.1.1.

Many different types of DCs exist including plasmacytoid DCs, myeloid DCs, Langerhans cells (skin-resident DCs) (Schulz et al., 2012) and interstitial DCs (Shortman and Liu, 2002). As previously mentioned these cells are known for their stellate morphology but also for their high expression of markers such as

MHCII (key to antigen presentation) and CD11c (Nussenzweig et al., 1981, Nussenzweig and Steinman, 1982).

1.1.1.7 Other Granulocytes

Eosinophils are a key innate immune cell in host defence against parasitic infections. Upon activation, eosinophils have been shown to release key inflammatory mediators such as leukotrienes and prostaglandins (Bandeira-Melo et al., 2002, Luna-Gomes et al., 2011). Eosinophils make up around 5% of white blood cells and have been shown to play a role in airway inflammatory processes such as asthma (Stokes et al., 2015).

Basophils store immune modulators in granules that are released upon activation. The modulators include histamine, which increases vascular permeability and activates local endothelial cells (van Nieuw Amerongen et al., 1998). Activation of basophils induces the release of IL-4 which again implicates these cells in allergy (Min et al., 2004). Finally, it has been suggested that these 'bi-lobed' nuclear granulocytes have the ability to act as antigen presenting cells for haptens (Otsuka et al., 2013) and during parasitic infection (Perrigoue et al., 2009).

1.1.1.8 Natural Killer Cells

Natural Killer (NK) cells come from lymphoid progenitors and function in a very similar fashion to cytotoxic T cells, which will be mentioned in more detail below (section 1.1.2.1.2). These cells have an essential role in host defence against tumours and virus-infected cells (Campbell and Hasegawa, 2013, Zhang, Basher and Wu, 2015). These cells contain granules that are 'cytolytic' as they can cause the rupture of cell membranes. One of the ways that the adaptive, and innate, immune systems interact is through a process known as antibody-dependent cell-mediated cytotoxicity (ADCC). This antibody-mediated process results in the F_c portion of IgG binding to the $F_{c\gamma}RIII$ receptor on the NK cell. Following this binding the NK cell releases the granules mentioned above and induces the destruction of the target cell.

All of the cells, mentioned so far, combine to defend the host from many pathogens. One drawback of the innate immune system is that, although very effective, it does not have the ability to form immunological memory. The other arm of the immune system, the adaptive immune system, can augment the response of the innate immune system. This network of cells has a more antigen-specific function.

1.1.2 Cells of the Adaptive Immune System

The cells of the innate immune system are not antigen specific and whilst the innate arm of the immune system probably does not have the ability to form 'memory', the adaptive immune system does. It is worth noting, however, that some evidence has emerged of 'innate memory' formation (Saeed et al., 2014). The adaptive immune system is made up of cells from the lymphoid-arm of the haematopoietic system that gives rise to T and B cells. T cells develop in the bone marrow but then migrate to the thymus (hence T cell) to undergo further development, and stringent selection, to ensure that these cells are not 'autoreactive' once they are released into circulation.

1.1.2.1 T Cells

1.1.2.1.1 CD4⁺ T Cells

A variety of T cells exist and each performs differing functions that are key to efficient immune responses. Naïve T lymphocytes, or T cells, circulate through the lymph nodes and tissues of the body and 'look' for antigens that are presented by APCs. Antigens, presented by APCs on the MHCII complex binds to the T cell receptor (TCR) on CD4⁺ T cells. If the antigen expressed on this complex is the cognate antigen for the TCR the first signal required for T cell activation is satisfied. This is called the recognition stage. It is now known that for complete activation of T cells multiple signals are required. Signal two, known as verification, comes from the so-called co-stimulatory molecules CD80 and CD86 present on the APC binding to CD28 on the T cell (Caux et al., 1994). This binding event induces the release of IL-2 from the T cell itself to work in an

autocrine fashion to promote cell survival and clonal expansion (Ballesteros-Tato et al., 2012).

CD4⁺ T cells, also known as T helper (T_H) cells, have a major role in shaping the antibody-driven response brought about by B cells. Depending on the conditions in which antigen is presented T_H cells can take different forms. Five main subsets exist known as T_H1, T_H2 (Tada et al., 1978), T_H17 (Bettelli et al., 2006) and the more recently described T_H9 (Dardalhon et al., 2008) and T_H22 (Eyerich et al., 2009). The cell subsets are distinguished by the cytokines that they release upon activation. T_H1 cells secrete some of the classical inflammatory cytokines such as interferon (IFN)- γ , TNF- α , IL-1, IL-6 and IL-12. T_H2 cells are often associated with more allergic-type contexts and release many cytokines that are known to limit inflammation such as IL-4, IL-5 and IL-13. T_H17 cells are, like T_H1 cells, pro-inflammatory in nature. The defining cytokine released by this cell-type is IL-17A/F (hence the name) as well as IL-6, IL-22 and TNF- α . T_H9 cells are named due to their production of IL-9 (Dardalhon et al., 2008). These cells play key roles in the defence against intestinal worms (Licona-Limón et al., 2013) and allergy (Chang et al., 2010) and also play key roles in autoimmunity (Murugaiyan et al., 2012). T_H22 cells are a newly described CD4⁺ T cell subset which were named, like T_H17 and T_H9 cells, in response to the main cytokine they produce, IL-22 (Eyerich et al., 2009). These cells have been shown to play a role in the skin in aiding the process of wound healing (Eyerich et al., 2009) and in psoriasis (Kagami et al., 2010, Luan et al., 2014). It has been suggested that skin-resident Langerhans cells play a major role in the induction of the T_H22 cell type (Fujita et al., 2009).

CD4⁺ T cells can take another form too. Unlike the helper T cells mentioned above which tend to be involved in driving immune responses, regulatory T cells (T_{REGS}) are key immune suppressor cells. They play key roles in the maintenance of peripheral immunological tolerance and preventing autoimmunity (Sakaguchi et al., 2006). Immunological tolerance involves the cellular recognition of 'self' and the disabling of cells that may be autoreactive through anergy or apoptotic induction.

1.1.2.1.2 CD8⁺ T Cells

The other main subset of T cells is the CD8⁺ T cell (cytotoxic T cell). These lymphocytes recognise their cognate antigen expressed on MHC I and undergo activation. MHC I is expressed by all nucleated cells and is the main way by which antigens from pathogens that have infected a cell are presented. Under normal conditions self-peptides are constantly expressed on MHC I molecules on the cell surface. If a cell becomes infected it then starts to express antigenic peptides on its surface and this in turn alerts cytotoxic T cells to a possible problem. Upon infection many pathogens and tumour cells also stop the process of MHC I expression by host cells in an attempt to evade the immune system. Effector cytotoxic T cells or NK cells usually kill any cell expressing no MHC I molecules. Upon activation these cells release cytolytic toxins that form holes in the membranes of target cells and result in its destruction.

1.1.2.2 B Cells

These cells are so called because in birds they are found in the Bursa of Fabricius. The main role of B cells is to produce antibodies. At around 150kDa, antibodies are fairly large proteins that carry out many important functions in immune defence. These include neutralising toxins, opsonising pathogens, preventing the entry of pathogens into host cells and activating the complement system.

As mentioned in the previous section, T helper cells interact with B cells to help activate them. B cells can express MHC II molecules and present antigen to T cells in the lymph node. Given the interaction between the MHC II-presented peptide on the B cell, the correct TcR on the T cell and appropriate costimulation, the B cell can become activated and clonally expand. The result of this is a pool of specific antibody-producing B cells that can aid in the immune response. Upon the resolution of the immune response a pool of memory B cells and plasma cells (the name given to an antibody-producing B cell) are retained to quickly respond to reinfection with the same pathogen.

1.2 Chemokines

There are two forms of chemically induced cell movement, chemokinesis and chemotaxis. Chemokinesis is random and undirected movement whereas chemotaxis is directed movement towards a chemical signal. Chemokines are small specialised cytokines that initiate chemotaxis in target cells. Their name derives from the amalgamation of the words chemotactic cytokines (Zlotnik and Yoshie, 2000). The chemokine family is made up of small (8-10 kDa) homologous proteins that are highly conserved throughout vertebrate evolution (Zlotnik and Yoshie, 2000). Chemokine nomenclature was historically confusing and poorly regulated. As a result of this, a standardised nomenclature was devised based on chemokine structure (Zlotnik and Yoshie, 2000).

CC — C — C —	CCL1-28
CXC — C — C —	CXCL1-17
C — — C —	XCL1
CXXXC — C — C —	CX ₃ CL1

Table 1.1- The cysteine motifs of chemokine subfamilies- A diagrammatic illustration of the conserved cysteine motif that makes up the chemokine subfamilies.

Four structural variants are clear within the family of chemokine ligands, these are depicted in Table 1.1. The determining factor is the presence, and relative position, of highly conserved cysteine (C) residues expressed at the amino terminus of the peptide (Zlotnik and Yoshie, 2000). These conserved cysteines form disulphide bonds and so influence the structure of chemokines (these interactions are depicted in figure 1.3). Chemokines are then numbered in order of when the sequence was added to the gene databases. There are two major, and two minor, chemokine subfamilies.

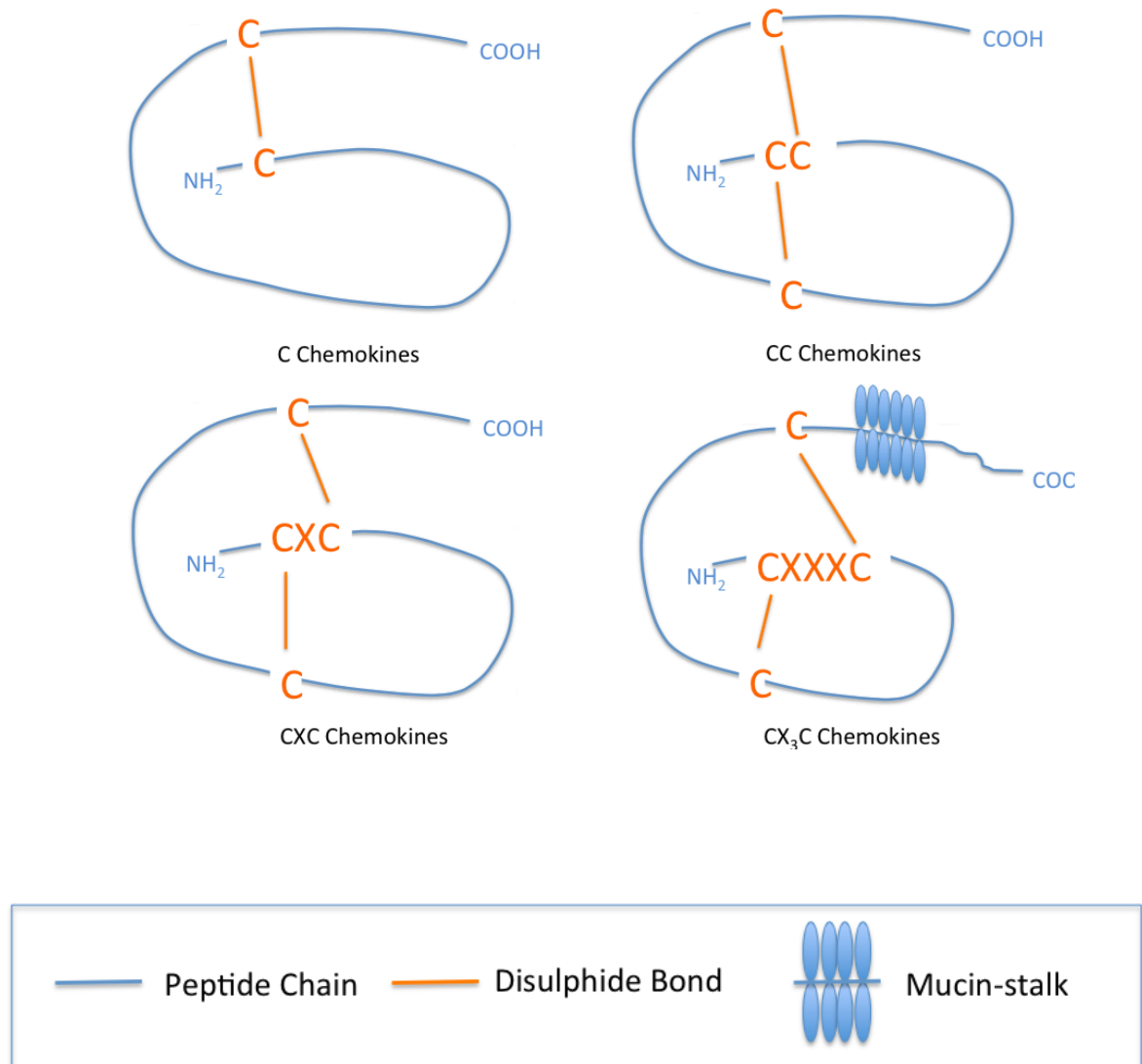


Figure 1.3- The relationship of cysteine motifs in chemokines. The above figure depicts two-dimensional representations of the interactions between the cysteine residues in the four subfamilies (Zlotnik et al. 2006) .

1.2.1 CC Chemokines

The first, and largest group is known as the beta (β), or CC, chemokine subfamily as two of the key cysteine residues sit adjacent to one another at the amino terminus of the sequence. There are twenty-eight ligands in this group (CCL1-CCL28) and these chemokines tend to be chemoattractive to monocytes, lymphocytes, and some granulocytes (Alam et al., 1994, Lee et al., 2000, Rot and Andrian, 2004).

CCL3, previously known as macrophage inflammatory protein (MIP) 1- α , is a key recruiter of cells such as monocytes, macrophages, dendritic cells and both CD4⁺

and CD8⁺ T cells during the acute immune response. It has been shown that neutrophils can release this chemokine in order to recruit these effector cells (Charmoy et al., 2010). CCL2, CCL7, CCL8 and CCL13 have roles in the recruitment of monocytes to inflammatory sites (Shi and Pamer, 2011). Whereas CCL20 has been shown to recruit T_H17 cells.

1.2.2 CXC Chemokines

The second major group is the alpha (α), or CXC, chemokines that have the same cysteine groups at the N-terminal but with the addition of an intervening amino acid. There are seventeen ligands in this group (CXCL1-CXCL17). These chemokines are functionally divided into ELR positive, and ELR negative, groups depending on the presence, or absence, of a glutamic acid (E)- leucine (L)- arginine (R) motif that lies before the first cysteine of the CXC motif. ELR positive chemokines in this group act on neutrophils specifically and are angiogenic (Strieter et al., 1995). CXC chemokines without the ELR motif have a more diverse target cell repertoire and tend to be more angiostatic (Zlotnik and Yoshie, 2000). Of the CXC chemokines, CXCL8 is the most potent attractor of neutrophils. Pre-formed molecules of this chemokine are stored within the Weibel-Palade bodies of endothelial cells and released upon their activation (Wolff et al., 1998). During the inflammatory response this chemokine is released in large quantities to rapidly recruit neutrophils to the site of trauma. Mice do not have this gene but do have CXCL1 and CXCL2 which both bind to the receptor CXCR2 for neutrophil recruitment (Hol, Wilhelmsen and Haraldsen, 2010).

1.2.3 XC Chemokines

The penultimate group is the XC or gamma (γ) chemokines. This group is unique as its members only have two of the four conserved C residues. The lymphotactins (α and β) are the only two proteins that fit into this subdivision as XCL1 (lymphotactin α) and XCL2 (lymphotactin β). Originally these chemokines were associated with the T_H1 response (Dorner et al., 2002). Activated CD8⁺ T cells in the thymus and peripheral blood have been shown to express XCL (Kennedy et al 1995). It has also recently been suggested that the XCL1 released

from these CD8⁺ cells may inhibit HIV-1 infection (Guzzo et al., 2013). One of the key roles of the lymphotactins is in DC biology (Dorner et al., 2009). In the thymus XCL1 mediates the accumulation of thymic DCs which, interact with and are key to, the development of natural regulatory T cells (Lei et al., 2011).

1.2.4 CX₃C Chemokine

The fourth, and final, structural form that makes-up the family is the CXXXC (CX₃C) chemokine. This is a single entity, rather than a group, and the most recently discovered (Bazan et al., 1997). CX₃CL1, or fractalkine, is an interesting chemokine as it can exist as both a soluble ligand and as a membrane-anchored ligand (Bazan et al., 1997). The anchor is a mucin stalk and it is cleaved by TNF- α converting enzyme (TACE) or a disintegrin and metalloproteinase (ADAM)17 in order to release the soluble chemokine ligand (Garton et al., 2001). Other than CX₃CL1, only CXCL16 is thought to exist in this form (Matloubian et al., 2000). CX₃CL1 is thought to play a role in the brain and has been shown to be expressed in the hippocampus and may play a role in glutamate signalling (Sheridan et al., 2014).

Chemokines can be characterised by functional as well as by the structural divisions mentioned above. These proteins can be either constitutive or inflammatory depending on the context with which they are most often expressed and function. There are also some 'dual function' chemokines that can play constitutive and inflammatory roles.

1.2.4.1 Inflammatory Chemokines

Inflammatory chemokines are expressed in such abundance after tissue damage that they were discovered and characterised early in the history of chemokines. These chemokines are inducible upon stimulation of cells by inflammatory signals. Interestingly, the genes for the inflammatory chemokines in the CC family are mainly clustered on chromosome 17 in the human (Naruse et al., 1996, Nomiyama et al., 1999) whilst the counterpart CXC chemokine genes are clustered on chromosome 4 (O'Donovan, Galvin and Morgan, 1999). It is thought that this has been caused by gene duplication in response to specific pathogen

pressures on different organisms. Inflammatory chemokines can be expressed virtually anywhere in the body, in fact CCL2 can be expressed by all nucleated cells and acts as a warning signal to attract cells to sites of damage/trauma. Unlike their homeostatic relatives, inflammatory chemokines are said to be promiscuous and their receptors unfaithful (Bachelier et al., 2014). This phenomenon seems to act as an immunological 'skeleton key' allowing inflammatory cells into almost any tissue in order to protect the host. That is to say that it would seem the inflammatory chemokine response promotes the recruitment of an array of effector cells in order to quickly resolve local trauma. This may seem like a very blunt and non-specific response but the field is now moving towards investigating if there is, in fact, more subtlety into how this system functions (Zweemer et al., 2014).

1.2.4.2 Homeostatic Chemokines

These proteins are involved in basal leukocyte trafficking and tend to have a much more specific ligand-receptor relationship than their inflammatory counterparts. This group of chemokines can be extremely tissue-specific. CCL25 is expressed in the small intestine and so, under homeostatic conditions, CCR9 expression is required for trafficking to this location. This relationship is also key to thymic migration. Other constitutive chemokines include CCL19, CCL21, CCL27, CXCL12 and CXCL13. The roles of some of these chemokines will be discussed in more detail below.

1.3 Chemokine Receptors

1.3.1 Receptor Structure

Chemokine receptors are seven-transmembrane spanning proteins that are between 340 and 370 amino acids in length and are part of the Rhodopsin family of G-protein coupled receptors (GPCRs). Chemokine receptors have highly conserved structural and functional properties although they have moderately variable amino acid sequences (Clark-Lewis et al., 1995). One of the key examples of this is known as the DRYLAIV or DRY motif. All classical chemokine receptors contain the DRY motif in the second intracellular loop. DRYLAIV- D-

Aspartate, R- Arginine, Y- Tyrosine, L- Leucine, A- Alanine, I- Isoleucine, V- Valine. This motif seems to be indispensable for the ability to signal, as without this motif a calcium flux is not induced in the cell following ligand binding (Graham et al., 2012). The DRY motif is the main site for G-protein coupling in the chemokine receptors.

These GPCRs are made up of a flexible N-terminus that is on the extracellular side of the cell membrane. This exposed section of the protein is responsible for initial ligand binding. It has been shown that chemokines bind their cognate receptor in a two-step mechanism (Montecarlo and Charo, 1996, Crump et al., 1997). Firstly, the ligand binds the extracellular N-terminus of the receptor. This is a high-affinity bond that arrests the chemokine in position allowing the non-bound portion of the chemokine to start making low-affinity bonds with the transmembrane spanning regions of the receptor. This results in receptor activation and down-stream signalling events. The C-terminus of chemokine receptors also contains many serine and threonine residues that can act as phosphorylation sites on the receptor. A two-dimensional cartoon depiction of a chemokine receptor is shown in figure 1.4.

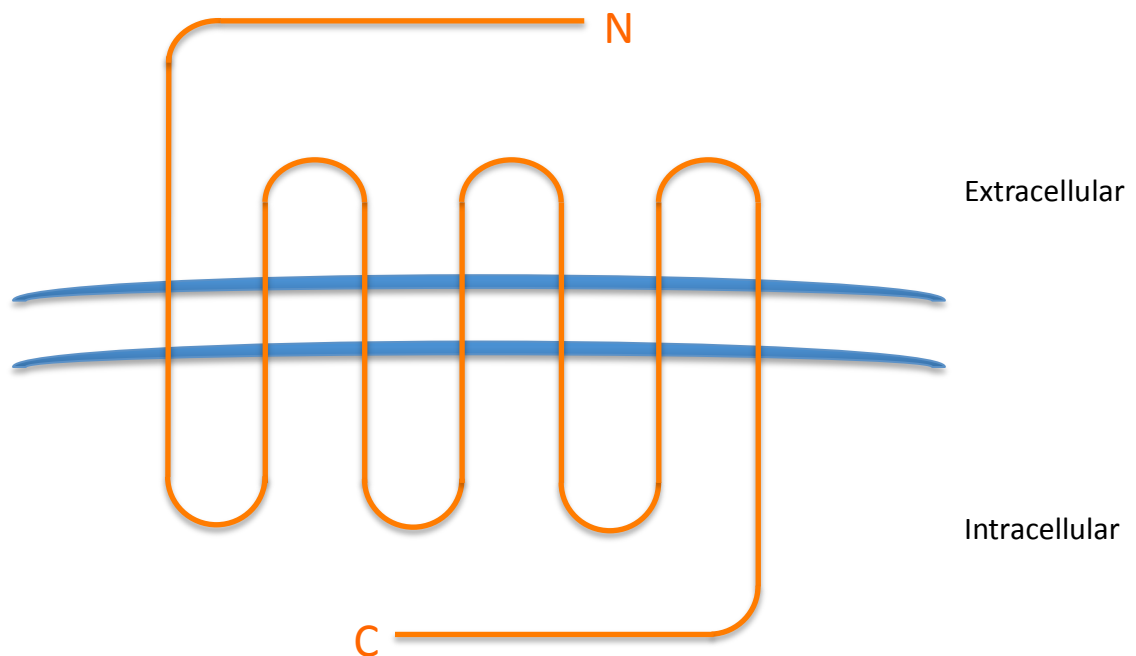


Figure 1.4- 2-dimensional chemokine receptor basic structure- This diagram depicts a simplified illustration of the basic two dimensional structure of a chemokine receptor. The N' terminus of the protein is key in ligand binding and immobilisation. The C' terminus plays important roles in receptor signaling upon ligand binding.

Working out the three-dimensional structures of the chemokine receptors has proved technically difficult, however, in recent years the structure of CXCR4, CXCR1 and CCR5 have been elucidated. These studies helped confirm many of the hypothesised structural characteristics of chemokine receptors. The structures of CXCR4 and CCR5 were revealed using X-ray crystallography and, in the case of the former, confirmed the ability of the receptor to homodimerise (WU et al 2010). The structure of CCR5 was elucidated in association with the small molecule inhibitor of the receptor Maraviroc (Tan et al 2013). NMR spectroscopy was utilised to characterise the structure of CXCR1 (PARK et al 2012). The studies mentioned here have been invaluable in shedding light on the nature of chemokine receptor interactions with ligands and other receptors.

1.3.2 CC Chemokine Receptors

As for the ligands, four receptor subgroups have been defined (Bachelierie et al., 2014). The first of the chemokine receptor groups are the CC receptors. These receptors are responsible for binding CC chemokine ligands. There are ten identified receptors in this group (CCR1-10). Again chemokine receptors are numbered in chronological order of discovery and lower numbers tend to be inflammatory receptors. CCRs 1-3 and CCR5 are considered to be the classical inflammatory CC chemokine receptors. Like their CXC counterparts, these receptors each have many ligands. CCR2 is expressed on a subset of inflammatory monocytes and plays an important role in their recruitment into tissue. However, in the absence of CCR2 monocyte recruitment still takes place thanks to the receptors CCR1 and CCR5 which seem to act as substitutes in this case (Dagkalis et al., 2009). This is an example of the marked redundancy that appears to be present in the inflammatory chemokine system (Rot and Andrian, 2004).

CCR7 is a key homeostatic CC chemokine receptor. It has been shown that in mice lacking this receptor there is a catastrophic breakdown in organisation of cells in secondary lymphoid organs such as the lymph nodes and the spleen (Förster et al., 1999). CCR7 appears to play a key role in directed cellular movement during many key immunological processes. These include the exit of dendritic cells and T cells from the peripheral tissues to the lymph nodes, entry

of T cells to the lymph nodes via high endothelial venules (HEV) and subsequent organisation of these cells within these tissues. CCR7 has only two known ligands, CCL19 and CCL21 (Vander Lugt et al., 2013). A mouse strain with a mutation known as paucity of lymph node T cell (plt) has functional CCR7 but lacks expression of both of the receptors ligands in secondary lymphoid organs (Nakano et al., 1998). Naïve T cells and activated dendritic cells in these mice have an impaired ability to traffic to the lymph nodes. As a result of this mutation these mice show delayed but actually enhanced adaptive immune responses (Mori et al., 2001). In fact it was shown that plt mice express IL-2, a key cytokine for T cell proliferation, for around 8 weeks compared to around 20 days in WT mice. The relationships between the CC chemokine receptors and their cognate ligands are depicted in figure 1.5.

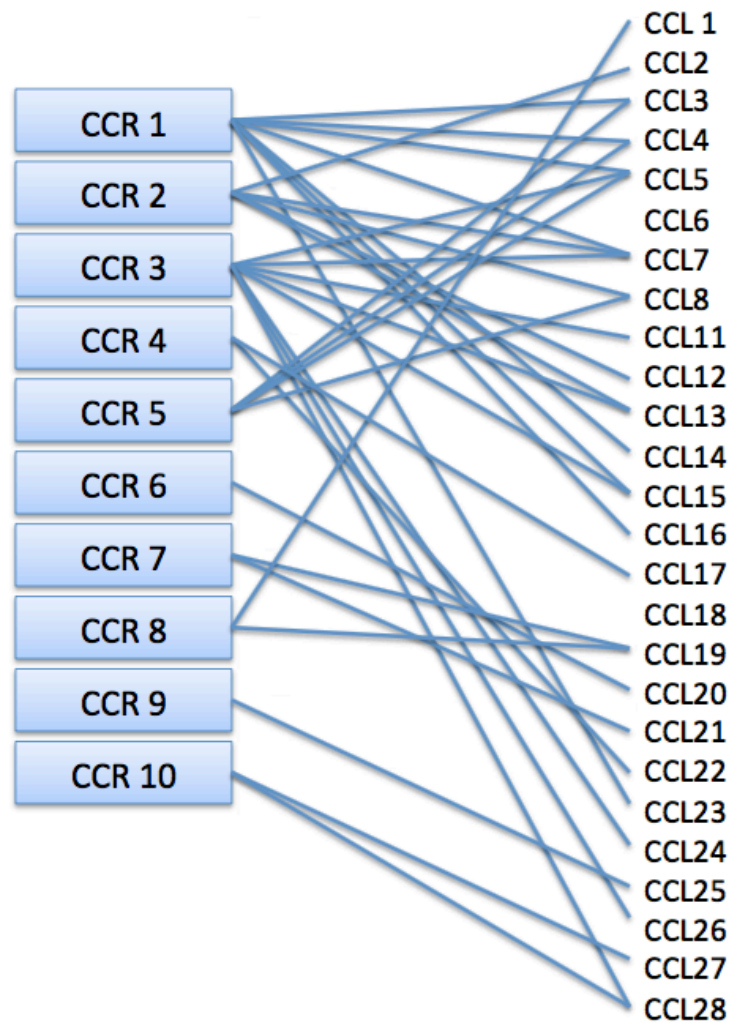


Figure 1.5- CC chemokine ligands and receptor binding relationships- The above diagram illustrates the complexity of the relationship between CC chemokine ligands and their receptors. Interestingly the inflammatory receptors (CCRs1-5) have more ligands than the constitutive receptors (CCRs 6-10).

1.3.3 CXC Chemokine Receptors

Six classical CXC chemokine receptors exist (CXCR1-6). These receptors are mainly found on neutrophils and lymphocytes. Just like CC chemokine receptors, these receptors can be functionally characterised as either inflammatory (CXCR1-3) or homeostatic (CXCR4-6). CXCR1 and 2 have well defined roles in the migration of neutrophils during inflammation. The ligand CXCL8 is rapidly and abundantly released at sites of trauma in humans. Mice lack CXCL8 but appear to

use the CXCR2 receptor, along with CXCL1 and CXCL2, to coordinate movement of these key inflammatory cells during inflammation.

CXCR3 interacts with the ligands CXCL9, 10 and 11 and is associated with T_H1 and NK cell recruitment. Two variants of this receptor exist, known as CXCR3-A and CXCR3-B. The latter binds all of the ligands previously mentioned with the addition of CXCL4 (Struyf et al., 2011). CXCR3 has been shown to be important for CD8 T cell responses and subsequent memory cell formation whilst CXCR3 is expressed along with CCR5 and is a key mediator of the T_H1 cell response. CXCR3 has been implicated in many inflammatory diseases such as type-1 diabetes mellitus (Frigerio et al., 2002) and rheumatoid arthritis (Laragione et al., 2011).

CXCR4 is a chemokine receptor of utmost importance. This is illustrated best by the fact that mice lacking CXCR4 die perinatally (Zou et al., 1998). Along with its cognate ligand, CXCL12, this receptor is involved in many key processes. In fact, by functionally deleting this receptor, abnormalities in the development of the immune system, the reproductive system, the kidneys, the lungs and the brain result. The interaction between CXCL12 and CXCR4 has been shown to be essential in the retention of haematopoietic stem cells in the bone marrow niche (Zou et al., 1998). Intravenous injection of AMD3100, a small-molecule inhibitor of CXCR4, results in the mobilisation of progenitors from the bone marrow and into the peripheral blood (Liles et al., 2003). This has brought about a significant advance in the field of stem cell transplantation. CXCR4 is also a co-receptor for the HIV-1 virus (Feng et al., 2011) and it has been shown that plerixafor (the trade name of AMD3100) can block viral entry of HIV-1 into host cells (Donzella et al., 1998).

CXCR5, like CCR7, plays a key role in the organisation of secondary lymphoid tissues as well as migration of leukocytes within these structures (Breitfeld et al., 2000). CXCL13 is the only identified ligand for CXCR5 and is produced by follicular dendritic cells of the B cell zone to attract B cells (Yu et al., 2002). B cells that reside in the follicles of secondary lymphoid structures express CXCR5 whilst T helper cells upregulate CXCR5 in order to interact with B cells to initiate the humoral immune response. CXCR6 also has only one known ligand,

CXCL16. Current literature suggests that this axis may have a minor role in both cancer development and HIV-entry (Paust et al., 2010).

1.3.4 XC and CX₃CR Chemokine Receptors

Only one receptor has been discovered for the gamma chemokines, this is known as the lymphotactin receptor (XCR1) (Zlotnik and Yoshie, 2000). Like many chemokine receptors, XCR1 has been associated with rheumatoid arthritis. T cells within the synovium have been shown to express XCL1 and it is thought that this initiates the infiltration of XCR1⁺ mononuclear cells (Blaschke et al., 2003). It is also suggested that a population of dendritic cells express this receptor (Dorner et al., 2009).

The final receptor to be mentioned is the fractalkine receptor or CX₃CR1. As this is the cognate receptor for the membrane-anchored chemokine CX₃CL1 it can function as an adhesion molecule (Haskell, Cleary and Charo, 2000). CX₃CR1 has been found on NK cells, T cells, monocytes and microglial cells of the central nervous system. In the case of monocytes this molecule appears to play an important role in cell survival (Landsman et al., 2009). The CX₃CL1/R1 axis is important for the engulfment of apoptotic cells by macrophages (Truman et al., 2008).

1.3.5 Chemokine Receptor Signalling

Chemokine receptor signalling is a complex process that begins with the binding of a ligand to its cognate receptor. As already mentioned, chemokine receptors are heptahelical molecules that bind heterotrimeric G proteins that are associated with guanosine diphosphate (GDP) and are inactive as a result. Upon ligand binding there is a conformational change in the transmembrane domain of the receptor that allows binding of G α proteins. This allows for the exchange of bound GDP for guanosine triphosphate (GTP) at the G α subunit. This then allows the G α /GTP complex to dissociate from the G $\beta\gamma$ subunits and leads to downstream signal transduction and a calcium ion flux (Wu, LaRosa and Simon, 1993, Oldham and Hamm, 2008). The receptor is typically internalised and

desensitised before being recycled to the cell surface free of ligand. A simplified depiction of this is shown in figure 1.6.

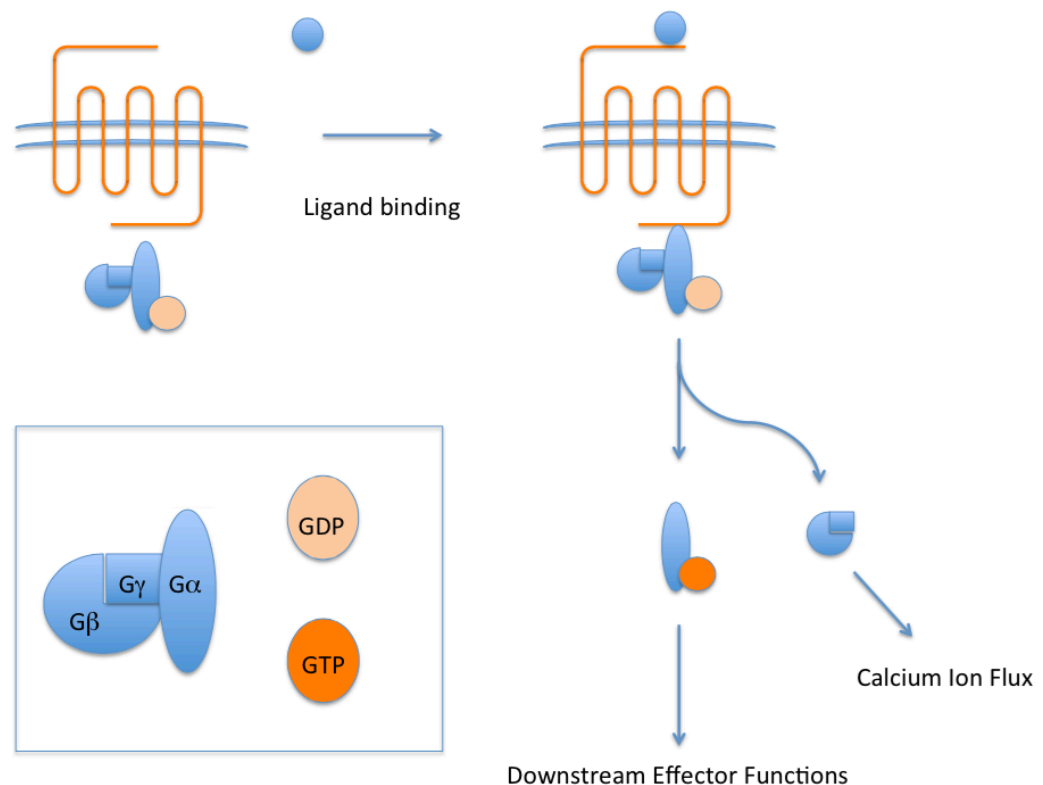


Figure 1.6- The downstream signalling following chemokine receptor binding- Above is a simplified illustration of the downstream events following the binding of a chemokine to its cognate receptor. The G-protein subunits exist as a heterotrimer ($G\alpha$, $G\beta$, $G\gamma$) and associate with the receptor. Guanosine diphosphate (GDP) binds the $G\alpha$ subunit. Upon ligand binding the GDP is released from the heterotrimer. The G-proteins stably bind the receptor and then guanosine triphosphate binds the $G\alpha$ subunit. This causes a destabilisation of the heterotrimer and the $G\alpha$ subunit dissociates from the $G\beta/\gamma$ complex. Both of the newly formed products induce downstream functions via interactions with effector proteins. The signal is terminated through hydrolysis of GTP to GDP by $G\alpha$ which then binds the $G\beta/\gamma$ complex to reform the heterotrimer and then binds the receptor. (Oldham & Hamm 2008).

Ligand binding of conventional chemokine receptors can induce a multitude of downstream depending on the receptor, the ligand and the context in which the ligation occurs (Rot and Andrian, 2004). Upon ligand binding many downstream effector proteins are activated including members of the Janus kinase/signal transducer and activation of transcription (JAK/STAT) family (Soriano et al., 2003). The $G\alpha$ subunit activates Rho family GTPases such as Rac1 to induce the formation of lamellipodia formation at the leading edge of the cell and subsequent movement (Gu et al., 2003).

1.3.5.1 Chemokines and Chemokine Receptors in Disease

1.3.5.1.1 Cancer

Along with malignant cells there are also many leukocytes at tumour sites. Cancers are known to move to secondary sites in a process known as metastasis and they also require a blood supply and so promote angiogenesis. The chemokine network is essential to all three of these aforementioned features of cancer biology (Balkwill, 2012). One of the main leukocytes found at cancer-sites are known as tumour associated macrophages (TAMs) and the levels of CCL2 and CCL5 correlate with the number of these cells present in the tumour (Mantovani et al., 2008).

The GTPase Ras has been shown to be mutated in many cancers. Oncogenic Ras is a constitutively active form of the enzyme which can induce the production of CXCL8 and CXCL1 both of which have been shown to recruit neutrophils and increase tumour-associated angiogenesis (Sparmann and Bar-Sagi, 2004).

The process of metastasis is a feature of malignant cancers and is key to disease progression (Joyce and Pollard, 2009). The chemokine receptor CCR7 and CXCR4 have been shown to play a role in this process (Müller et al., 2001, Mashino et al., 2002, Günther et al., 2005). CCR7, as discussed in 1.3.2, is the key receptor involved in the trafficking of cells to lymph nodes (Gosling et al., 2000). This receptor is upregulated in many cancers, including colorectal and gastric carcinomas, which metastasise to the lymph node via the CCL19/CCL21/CCR7 axis (Mashino et al., 2002, Günther et al., 2005).

Tumours are sites of hypoxia and, in many cancers including breast cancer, this results in an increase in the expression of CXCR4. This results in metastatic migration towards CXCL12-expressing sites like the liver, the lung and the lymph nodes (Müller et al., 2001).

1.3.5.1.2 Human Immunodeficiency Virus (HIV)

The retrovirus HIV was discovered in 1983 and is known to target and destroy the immune system of the host (Gallo and Montagnier, 2003). HIV infection leads to

the development of Acquired Immunodeficiency Syndrome (AIDS) (Gallo and Montagnier, 2003, Costin, 2007). The molecule glycoprotein (gp) 120 on the viral envelope of the virus binds to CD4 and so helps it infect CD4⁺ T cells (Dalglish et al., 1984). This binding event leads to a conformational change in the viral envelope, which exposes chemokine receptor binding regions. It was also shown that the chemokine receptors CCR5 and CXCR4 facilitate the entry of the virus to the host cell (Deng et al., 1996, Dragic et al., 1996).

M-tropic HIV, the initial stage of infection, uses CCR5 as a co-receptor for cell entry to target monocytes, macrophages and CCR5⁺ T_H1 cells. This stage is followed by the T-tropic stage of the infection which uses CXCR4 as a co-receptor to infect lymphocytes. This results in a vast reduction in the CD4⁺ T cell count of the host and leads to great susceptibility to opportunistic infections and the onset of AIDS. CCR5 ligands have been shown to reduce the ability of the virus to infect cells *in vitro* (Cocchi et al., 1995, Nibbs et al., 1999). To add to this, patients with high levels of CCL5 have been shown to be refractory to the progression of HIV infection to AIDS (Clerici et al., 1996).

Around 1% of people of northern European descent have no functional CCR5 receptor activity (Huang et al., 1996). People who are homozygous null for CCR5 have what is known as the CCR5delta32 (CCR5Δ32) mutation. This results in a 32 base pair deletion in the second extracellular loop of the receptor and renders the receptor non-functional. This deletion appears to be advantageous to the host in many pathologies. For example, patients who are CCR5Δ32 homozygous are highly resistant to infection by the human immunodeficiency virus (HIV)-1 (Huang et al., 1996). In fact it was this property of the mutation that led to its discovery (Huang et al., 1996). It has also been shown that if a Δ32 heterozygous person does become HIV-positive they take longer to develop acquired immunodeficiency syndrome (AIDS) (Huang et al., 1996).

This mutation has also been shown to be protective for graft rejection and many inflammatory disorders (Zapico et al., 2000, Pokorny et al., 2005, Bettencourt et al., 2014). In many ways CCR5Δ32-positive individuals are less prone to inflammatory disease. There is, however, one case in which this 32 base pair deletion is non-protective. Specifically if a CCR5Δ32-positive host becomes

infected with West Nile virus they are at greater risk of developing lethal encephalopathy than a patient with wild-type CCR5 (Glass et al., 2006).

1.4 Atypical Chemokine Receptors

The receptors mentioned so far are conventional receptors as they convey intracellular messages downstream of ligand binding and induce cell movement. However another receptor type exists. Atypical chemokine receptors tend to be structurally homologous but fail to induce a typical signalling response downstream of chemokine binding (Graham et al., 2012). For this reason, they are sometimes known as ‘silent’ or scavenging receptors- both of these aliases giving a good description of some of the function of these molecules.

They are known as silent as they fail to induce the canonical response from the target cell after receptor ligation. The chemokine ligand is internalised by the receptor and efficiently degraded, hence the scavenger synonym. Currently, there are four members of this receptor group. These are the Duffy antigen receptor for chemokines (DARC), CCX-CKR, CXCR7, and D6, which will be the focus of this work. In 2014 the atypical chemokine receptors were given a standardised nomenclature in much the same way as the classical chemokines and receptors. They are now named ACKR (atypical chemokine receptor) and numbered in order of their discovery. As a result DARC is ACKR1, D6 is ACKR2, CXCR7 is ACKR3 and CCX-CKR is now ACKR4 (Bachelier et al., 2014).

These chemokine receptors express variations of the DRY motif mentioned earlier. For example, ACKR2 has the amino acid sequence DKYLEIV in the second intracellular loop of its structure (Nibbs, Wylie, Pragnell, et al., 1997, Nibbs, Wylie, Yang, et al., 1997). Different atypical receptors express variations of the DRYLAIV motif but the variation of these amino acids is key to the inability of these receptors to mount classical signalling responses. These motif variations are shown in figure 1.7. Notably, studies have been carried out on ACKR2 in which the altered DKY motif was changed to DRYLAIV in an attempt to restore signals. This alteration imparted signalling activity (Smit, Lira and Leurs, 2010).

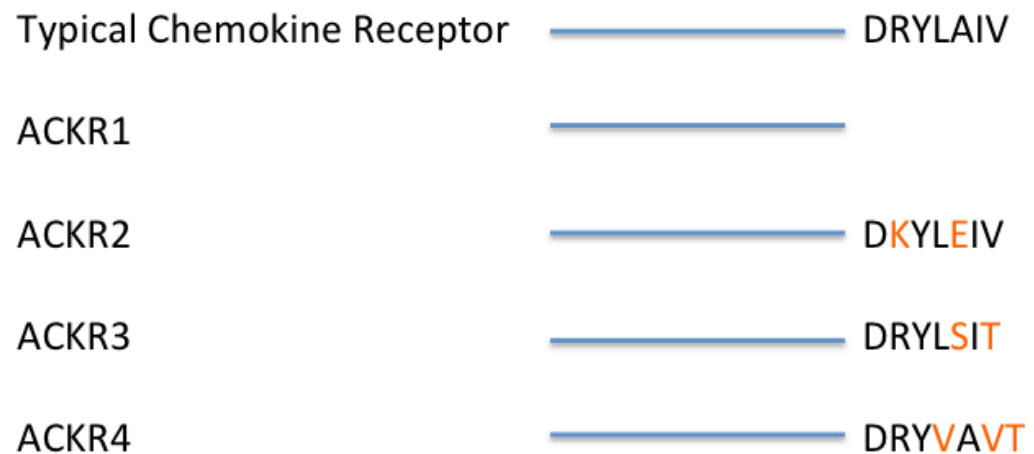


Figure 1.7- Variations of the DRY motif in atypical chemokine receptors- This figure is a comparison of the DRY motif, of typical chemokine receptors, and it's variations in atypical receptors.

1.4.1 ACKR1

The first atypical chemokine receptor to be discovered was the Duffy antigen receptor for chemokines, now known as ACKR1. Like all chemokine receptors this is a seven-transmembrane spanning receptor but unlike the canonical receptors it lacks the DRYLAIV motif and fails to induce a calcium flux upon ligand binding (Horuk et al., 1996). The primary amino acid sequence of ACKR1 is unlike any other chemokine receptor and in fact is almost unrecognisable as a member of this family in this respect (Graham et al., 2012). Originally this molecule was found on red blood cells and is the most promiscuous of all chemokine receptors as it can bind both CC and CXC inflammatory chemokines (Novitzky-Basso and Rot, 2012). ACKR1 is the protein determinant in the Duffy antigen blood group system (Hadley and Peiper, 1997).

It is also a receptor for the human malarial parasites *Plasmodium vivax* and *Plasmodium knowlesi* (Horuk et al., 1993). Individuals with an ACKR1 polymorphism in the binding site for the erythroid transcription factor, GATA-1, have been shown to express no ACKR1 on their erythrocytes (Tournamille et al., 1995). These same individuals with what is known as a Duffy-null polymorphism,

are highly resistant to malarial infection (Tournamille et al., 1995). It is worth noting that these individuals only lack erythrocyte expression but retain expression on blood endothelial cells (BECs). As a chemokine receptor, this molecule has been described as having two main roles. The first is in chemokine ligand transcytosis. In *in vitro* studies using polarized cells that had been transfected with ACKR1 it was shown that ligand could be internalised by the receptor on one face of the cell and presented, intact, on the opposite side (Middleton et al., 1997, 2002). This gives ACKR1 the ability to act as a ligand presentation receptor to leukocytes for example. It is important to note that although ACKR1 internalises chemokines it does not degrade them and so is not considered to be a scavenging receptor like some of the other atypical chemokine receptors (Middleton et al., 1997, 2002, Pruenster et al., 2009). ACKR1 has also been reported to be expressed by endothelial cells, especially blood endothelial cells, and in the cerebellum (Horuk et al., 1996). Due to the nature of the CXC chemokines that ACKR1 has specificity for it is suggested that it has an anti-angiogenic ability. An *in vivo* study using ACKR1 transgenic mice that over-expressed the receptor in a melanoma model showed a reduced angiogenic capability when compared to WT mice (Horton et al., 2007).

The other function that ACKR1 on erythrocytes is thought to serve is that of a chemokine buffer in the blood. As inflammatory chemokines are released into the bloodstream they are thought to become associated with ACKR1 on the surface of erythrocytes. These bound chemokines can then be released as the inflammation subsides and so this help prevent large fluctuations in blood-chemokine levels (Novitzky-Basso and Rot, 2012). ACKR1-null mice take longer to clear peripherally administered chemokines compared to WT mice. It was also shown that, in a model of LPS-driven peripheral inflammation, null mice had an increased leukocyte infiltrate into the liver and the lungs (Lee et al., 2006).

One of the most striking human phenomena associated with ACKR1 is that of benign ethnic neutropenia. It has been shown that individuals of European descent tend to have a higher peripheral blood leukocyte (PBL) count than those of an African background (Kulkarni et al., 2009). It has been shown, by genome-wide association studies, that these findings correlate very strongly with the Duffy-null polymorphism (Kulkarni et al., 2009).

1.4.2 ACKR3

The atypical chemokine receptor ACKR3 was originally thought to be a member of the classical chemokine receptor group and was in fact designated as CXCR7 until quite recently. The receptor was originally known as RDC1 and was cloned and characterised from mouse genomic DNA and the murine receptor has 90% amino acid similarity to the human receptor (Heesen et al., 1998). It was 'de-orphanised' when the ligands CXCL11 and CXCL12 were shown to bind the receptor (Heesen et al., 1998). It is worth noting that CXCL12 binds ACKR3 with higher affinity than it binds CXCR4.

ACKR3 expresses a two amino acid variation of the DRY motif and has DRYLSIT in its place. The functional result of this is, of course, that ligand binding by ACKR3 does not induce a calcium flux in the cell. Chemokine receptors mainly mediate their signal via G-proteins upon ligand binding. ACKR3, however, is a beta-arrestin biased chemokine receptor (Rajagopal et al., 2010). Unlike ACKR1, ACKR3 has been shown to be a chemokine scavenging receptor capable of scavenging and therefore regulating the availability of both of its ligands (Wang et al., 2012).

ACKR3 is not widely expressed. It has been shown on marginal zone B cells (Wang et al., 2012), a subset of monocytes (Infantino, Moepps and Thelen, 2006) BECs of the CNS (Cruz-Orengo, Holman, et al., 2011), cells of the foetal mouse liver, and lymphatic endothelial cells (LECs) in normal tonsil and kidneys during acute graft rejection. The expression of ACKR3 in the adult is very low. However it has been found in many tumour environments. ACKR3 is expressed both in the neovasculature of tumours and can be expressed by the tumour itself (Miao et al., 2007, Wang et al., 2008).

Mice that lack ACKR3 tend to die perinatally (Sierro et al., 2007). The cause of this is thought to be largely due to defective heart development involving thickened semilunar valves and ventricular septal defects. These mice show normal haematopoiesis. The expression of ACKR3 in the microvessels of the developing heart was key to the cardiac phenotype observed by Sierro et al. To demonstrate this they generated mice with a specific deletion of ACKR3 in the

endothelium. This resulted in development of the same phenotype (Sierro et al., 2007).

Like many chemokine receptors ACKR3 has the ability to form dimers. Studies have shown ACKR3 to form homodimers and also heterodimers with CXCR4. When ACKR3 heterodimerises with CXCR4 it has a modulating effect on CXCL12 driven responses. Binding of the heterodimer to CXCL12 causes bias in favour of signalling via β -arrestin and not G proteins (Décaillot et al., 2011).

In a model of zebrafish embryogenesis a study found an example of CXCR4 and ACKR3 working together for efficient cellular migration along the lateral line. What was observed in this work was that a CXCL12 gradient was formed along the lateral line of the developing organism that triggered CXCR4 signals at the leading edge of the cells of the migrating primordium. This response was driving the migration of these essential cells. The most interesting observation here was that at the trailing end of the primordium there was expression of ACKR3 which was scavenging the CXCL12 ligands and blunting migratory responses in the trailing cells. This then allows 'pockets' of the cell to become immobile and to be deposited at defined points along the lateral line (Dalle Nogare et al., 2014).

ACKR3 has been investigated as a potential therapeutic target. In one model of collagen-induced arthritis (CIA), the murine model of human rheumatoid arthritis, it was shown that prophylactically and therapeutically applied antagonists of the receptor suppressed the symptoms of the disease (Watanabe et al., 2010). This study showed a reduction in angiogenesis in the inflamed joints that was considered a contributor to the reduced pathology. It is also worth noting that in human rheumatoid arthritis ACKR3⁺ blood vessels can be detected in the inflamed joint (Watanabe et al., 2010). Another mouse model of human disease in which ACKR3 has been shown to have a role is CNS disease. In the mouse model of experimental autoimmune encephalitis (EAE) antagonism of ACKR3 reduced CNS leukocyte infiltration compared to WT mice (Cruz-Orengo, Chen, et al., 2011).

1.4.3 ACKR4

ACKR4 has had many names since its original discovery including CCX-CKR (ChemoCentryx Chemokine Receptor), CCR11 and CCRL1 (Gosling et al., 2000, Khoja et al., 2000, Schweickart et al., 2000). Like ACKR3, this receptor was originally considered to be a classical receptor and was known as CCRL11 for some time. It is a receptor for three of the homeostatic CC chemokines CCL19, CCL21 and CCL25. As previously mentioned, CCL19 and CCL21 are essential in the efficient migration of cells from tissue to draining lymph nodes and CCL25 has roles in the development of thymocytes and cell-trafficking to the small intestine. Until the characterisation of ACKR4 it was thought that CCR9 was the only receptor for CCL25 (Zaballos et al., 1999).

In 2002 a study by Townson and Nibbs set out to compare mouse ACKR4 to the human homologue. In this study they demonstrated that upon ligand binding the receptor failed to induce a calcium ion flux in HEK 293 cells and so confirmed it as a member of the atypical chemokine receptor family. In this study it was shown that the receptor was strongly expressed in the heart and lungs and that transcript was expressed in most organs. This finding was also confirmed in human tissue (Townson and Nibbs, 2002). Expression of the receptor has also been shown in the small intestine (Gosling et al., 2000).

Like ACKR2 and ACKR3, ACKR4 has a scavenging function for its ligands. Upon binding of ligand the receptor internalises (Graham et al., 2012). This internalisation process has been shown to require the GTPase dynamin to induce endocytosis. The calveolar network is also important in this process. Other known mechanisms of receptor internalisation including rearrangement of β -arrestins and clathrin-coated pit formation are not involved in this process (Comerford et al., 2006). A study in 2013 has suggested that ACKR4 may have the ability to recruit β -arrestins upon activation (Watts et al., 2013).

Recent work by Ulvmar et al uncovered a key function for ACKR4 in the generation and maintenance of CCL21 gradients in lymph node margins. This was shown to be essential for the efficient propagation of adaptive immune responses (Ulvmar et al., 2014). This study showed that lymphatic endothelial cells that lined the roof of the subcapsular sinus of the lymph node expressed

ACKR4 and that these cells were key to this gradient-formation. Another study which relates to the last study showed that mice lacking ACKR4 had fewer CD11c⁺ MHCII⁺ dendritic cells in the draining lymph nodes under non-inflammatory conditions (Heinzel, Benz and Bleul, 2007).

In a separate study, the Bleul group showed that overexpression of ACKR4 on thymic epithelial cells resulted in a reduction in migration of haematopoietic thymic precursors to the embryonic anlage at embryonic day 12.5. They also showed that this did not seem to have an effect on the number of T cells developing in this mouse (Heinzel et al., 2007).

ACKR4 has been shown to play a role in human pulmonary sarcoidosis. This disease results in the formation of granuloma in the lungs of patients and increased CCL19 levels (Kriegova et al., 2006). The authors harvested bronchoalveolar lavage cells (BAL) from both sarcoidosis patients and healthy controls and used real-time PCR to compare levels of ACKR4 transcripts between the two groups. They found that ACKR4 was upregulated in the sarcoid patients and, using chest X-ray, they showed that this correlated with disease stage (Kriegova et al., 2006).

1.4.4 ACKR2

The focus of this thesis will be on the inflammatory CC chemokine receptor ACKR2 which is an atypical chemokine receptor with key, non-redundant, roles in mammalian biology.

ACKR2 was first cloned and characterised in 1997 (Nibbs, Wylie, Pragnell, et al., 1997, Nibbs, Wylie, Yang, et al., 1997) and was shown to limit *in vivo* inflammatory responses via the scavenging activity previously mentioned (Jamieson et al., 2005). ACKR2 binds all of the inflammatory CC chemokines including CCL2, CCL3, CCL3L1, CCL4, CCL5, CCL7, CCL8, CCL11, CCL12, CCL13, CCL14, CCL17, CCL22, CCL23 and CCL24. ACKR2 does not bind any of the homeostatic chemokines nor does it bind ligands from any of the other chemokine subfamilies. The *Ackr2* gene can be found on chromosome 3 in humans and chromosome 9 in mice. These chromosomal locations are major sites

of CC chemokine receptor encoding genes and the homology of the receptor with other chemokine receptors make it clear that ACKR2 has evolved from within this family (Nomiya, Osada and Yoshie, 2013). The molecule was first reported in mice then in man. Human ACKR2 is 71% identical to mouse ACKR2 and both of these receptors express a DKYLEIV motif instead of the conventional DRYLAIV equivalent (Nibbs, Wylie, Pragnell, et al., 1997). It is worth noting that the DKYLEIV motif seen in ACKR2 is conserved across mammals, suggesting that this may be functionally important (Patel, McInnes and Graham, 2009). Upon purification of the human protein it was shown to be 49kDa in size with an N-terminus that is both sulphated and glycosylated (Blackburn et al., 2004).

Like all chemokine receptors, the N-terminal end of the molecule is crucial for ligand binding. An elegant study by Hewit et al showed that the N-terminus of ACKR2 contains a conserved tyrosine motif that is indispensable for ligand binding, internalisation and scavenging. It was also demonstrated in this study that a staphylococcus-derived protease, Staphopain A, has the ability to cleave the N terminus of ACKR2 and significantly reduce its chemokine-binding ability (Hewit et al., 2014).

Interestingly, it has been shown that the majority of ACKR2 protein in cells (>97%) is in intracellular vesicles including early and recycling endosomes (Weber et al 2004, McCulloch et al 2008)(Blackburn et al., 2004). During inflammation this proportion of intracellular receptors changes and more ACKR2 molecules are expressed on the surface of the cell (Weber et al., 2004, Bonecchi et al., 2008).

1.4.4.1 Expression of ACKR2 and Regulation of Expression

Some of the main tissue types that express ACKR2 are mucosal and barrier tissues. In the mouse, ACKR2 is widely expressed and has been shown in the lung, the liver, the skin, the ovary, the heart, the muscle, the kidney and the thymus (Nibbs, Wylie, Yang, *et al.*, 1997).

The cells that express ACKR2 are varied in function and anatomical location. Some of the particular cell-types that have been shown to express ACKR2 are the trophoblasts of the human and mouse placenta (Martinez de la Torre et al.,

2007, Madigan et al., 2010) and lymphatic endothelial cells (Nibbs et al., 2001, McKimmie et al., 2013). Expression has been shown on astrocytes of the brain (Neil et al., 2005), some leukocytes including innate-like B cells, plasmacytoid dendritic cells (pDC) and a subset of neutrophils (McKimmie et al., 2008a). The study by McKimmie et al used flow cytometry and this also suggested expression of ACKR2 on T cells, mast cells and macrophages. Work by Hansell et al suggested that ACKR2 expressed on innate-like B1b cells was biologically active under resting conditions (Hansell et al., 2011). It is also expressed on keratinocytes (Singh et al., 2012).

1.4.4.2 ACKR2 Signalling

Like all of the atypical chemokine receptors, ACKR2 does not mount classical chemokine receptor signalling responses. In the literature it has often been referred to as a 'silent' receptor which implies an absence of signalling activity upon ligand binding. Whilst this is a slight misnomer, what has been shown is that no calcium ion flux is induced up ligand binding and therefore it can be said that ACKR2 is unable to convey classical signalling upon ligand binding. The physiological trafficking of ACKR2 to and from the cell surface is one of the major contributors to its function as a scavenging receptor (Weber et al., 2004). ACKR2 can internalise in a ligand-independent manner and no receptor desensitisation occurs (Weber et al., 2004, McCulloch et al., 2008). Interestingly, it has been shown that the vast majority of ACKR2 molecules are stored in vesicles within the cytosol and cell surface expression tends to be low under normal conditions (Blackburn et al., 2004). This is a phenomenon that is unique to ACKR2 as most classical chemokine receptors become desensitised upon binding and recycling. In fact, even upon ligand binding and recycling no desensitisation occurs and in the presence of high chemokine concentrations more ACKR2 molecules are expressed on the surface of cells (Comerford and Nibbs, 2005, Bonecchi et al., 2008).

Internalisation of ACKR2 is mediated by clathrin-coated pits and is initiated by activation and re-localisation of β -arrestin molecules. The receptor becomes enveloped in Rab-5 positive clathrin-coated pits then Rab-4 and Rab-11 dependent pathways target the molecule for transit in early endosomes then

recycling to the cell surface, respectively (Weber et al., 2004, Bonecchi et al., 2008). The activated receptor becomes associated with clathrin-coated pits which drives the internalisation of the receptor from the membrane. Dynamin-dependent fission then allows for the complete envelopment of the molecule and allows endocytosis to take place. At this stage the chemokine is sequestered from the receptor and is targeted for lysosomal degradation. At this point one of the unique features of ACKR2 comes into effect. The now ligand-free receptor recycles to the cell surface and is not desensitised. One of the key regulatory mechanisms of classical chemokine receptors is that when they are recycled to the cell surface its binding affinity for ligands is reduced. This does not seem to occur in the case of ACKR2 and increases its efficiency as a chemokine scavenger. This is depicted in figure 1.8.

In 2013 a study was published that revealed a novel-signalling pathway that was activated when CC chemokines bind to this receptor (Borroni et al., 2013). This work showed that ligand binding induced the activation of the cofilin pathway and that this was essential for the scavenging function of the receptor. Cofilin is a ubiquitously expressed actin-binding protein that has been shown to reorganise actin filaments.

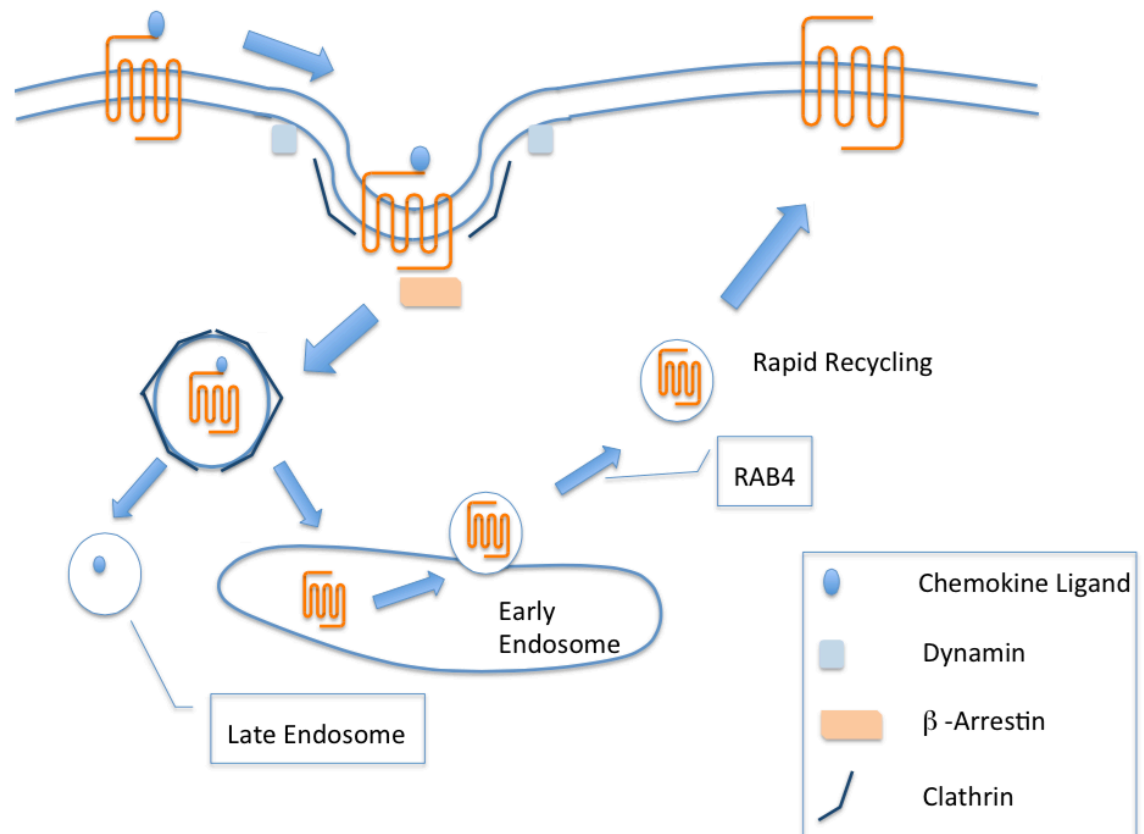


Figure 1.8- The internalisation and recycling of ACKR2 following ligand binding- The diagram above shows the events that take place upon ACKR2 binding a cognate ligand. The receptor is internalised in clathrin-coated pits and recycled to the cell surface via a Rab-4 dependent pathway.

1.4.4.3 In vivo function of ACKR2

In vivo models, using ACKR2 null mice, have demonstrated indispensable physiological and pathological roles for this receptor. In a model of cutaneous inflammation the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) was used to induce inflammation on dorsal skin. In this study ACKR2 null mice showed increased cutaneous inflammation. Further investigation into the phenotype showed an increase in inflammatory chemokines within the skin of the ACKR2 null mice compatible with a lack of scavenging. The histological phenotype closely resembled that of human psoriasis (Singh et al., 2012). Wild-type mice in this study displayed a transient and resolving inflammatory pathology unlike knockout mice, which displayed delayed inflammatory resolution (Jamieson et al., 2005). In a related model where the mutagen 7,12-dimethylbenz(a)anthracene was applied to the skin before TPA painting, ACKR2 null mice developed papillomas more frequently than WT mice (Nibbs et al.,

2007).

ACKR2 expression on lymphatic vessels has been an area of intense study in more recent years. Lymphatic endothelial cells (LEC) are the major cells expressing ACKR2 in tissue (Nibbs et al., 2001) which does not completely fit in with the anti-inflammatory function of ACKR2. In a series of experiments using wild type and ACKR2 null mice, it was shown that this molecule scavenges inflammatory chemokines that are expressed on the LECs. Chemokine ligands can be expressed on endothelial surfaces and presented on glycosaminoglycans (GAGs). During inflammation inflammatory chemokines that are not scavenged could be presented on LEC surfaces via GAGs as these interactions are not as specific as ligand/receptor interactions. Indeed fluorescently labeled chemokines injected into ACKR2-deficient mice accumulated around the lymphatic endothelium and this phenomenon was not seen in WT counterparts. It is thought that a lack of clearance of inflammatory CC chemokines from the LEC surface leads to inflammatory cells accumulating around these vessels and disrupting the efficient flow of APCs and fluid into the vessels. This work suggested, for the first time, a role for ACKR2 in the free movement of fluid and cells into the draining lymphatics and maintaining the efficiency of an adaptive immune response (Lee et al., 2011).

Another recent finding on ACKR2 function related to lymphatic vessel formation and development. Work by Lee et al. suggested a reciprocal relationship between ACKR2 and CCR2 in the regulation of lymphatic vessel network density. In this work it was shown that ACKR2 null mice had a denser lymphatic vessel network and CCR2 null mice had a less dense network when compared to wild-type mice. The proposed mechanism behind this phenotype relates to macrophages and inflammatory chemokines. The hypothesis is that in mice CCL2 concentration in the proximity of the LEC guides CCR2-expressing prolymphangiogenic macrophages to the vessels thus higher CCL2 levels in ACKR2 null mice cause increased branching and a denser network. The opposite phenotype occurs in CCR2KO mice. In this case the lack of CCR2 on the macrophages results in a reduction in the ability of these cells to respond to CCL2 (Lee et al., 2014).

One of the cell types that expresses the most ACKR2 are the syncytiotrophoblasts of the placenta (Borroni et al., 2006, Martinez de la Torre

et al., 2007, Madigan et al., 2010). The functional relevance of this was investigated in WT and ACKR2-null pregnant mice that were treated systemically with an inflammatory stimulus. It was shown in this work that mice lacking the chemokine-scavenging molecule had a higher incidence of miscarriage upon inflammatory stimulation. The data from this study suggested that there was an increased presence of inflammatory CC chemokines in ACKR2 null when compared to WT counterparts. The cellular response to the increased inflammatory signals in the placenta included an increase in migration of macrophages and T cells which was ameliorated using neutralising antibodies to inflammatory chemokines. Interestingly, the levels of inflammatory CC chemokines in the placenta were similar between the two genotypes of mice before inflammation was induced (Martinez de la Torre et al., 2007). Madigan et al took these findings further using placental cell lines in an attempt to elucidate mechanistic details underpinning this physiological process. They suggested that interactions between CCL2 and ACKR2 may be responsible for the phenotype in the de la Torre et al study and also went to show that CCL2 and ACKR2 are associated in the placental tissue (Madigan et al., 2010).

A more recent study took the findings of ACKR2 in the placenta further. This work provided evidence that cultured primary human trophoblasts expressed ACKR2 at significantly higher levels than conventional chemokine receptors. It was also suggested in this work that trophoblast ACKR2 was able to scavenge chemokines. In mice lacking ACKR2 it was shown that there was increased instances of neonatal death, decreased foetal weight and structural defects in the placenta. These phenotypes led to increased neonatal mortality (Teoh et al., 2014).

In a model of murine hepatic inflammation using CCl₄ induced damage it was shown histologically that mice-lacking ACKR2 developed more extensive liver damage than WT mice (Berres et al., 2009). As was also the case in the placental example discussed previously, the livers of the ACKR2 null mice showed increased leukocyte infiltration compared to WT.

1.4.5 ACKR2 in Disease

ACKR2 in Cancer

During the development of tumours the inflammatory process is frequently key to the growth of the neoplasm. As inflammatory cells arrive at the tumour site they provide the tumour with growth factors which help develop the blood supply which is vital to the overall survival of the cancer. Given that inflammation can aid tumour survival, ACKR2 and its ability to scavenge inflammatory chemokines, has been shown to play a role in tumour development. When the skin of ACKR2-null mice were painted with a mutagen (7,12-dimethylbenz(*a*)anthracene (DMBA)) and an inflammatory agent (12-O-tertadecanoylphorbol-13-acetate (TPA)) they developed papillomas more frequently than wild-type mice (Nibbs et al., 2007).

In a clinical study of breast cancer it was shown that levels of ACKR2 expression had a negative correlation with metastasis to the lymph nodes (Wu et al., 2008). This study also showed that the transcript levels of *Ackr2* in breast cancer cell lines negatively correlated with the invasiveness of the cancer when injected into mice.

In colonic adenocarcinomas it was reported that there is a reduction in ACKR2 expression. This study suggested that this was having an effect on inflammatory CC chemokine levels in the local environment and aiding the survival of the tumour (Langenes et al., 2013). To continue with the setting of the gut, ACKR2 has been shown to play a role in the pathology of inflammatory bowel disease (IBD) and IBD-associated colon cancer (Collins et al., 2010). The results of this study suggested that in acute colonic inflammation the pathology could be controlled by ACKR2 expressed on gut lymphatic epithelial cells rather than haematopoietic cells (Vetrano et al., 2010). Another mucosal barrier tissue of high ACKR2 activity is that of the lung.

ACKR2 in Cutaneous Disease

ACKR2 is highly expressed in barrier tissues and has been reported to have key roles in multiple human pathologies. One of example of this is in the

inflammatory skin condition psoriasis. In this pathology it has been reported that ACKR2 is expressed at higher levels in the epidermis of uninvolved, or plaque-free, skin than in lesional skin. There is also an upregulation of ACKR2 in the peripheral blood leukocytes of psoriasis patients (Singh et al., 2012).

In systemic sclerosis (SSc), a chronic autoimmune disease that can cause the thickening of the skin and multiorgan fibrosis due to aberrant collagen deposition, there is involvement of ACKR2. In this condition it was shown that patients have increased transcript and protein levels of ACKR2 in peripheral blood mononuclear cells (PBMCs) (Codullo et al., 2011). In this study the levels of some systemic inflammatory CC-chemokines negatively correlated with the levels of ACKR2, suggesting a possible controlling function for ACKR2 on these ligands (Codullo et al., 2011). Skin is not the only barrier tissue that ACKR2 is involved in the pathology of however.

ACKR2 in Cardiovascular and Pulmonary Disease

ACKR2-null mice have also been shown to be more susceptible to cardiac rupture following myocardial infarction (MI) (Cochain et al., 2012). In human disease it was found that following MI the undamaged tissue was ACKR2-negative whilst area of inflamed infarcted tissue had ACKR2 expression (Cochain et al., 2012).

In advanced chronic obstructive pulmonary disease (COPD) it has been shown that there is an increase in the expression of ACKR2 on local lymphatic vessels (Mori et al., 2013). Another finding in COPD is that there is an increase in ACKR2-expressing alveolar macrophages. In this study, this increase in expression correlated with increased immune activation and functional impairment (Bazzan et al., 2013).

Another ACKR2-related finding involving the lung was after infection with *Mycobacterium tuberculosis*. In this work mice lacking ACKR2 had significantly higher mortality rates than wild type mice (Di Liberto et al., 2008). This study showed a higher influx of inflammatory cells, mainly macrophages, DCs and T cells, to the lung tissue and higher levels of CC chemokines in the serum and bronchoalveolar lavage (BAL) fluid of ACKR2-null mice when compared to wild type animals after infection (Di Liberto et al., 2008). It was of note that the

bacterial load present in each genotype was similar which suggested that the pathology was due to an exaggerated and prolonged inflammatory response. This study suggested that increased concentrations of CC chemokines, due to a lack of ACKR2, resulted in enhanced recruitment of inflammatory cells resulting in increased liver and kidney damage and eventually increased mortality when compared to wild type mice (Di Liberto et al., 2008).

ACKR2 in HIV-Infection

It is not just in barrier tissue pathologies that ACKR2 has a role. It has also been shown that ACKR2 may be a functional co-receptor for primary isolates of dual-tropic human immunodeficiency virus (HIV)-1 and -2 in the brain (Neil et al., 2005). Dual-tropic HIV means that the virus can use the both CCR5 and CXCR4 as co-receptors. HIV-infection results in progression to acquired immune deficiency syndrome (AIDS) and one of the most common symptoms of this progression is the presence of Kaposi's sarcoma (KS) lesions. The viral presence in these tumours causes an increase in the expression of the viral IL-6 homologue which increases the expression of ACKR2 (Nibbs et al., 2001, McKimmie et al., 2013).

ACKR2 in Liver Disease

Another human tissue that has been shown to express high levels of ACKR2 is the liver (Nibbs, Wylie, Pragnell, et al., 1997). It has been shown that single nucleotide polymorphisms (SNPs) of ACKR2 exist and affect the function of the receptor. Patients with these SNPs tended to present increased inflammation after hepatitis C viral infection (Wiederholt et al., 2008).

1.5 Aims

The overall aim of this work was to determine the role that ACKR2 plays in tissue-specific inflammatory responses. Using three anatomically distinct types of mouse tissue this work looks at how a lack of ACKR2 can impact on a variety of inflammatory models. There is also an attempt to take these findings into the human clinical setting in order to provide some translational relevance to the findings presented.

Previous work carried-out in our lab has suggested important roles for ACKR2 in cutaneous inflammation. It has also been shown to have an affect on wound healing in the skin. The first part of the work performed in this thesis attempted to provide insights into how ACKR2 can affect cutaneous wound healing during all stages of the response. Using alternative, well-characterised, models of cutaneous inflammation this part of the research also looks into what other aspects of skin pathology ACKR2 may play a role in.

The middle portion of work in this thesis studies the eye tissue. Certain tissues of the eye have been shown to be highly immunomodulatory and so it seemed a good target for ACKR2 studies. Using a model of experimental autoimmune uveoretinitis (EAU) mice with, and without, ACKR2 were examined for levels of pathology in the ocular tissue.

The final body of work for this thesis was carried out to identify the potential role of ACKR2 in rheumatoid arthritis. Work done previously in our lab has shown an increase in the expression of ACKR2 on peripheral blood leukocytes harvested from patients diagnosed with rheumatoid arthritis. This work set out to look specifically at this finding in more detail.

1.5.1 Linked Research Questions

What role does the absence of ACKR2 play in inflammation brought about by the acute innate immune response?

What role does the absence of ACKR2 play in the pathology of models with involvement of both the innate and adaptive arms of the immune system?

Do the findings of the previous questions translate from work on mice to work on humans?

Chapter 2- Materials and Methods

Chapter 2 Materials and Methods

All work animal models carried out for this thesis was done in accordance with Home Office regulations and the principles of the 3Rs (replacement, reduction and refinement). The work was done under the PPL 60/4092 and the PIL 60/11984.

2.1 Cutaneous Inflammation

2.1.1 Wounding Model

Mice were shaved dorsally using an electric shaver (Wella) twenty-four hours before the wounds were made. They were anaesthetised with isofluorane (Abbott Laboratories) and cleaned using 70% ethanol (Fisher) before being wounded using 5mm biopsy punches (Stiefel, Brentford, UK). Mice were sacrificed at different time points depending on experimental requirements, usually days three, five, seven or twenty-one. Tissue was harvested on these days and stored in 10% NBF (Surgipath) until it was processed. The tissue was prepared for immunohistochemistry and histology according to methods listed in the section entitled 'Tissue Processing, Embedding and Sectioning'.

The sections were stained for MAC-2 (macrophages)(Cedarlane, Burlington, ON, Canada), astra blue (mast-cells)(Sigma-Aldrich), picrosirius red (total collagen)(Sigma-Aldrich), myeloperoxidase (neutrophils) and haematoxylin and eosin (general tissue architecture)(Cell Path, Powys, UK) (as seen in figure 3.3). Example images of MAC-2, MPO and astra blue staining can be seen in appendix IV).

Wounds were also flash frozen in liquid nitrogen (BOC Gases). The mRNA was extracted from this tissue (see 'RNA Extraction') (2.8) and, after making cDNA (see 'cDNA Synthesis') (2.9), subsequently analysed by quantitative polymerase chain reaction (QPCR). For process of selection of the primers used see 'Primer Design' section (2.10.1).

2.1.1.1 Wound Closure Analysis

A digital SLR camera (Fujifilm with Nikon lens, Surrey, UK) was attached to a rig and was set at a constant height of 40cm from the platform. The mice were placed atop the cage and pictures were taken at time-points to analyse wound closure rate. Pictures were uploaded to a PC (Dell, Berkshire, UK) for quantitative analysis of wound closure using Axiovision software (Zeiss, Cambridge, UK).

The wound sections were then stained with H&E (please refer to Haematoxylin and Eosin Staining section for details (2.6.1)) and analysed on the Axiostar light microscope (Zeiss). Pictures of the wound margins were taken at 50x magnification and the gap between wound margins was measured using Axiovision software (Zeiss).

2.1.1.2 Macrophage Depletion Study

Mice were treated with clodronate liposomes (courtesy of van Rooijen) in order to deplete them of macrophages. Control mice were injected with liposomes filled with PBS. A volume of 0.2ml of liposome emulsion was injected intravenously (i.v.) into the tail vein. Mice were treated 48 hours before being wounded then every 48 hours post-wounding to ensure absence of macrophages before and throughout the study. The wounding model described above in 2.1.1 was carried out. Mice were left until day 21 and wounds were harvested and analysed as previously described (see 'Wounding Model' section (2.1.2)).

2.1.1.3 Analysis of Tail Pathology

To the two groups above that were injected with liposomes, both clodronate- and PBS-filled, a group of WT and ACKR2-null mice were added to receive injections of PBS only. The animals were injected at the same time and under the same conditions as the other groups of mice but after injection had their tail girth measured. This was done using a dial micrometer (Mitutoyo, East Kilbride, UK) and measurements were taken at the same point on the base of each mouse's tail. The tail girth was measured for mice in all four groups and the data collected was graphed and analysed using Graphpad (Prism, La Jolla, CA, USA) software.

2.1.1.4 Mast-Cell Degranulation Model

The highly active mast-cell degranulator, compound 48/80 (Sigma Aldrich), was used for this work. 20 μ l of the reagent was injected intradermally into each mouse using a small volume syringe (Hamilton Needles, Bonaduz, Switzerland) and left for 48 hours.

C57BL/6J mice were injected with a concentration of 10mg/ml. Drug was administered with PBS as a diluent. Control animals for the experiment were injected with diluent only. After four days a 5mm biopsy punch (Stiefel) was taken of the injection site and surrounding area.

The mice were sacrificed and the skin was stored in 10% NBF (Surgipath) until processing. Once processed, sectioned and on microscope slides, the tissue was stained with H&E and astra blue to assess the reaction to the reagent. For more details on the staining protocols used please refer to the 'Histological Staining' section below (2.6).

Stained sections taken from the mice were blinded and photographed for further analysis. Oedema was also quantified using the Axiovision software program (Zeiss) to measure the thickness of the tissue sections. Six randomly selected view fields were taken for each sample and the thickest part of tissue in the field of view was measured. These numbers were then averaged to give a single value for each mouse involved in the study.

2.1.2 Allergic Contact Dermatitis Model

Mice on the FVB background were used in this model. Before priming the abdomen of the mice were shaved. Priming involved the hapten 2,4-dinitrofluorobenzene (DNFB or Sanger's reagent)(Sigma Aldrich) in a vehicle (1:4 mix of acetone (Fisher Scientific) and olive oil (Bertolli, London, UK). This DNFB and vehicle mix was painted onto the exposed abdominal skin on day 1.

Six days later, the challenge stage, the same DNFB/vehicle mix was painted onto the right ear of the mice. The left ear of the mice was painted with vehicle only as an internal control. The ears of the mice were measured using digital callipers

(Mitutoyo) every 24 hours. This was carried out up until 144 hours after the challenge.

2.2 Ocular Inflammation

2.2.1 Experimental Autoimmune Uveoretinitis (EAU) Model

C57Bl/6J mice were injected subcutaneously with 50µg complete Freund's adjuvant (CFA) (Difco from BD) and 600µg of interphotoreceptor retinoid binding protein (IRBP) 1-20 (New England Peptides, Gardner, MA, USA) in the dorsal skin (the mix of the CFA and IRBP 1-20 was sonicated until it was a thick paste-like consistency). This dose was split and injected into the rump at each side of the animal in two instalments. The mice were then injected intraperitoneally with 500ng of pertussis toxin (Alexis Biochemicals from Enzo Life Sciences, Exeter, UK). Control mice were injected with a sonicated mix of PBS, CFA, and pertussis toxin.

After twenty-eight days the mice were euthanised with CO₂ and harvested eyes were frozen in optimal tissue cutting (OCT) (Sakura Finetek, Alphen aan den Rijn, Netherlands) media on dry ice. Eyes were sectioned at 8µm using a cryostat (Thermo Scientific) and three samples from each eye were taken in order to investigate pathology throughout the eye. Tissue was placed on polylysine slides (VWR International, Lutterworth, UK) and left to air dry. Once all of the eyes were cut the book housing slides (Raymond Lamb from Thermo Scientific) was wrapped in tin foil (Caterwrap, Shropshire, UK) and put into a -80°C freezer (Thermo Scientific) for storage. Ice-cold acetone (Fisher Scientific) was used for fixing the tissue. For the following steps taken in this protocol please refer to 'Haematoxylin and Eosin Staining' (2.6.1).

We repeated this model using our collaborators from the University of Aberdeen's protocol. For this model 500µg of IRBP was combined with 50µg of CFA and also had an extra 25mg of *Mycobacterium tuberculosis* supplementing the CFA. This mixture was hand emulsified using linked syringes (Hamilton) rather than sonication. The mix was injected subcutaneously, as before, in two instalments into the rump of the mouse. An intraperitoneal injection of pertussis toxin (1µg) was also given immediately after the interphotoreceptor retinoid

binding protein (IRBP) injection. As before, control mice were injected with PBS in place of IRBP.

For one study in section 4.2.1.4 the B10.BR/III mouse strain was used as opposed to C57BL/6 as per all other studies in this chapter. This particular study was carried out by our collaborators at The University of Aberdeen and we were sent the laser-captured tissue for analysis. This model was also carried out over 70 days rather than 28 as per other studies in this section.

2.2.2 Histopathological Grading

Once the eyes were stained they were analysed using a customised histopathological grading system shown below:

Customised histopathological grading of EAU

Cellular infiltration			Structural/morphological changes		
Anterior segment			Posterior segment		
Iris	Infiltrating cells	1	Rod outer segments	Cell infiltrate	1
	Thickening of iris	2		Partial loss	2
Anterior chamber	Cells ≤ 20	1		Moderate loss	3
	Cells >20	2		Subtotal loss	4
Cornea	Infiltrating cell/ thickening	1		Total loss	5
Posterior segment			Neuronal layers		
Ciliary body	Cell infiltrate <5 cells	1		Cell infiltrate	1
	Mild thickening	2		Partial loss	2
	Moderate thickening	3		Moderate loss	3
	Gross thickening	4		Subtotal loss	4
Vitreous	Cells <5	1	Retinal morphology	Total loss	5
	Cells 5--25	2		Folds	1
	Cells 25--50	3		Focal detachment	2
	Cells 50--100	4		Subtotal detachment	3
	Cells >100	5		Total detachment	4
Vasculitis (mural or extravascular cells)	<10% vessels involved	1	SRNVM (SRNVM: subretinal neovascular membrane)	1--3	1
		2		>3	2
		3	TOTAL		
		4	Infiltrative	35	
		5	Structural	16	
	Cells in or around wall	1	Grading: Infiltrative		
		2	1	1--4	
		3	2	5--9	
		4	3	10--14	
		5	4	15--19	
Rod outer segments	<10% involved	1	5	20--24	
		2	6	25--29	
		3	7	>=30	
		4	Grading: Structural		
		5	1	1--3	
Choroid	Cell infiltrate	1	2	4--6	
	Mild thickening	2	3	7--9	
	Moderate thickening	3	4	10--12	
	Gross thickening	4	5	13--16	
	Granulomas 1	1			
	Granulomas 2--5	2			
	Granulomas >5	3			

Table 2.1-Customised Histopathological Grading System for EAU- The table above details the factors taken into consideration for quantifying the pathology presented by the animals in the EAU model (Xu et al. 2008).

2.2.3 EAU QPCR

Mice were culled using carbon dioxide (BOC Gases) and eyes were removed from 5 C57/BL6J mice using forceps (Fisher Scientific). The eye structure was to be

analysed for ACKR2 transcript levels. RLT buffer (Life Technologies) was added to the eyes and the TissueRuptor (Qiagen, Crawley, UK) was used to homogenise the sample. The homogenate was spun at 17,000g (Fisher Scientific) for 3 minutes and the supernatant was harvested. The process of RNA extraction is listed below in the 'RNA Extraction' section, for more protocol details please refer to this.

To a tube of PCR master mix (Rovalab, Teltow, Germany) 3 μ l of template (cDNA from the remaining eye structure sample) with was added along with 0.5 μ l of both forward and reverse primers (IDT DNA, Interleuvenlaan, Belgium). Once this assay had been completed a 50ml 2% agarose gel (Roche, West Sussex, UK) with 5 μ l ethidium bromide (Sigma Aldrich). The gel was run at 100V (BioRad, Hemel Hempstead, UK) for 45 minutes. A molecular weight ladder (Hyper Ladder IV (Bioline, London, UK)) was included on the gel.

2.2.4 Western Blot of ARPE-19 Cell Line for ACKR2 Expression

ARPE-19 cells were lysed for western blot analysis (for details on the cell line please refer to section 2.11.2). Cells were lifted from the flask (Corning) with trypsin-EDTA (Life Technologies) and resuspended. After being resuspended in fresh medium (Life Technologies/Sigma) the cells were centrifuged (Thermo Scientific) and the cell pellet was resuspended in sterile PBS (Life Technologies) and re-centrifuged. The cell pellet was then resuspended in Mammalian Protein Extraction Reagent (MPER) Lysis Buffer (Pierce from Thermo Scientific) to lyse the cells. Originally 400 μ L of MPER was used to prepare the cell lysate, however after carrying out the western blot this created a lysate that was too dilute. As a result, 200 μ L of the lysis buffer was used to create the second lysate. The lysates were stored at -80°C (Thermo Scientific).

2.5 μ L of sample buffer (NuPAGE LDS Sample Buffer (4x)(Life Technologies)) was added to 7.5 μ L of cell lysates. This was carried-out in accordance with Blackburn et al (Weber et al., 2004) This was incubated at room temperature for around 10 minutes, after this, 2 μ L of NuPAGE sample reducing agent (10x)(Life Technologies) was added to the lysates. Along with the cell lysate of ARPE-19 cells, Human Embryonic Kidney (HEK)-293 cell lysates were used as a negative

control and ACKR2-transfected HEK-293 cells as a positive control (all cell lines from ATCC, Manassas, VA, USA). The lysates were added to a NuPAGE 12 well 4-12% Bis-Tris gel (Life Technologies) electrophoresed at 100V in NuPAGE MES SDS running buffer (20x) (Life Technologies) for an hour and a half. A pre-stained protein ladder was used to estimate the size of the proteins on the gel (Life Technologies).

Electrophoresed proteins were transferred onto a nitrocellulose membrane (Life Technologies) using the iBlot system (Life Technologies). The transfer took 7 minutes. After transfer the membrane was washed in phosphate buffered saline with tween 20 (PBST) (Life Technologies/Sigma) (0.05%) 3 times for 5 minutes per wash. Once washed the membrane was blocked using 10% milk (Marvel, Dublin, Ireland) in PBST for 1 hour at room temperature.

After blocking, the primary antibody was added. The mouse anti-human ACKR2 antibody (made in-house) was applied at a dilution of 1:5 in the PBST/milk solution. The primary antibody was incubated on the membrane overnight at 4°C on a shaker. After this incubation the antibody solution was removed and the membrane washed 3 times in PBST for 5 minutes each.

The secondary antibody (HRP-conjugated sheep anti-mouse IgG) (GE Healthcare) was then added at a dilution of 1:10,000 in 5% milk PBST and incubated at room temperature for 1 hour. After this the membrane was washed in PBST 4 times for 15 minutes. The development reagent, WestFemto (Pierce) was prepared by adding equal amounts of solutions 1 and 2 contained in the kit for a total volume of 1.5mL.

X-ray film (Kodak, Geneva, Switzerland) was placed over the membrane and exposure allowed to proceed for various times. They were then developed using an XOMat developing machine (Konica-Minolta, Banbury, UK).

2.3 ACKR2 in Rheumatoid Arthritis

2.3.1 *In vivo Study Sample Collection*

2.3.1.1 Patient Criteria

After receiving ethical approval (see Appendix II) from the relevant bodies, this experiment was undertaken. Patients were selected by checking medical notes for a positive diagnosis. They were then consented before seeing the clinician. This involved a brief interview in which the project was explained and the necessary paperwork completed. Three groups of patients were used in this study along with healthy controls:

Clinic 1- Escalating RA: Patients whose current therapy regimen is no longer effective with disease diagnosed over 12 months before sample collection

Clinic 2- Established RA: Well-managed disease diagnosed over 12 months before sample collection

Clinic 3- Early RA: Diagnosed within 12 months of sample collection

Patients were ineligible for the study if they were undergoing any biological based therapies.

2.3.1.2 Blood Collection

18ml of blood (2 x 9 ml) was taken from participants and stored in EDTA Vacuettes (Grenier Bio-One, Stonehouse, UK).

2.3.1.3 Patient Data

From the clinicians we obtained the following details about the patient:

Initials

Age

Disease Activity Score (DAS)

Erythrocyte Sedimentation Rate (ESR)

C-Reactive Protein (CRP)

Rheumatoid Factor (RF)

2.3.1.4 Plasma and Cell Collection

Blood was pooled (approximately 18ml) in a 50ml centrifuge tube (BD) and centrifuged at 600g for 5 minutes (Thermo Scientific). Supernatant was aliquoted into 1.5ml tubes and spun at 17,000 g for 5 minutes. Supernatant was aliquoted into 1.5ml tubes and stored at -80°C. Cell pellet was resuspended in PBS (Life Technologies) with ethylenediaminetetraacetic acid (EDTA) (Life Technologies) and foetal calf serum (FCS) (PAA from GE Healthcare) and added to remaining blood.

Blood sample minus plasma was diluted 1:1 with PBS-EDTA-FCS (PEF) and layered carefully onto 5ml of Ficoll (GE Healthcare). It was then centrifuged at 600 g for 25 minutes.

The peripheral blood mononuclear cell (PBMC) layer was removed and placed in an RNase-free 1.5ml tube (StarLab) and centrifuged for 10 minutes at 200g. Supernatant was removed and the pellet was resuspended in PEF. This suspension was spun again at 200g for 5 minutes.

PBMCs were counted using trypan blue (Sigma Aldrich) and a haemocytometer (VWR International).

2.3.2 Patient Sample Analysis

2.3.2.1 QPCR

Cells were lysed in RLT lysis buffer (Qiagen) and placed a Qiashtredder (Qiagen) and centrifuged at 13000 x g for 2 minutes. After this, steps were taken in accordance with sections 2.8 'RNA Extraction', 2.9 'cDNA Synthesis' and 2.10 'QPCR Assays'. See these sections for full details.

2.3.2.2 Multiplex ELISA

A 30-plex ELISA (Life Technologies) was used with the plasma samples collected from patient peripheral blood samples. The targets of this assay are listed below:

Analyte	Family	Function
IL-1Ra	Cytokine Receptor	Pro-inflammatory
IL-1 β	Cytokine	Pro-inflammatory
IL-2	Cytokine	Pro-inflammatory
IL-2R	Cytokine Receptor	Pro-inflammatory
IL-4	Cytokine	Anti-inflammatory
IL-5	Cytokine	Anti-inflammatory
IL-6	Cytokine	Pro-inflammatory
IL-7	Cytokine	Pro-inflammatory
IL-10	Cytokine	Anti-inflammatory
IL-12	Cytokine	Pro-inflammatory
IL-13	Cytokine	Anti-inflammatory
IL-15	Cytokine	Pro-inflammatory
IL-17	Cytokine	Pro-inflammatory
IFN- α	Cytokine	Pro-inflammatory
IFN- γ	Cytokine	Pro-inflammatory
TNF- α	Cytokine	Pro-inflammatory
G-CSF	Cytokine	Pro-inflammatory
GM-CSF	Cytokine	Pro-inflammatory
CCL2	Chemokine	Pro-inflammatory
CCL3	Chemokine	Pro-inflammatory
CCL4	Chemokine	Pro-inflammatory
CCL5	Chemokine	Pro-inflammatory
CCL11	Chemokine	Pro-inflammatory
CXCL1	Chemokine	Pro-inflammatory
CXCL8	Chemokine	Pro-inflammatory

CXCL9	Chemokine	Pro-inflammatory
CXCL10	Chemokine	Pro-inflammatory
VEGF	Chemokine	Pro-inflammatory
FGF-basic	Growth Factor	Angiogenic
	Growth Factor	Angiogenic
EGF	Growth Factor	Angiogenic
HGF	Growth Factor	Angiogenic

Table 2.2- Analytes on 30-plex plate- The table above lists the molecules analysed using the multiplex ELISA kit for the patient plasma samples.

The assay was carried out in accordance with product literature. However as a quick overview, the wells were pre-wet with wash buffer and antibody-coated beads added. Samples and standards were added with incubation buffer and assay diluent. The plate was incubated and shaken for two hours then washed twice. Secondary/detector antibodies were then added to the wells and the same incubation, shaking and washing step was repeated. A streptavidin/phycoerythrin complex was added to the wells and the plate was incubated and shaken for 30 minutes. After three washes the plate was read using the xMap™ System (Luminex Corp).

2.4 Rheumatoid Arthritis and ACKR2- *In vitro* Studies

2.4.1 ACKR2 vs. Hypoxia QPCR

2×10^6 cells were seeded onto two 60mm dishes. These were then either placed in a hypoxia chamber filled with carbon dioxide or placed in a normal incubator. They were each incubated for 24 hours in their respective conditions. Once this was done the medium was removed from the plate, the plate was washed with PBS and cells lysed using RLT Buffer (Qiagen). Lysate was removed from the plate and taken for transcriptional analysis. QPCR was carried out in accordance with the previous section entitled 'QPCR Assays' (2.10).

2.4.2 Disease Modifying Anti-Rheumatic Drugs (DMARDs) vs. ACKR2

Peripheral blood mononuclear cells (PBMCs) were obtained from a buffy coat. The blood was separated using a Ficoll-Paque (GE Healthcare) gradient and PBMCs harvested. The monocyte fraction of the cells was isolated using CD14⁺ cell sorting.

From the cells obtained, half were used to generate dendritic cells (DC) and for macrophages. Cells were incubated in complete media with added growth factors in order to drive lineage commitment. DC and macrophage medium was made up of RPMI buffer was supplemented with 5% human AB serum (Life Technologies) and gentamicin (Sigma).

Both populations of cells had granulocyte macrophage colony-stimulating factor (GM-CSF) added to them (macrophages received 125 ng/ml and DC were treated with 50 ng/ml). Differentiated DC also required interleukin (IL)-4 (15 ng/ml).

The cells were incubated for ten days then were placed into two 12-well plates (one for macrophages and one for DCs) and given more growth medium (containing the appropriate growth factors). At this point the cells were treated with disease modifying anti-rheumatic drugs (DMARDs) methotrexate (20nM) (Nesher, Moore and Dorner, 1991, Thomas et al. 2011), dexamethasone (1 μ M) (Verhoef et al. 1999), or acitretin (100nM) (Papadimou et al. 1998). Doses were taken from publications.

Cells were incubated for six or twenty-four hours at 37°C then lysed with RLT buffer (Qiagen). Samples were then taken through the steps mentioned in the section entitled 'RNA Extraction' (2.8). Finally, a QPCR assay was carried out.

2.5 Tissue Processing, Embedding and Sectioning

2.5.1 Tissue Storage

Once harvested the skin was spread out dorsal side down on filter paper to help keep its shape and stored until it could be processed. This was done by placing

the sample in 10% neutral buffered formalin (NBF) (Surgipath) immediately after harvest.

2.5.2 Tissue Processing

Fixed tissue was placed in a tissue cassette (Simport from CamLab, Cambridge, UK) for treatment in a tissue processor (Thermo Scientific) using the following programme:

Station	Solution	Time
1	10% NBF	30 mins
2	70% Alcohol	1 hour
3	90% Alcohol	1 hour
4	95% Alcohol	1 hour
5	100% Alcohol	1 hour
6	100% Alcohol	2 hour
7	100% Alcohol	2.5 hours
8	Xylene	1 hour
9	Xylene	1 hour
10	Xylene	1.5 hours
11	Wax	4 hours
12	Wax	5 Hours

Table 2.3- Programme for Tissue Processing- The table above gives details of the steps taken in the tissue processing machine to prepare samples for histology.

2.5.3 Embedding Tissue

Once processed the tissue was embedded (an embedding suite (Thermo Scientific) was used). This involved orienting the sample in a tissue mould (Surgipath) in the way it was to be cut then surrounding it in liquid wax (VWR International).

2.5.4 Sectioning Tissue

Blocks of wax containing samples (Simport) were left on ice until cooled. Blocks were kept on ice during the process. Sections were cut, using a C35 blade (Feather from Cell Path), at 4-6µm on a microtome (Thermo Scientific) and floated in a water bath (Thermo Scientific). The water bath was kept at 40°C and sections were collected onto polylysine coated microscope slides (VWR International). Slides were left on a heating block (Thermo Scientific) at 60°C overnight.

2.6 Histological Staining

2.6.1 Haematoxylin and Eosin

This histological technique was used for both formalin fixed paraffin embedded (FFPE) and frozen tissue sections. As a result two slightly different protocols were required.

FFPE sections were dewaxed with xylene (VWR International) and rehydrated in 100%, then 70% ethanol (VWR International), then water. Frozen sections, on the other hand, were removed from storage at -80°C (Thermo Scientific) and brought to room temperature before being fixed in ice-cold acetone for five minutes (Fisher Scientific) then air-dried for twenty minutes.

After these stages the protocols became the same for both. Slides were stained with haematoxylin 'Z' stain (Cell Path) (seven minutes) and put in running water to remove excess stain. After this, 1% acid alcohol (Fisher Scientific) and Scott's tap water substitute (Cell Path) were used (two minutes incubation in each) for differentiation and were separated by rinsing in running water. 1% Eosin (Cell Path) in water was then used (four minute incubation) as the counter stain and slides were again rinsed to remove excess stain. Slides were dehydrated in 70%, 90% then 100% ethanol (Fisher Scientific), further cleared in xylene (VWR International) and finally mounted using xylene containing DPX mountant (VWR International)).

2.6.2 Picrosirius Red

Picrosirius red is a mix of Sirius red (Sigma) and saturated picric acid (Sigma) and stains collagen fibrils. Slides were dewaxed in xylene (VWR International) and rehydrated in 100%, 90% and 70% ethanol (Fisher Scientific) then water. After this slides were placed in picrosirius red solution (Sigma Aldrich) and left until the stain saturated the tissues (not less than one hour). Acidified water (5ml glacial acetic (Fisher Scientific) acid in 995ml water) was then used to prevent loss of stain intensity over time (ten minute incubation). Slides were dehydrated in three changes of 100% ethanol (Fisher Scientific) and cleared in xylene (VWR International) before being mounted with DPX (VWR International).

2.6.3 Astra Blue

Tissue was cleared in xylene (VWR International) and rehydrated in 100% and 95% ethanol (Fisher Scientific) and water before the astra blue stain (Sigma Aldrich) was added to the tissue. After removing the excess stain with water, 1% aqueous safranin (Sigma Aldrich) was added as a counter stain. This counterstain was removed with tap water and tissues were dehydrated in 70% and 100% ethanol (Fisher Scientific) before being cleared in three changes of xylene (VWR International) and mounted in DPX (VWR International).

2.6.4 MAC-2

For this immunocytochemical stain slides were cleared in xylene (VWR International), and rehydrated in 100% and 70% ethanol (Fisher Scientific) and running water. After this they were placed in distilled water (Triple Red, Bucks, UK) then rinsed with PBS (made in-house). 3% H₂O₂ (Fisher Scientific) was used as a peroxidase block and was rinsed off with PBS (Life Technologies). A 20% goat serum (Vector Labs)/PBS (in-house) solution was applied as block then the primary rat anti-mouse mac-2 antibody (1/6000 dilution) (Cedarlane) was applied in a 1% BSA (Sigma)/PBS (Life Technologies) blocking solution. The isotype control antibody was IgG2a (BD). This was added to slides and incubated overnight at 4°C prior to be removed with PBS washes (Life Technologies).

The secondary antibody used was a goat anti-rat IgG (Vector Labs, Peterborough, UK) that was suspended in blocking solution as mentioned above. This was removed with PBS as before and replaced with a tertiary antibody solution. Extravidin-peroxidase (Sigma) was then applied, diluted in blocking solution (Dako, Ely, UK).

A diaminobenzidine (DAB) substrate (Vector Labs) was left on the samples in the dark until sufficient time had passed to give appropriate levels of staining intensity. Sections were checked periodically for intensity using a light microscope (Zeiss). Slides were then soaked in tap water in order to quench the reaction. This was the substrate used for all MAC-2 staining except in the case of figure 4.6 in section 4.2.1.2. where red alkaline phosphatase (Vector Labs) had to be used instead. This was carried out as above for DAB but gave a red stain for positive staining instead of brown.

Haematoxylin was used as a counterstain and after this step slides were dehydrated in 70%, 90% and 100% ethanol (Fisher Scientific) and cleared in xylene (VWR International) before being mounted in DPX (VWR International) and covered (Menzel-Glasser, Braunschweig, Germany).

2.7 Microscopy

An AxioStar or AxioImager (both Zeiss) microscope with Axiovision or ZEN (both Zeiss) software, respectively, was used for this. During all microscopy work, where possible, images and analysis were carried out blind. Six random fields of view at 400x magnification were taken and cell types were counted within these fields. These counts were used to gain a percentage value for each cell type over total leukocytes.

For the wounding model the view fields were taken around the wound margin or the local cells just out-with the margin. Tissue sections were taken at different time-points throughout the healing process.

In the compound 48/80 model it was important to ascertain any differences in tissue integrity as well as cell numbers. Cell number figures were obtained using

methods described above and the area of interest, in this model, were the layers of skin just below the injection site. Measuring the area of broken epidermis and the extent of this breach of the top skin layer also assessed tissue integrity. Oedema in the tissue was also measured and this was carried out using Axiovision software (Zeiss) again. As described previously multiple measurements were made and a mean value was calculated from these figures.

The Experimental Autoimmune Uveoretinitis (EAU) model analysis had to adopt a very different approach, as required. The whole eye was assessed for inflammation and pathology. A customised grading guide was used during analysis of the eye tissue as mentioned earlier (section 1.2.2). The pathology of this condition has many varying outcomes/symptoms and so using this system allowed a fairer and qualitative way to analyse different animals in the experiment.

2.8 RNA Extraction

Samples that were to be analysed by QPCR were stored in RNA Later (Qiagen) at 4°C. They were then flash frozen in liquid nitrogen (BOC Gases) and crushed down to a fine powder using an RNase free pestle and mortar (Sigma Aldrich). The pestle and mortar was cleaned between samples and before use with 70% ethanol and RNase Zap (Ambion). The fine powder was placed into 1.5ml RNase free microcentrifuge tubes (Ambion) and RLT buffer (Qiagen) was added. The suspension was then passed through a blunt ended 20-gauge needle (BD).

The newly lysed tissue/RLT buffer suspension was filtered through an RNeasy mini-kit (Qiagen) column. An optional DNase (Qiagen) digest was carried out. The adherent RNA was cleaned using the wash buffers provided and eluted using an elution buffer also in the kit. For more details please refer to commercial product guidelines.

2.9 cDNA Synthesis

Using a commercial kit (Stratagene, Cambridge, UK) complimentary (c)DNA was made from the RNA extracted using the columns. 3µg of the total RNA (template) was added to 1µl of primers and RNase-free water was used to make

up the reaction volume of 15.7 μ l. This reaction mix was heated to 65°C to denature the poly(A) tail and cooled back to room temperature to allow the primers to anneal to the template.

This mix includes 0.8 μ l/sample of dNTP mix, 1 μ l/sample of reverse transcriptase, 2 μ l/sample of reverse transcriptase buffer, and 0.5 μ l/sample of an RNase block that, in conjunction with controlled temperature changes for DNA denaturing, annealing, and elongation of sequences, produces cDNA. For more details please read commercial product literature.

The reaction mix was placed into a thermal cycler (Applied Biosystems, Paisley, UK) at 25°C for 10 minutes then 55°C for 60 minutes. It was then heated to 72°C for 15 minutes to elongate the newly synthesised strands of DNA. Once the programme was complete the samples were cooled to 4°C to preserve the PCR products.

2.10 QPCR Assays

2.10.1 Primer Design

Sequences of genes of interest were obtained using Ensembl software (www.ensembl.org). In order to design and select optimal primer pairs for the QPCR assays 'Primer 3' computer software (<http://frodo.wi.mit.edu/>) was used.

For these assays two sets of primers were designed and used. The outer primers produce a large product to provide a target for the inner primers essential for the generation of a standard curve. The smaller inner primers are designed from sequence of the product produced by outer primers. The specifications for optimal inner primer design are given below.

Specification	Optimal Value	Acceptable Range
GC content	50%	40-65%
Sequence length	N/A	18-23 base pairs
Melting Temperature (T _m)	60°C	59.5-61°C
Maximum Self Complementarity	N/A	2
Maximum 3'Self Complementarity	N/A	1
Amplified Product Size (Inner)	150 base pairs	<150 base pairs
Amplified Product Size (Outer)	>300 base pairs	>300 base pairs

Table 2.4 Criteria for QPCR Primer Design. The table above shows the attributes used to design primers for the QPCR assays throughout the work in this thesis.

Other key criteria: sequences cannot contain more than three consecutive G or C bases, and two or more G or C bases must be avoided within the final five bases at the 3' end ('GC clamp').

Although the above criteria are important for optimal primer design some attributes can be altered if primers cannot be found. As an example, GC content can be relaxed to 35-70% and melting temperature range can be slightly extended to take in 58-62°C. Making these changes allow more difficult primers to be designed. Once primers had been selected using the software they were verified using the Basic Local Alignment Search Tool (BLAST) from NIH PubMed (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

2.10.2 Generation of a Standard Curve

To generate a standard curve we used cDNA from a positive control sample. 2µl of this cell lysate was added to a pre-made PCR master mix (Rovalab) along with 1µl of forward/reverse outer primer mix (IDT DNA). The PCR reaction was carried out using the following programme:

Step	Temperature (°C)	Time
1	95	3 minutes
2	95	15 seconds
3	60	20 seconds
4	72	40 seconds
Repeat 2-4 for 40 cycles		
5	72	7 minutes
6	4	Indefinitely

Table 2.5 End-point PCR Run Method. This table shows the timing and steps used during standard end-point PCR reactions.

The products of the reaction were separated out on a 2% agarose gel. Gel was made by adding 2g of agarose (Roche) to 100ml of tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer to which 7 μ l of ethidium bromide (Sigma) was added. A DNA ladder (type IV hyperladder (Bioline)) was added to the gel to allow estimation of product band size. The gel was run with 100V (BioRad) through it for around one hour.

The gel was observed under ultraviolet light and the band representing the specific PCR product was cut out with a scalpel (Swann-Morton, Sheffield, UK). This gel portion was then stored in a nuclease-free microcentrifuge tube (Ambion) and extraction of the product from the gel was carried out using a commercial kit (Qiagen). For full protocol details please refer to the product manual. In brief, however, the gel was dissolved in 'buffer QG' (provided in the kit) at 50°C for 10 minutes. Isopropanol (Fisher Scientific) was added at this stage then this mix was added to a QIAquick® (Qiagen) column provided in the kit which binds the DNA. This was spun (Thermo Scientific) and the flow-through was discarded. After a cleaning process to enhance the quality of the final product, the DNA was obtained using elution buffer supplied in the kit (Qiagen).

2.10.3 TOPO Cloning

Again, this method was carried out using a commercially available kit and full details can be obtained from manufacturer's literature. To briefly outline the

protocol, 1µl of TOPO® vector (Life Technologies) was added to 1µl of salt solution, a 3 times Molar excess of insert, plus nuclease-free water (Ambion) to make a total volume of 20µl.

This reaction mix was left for 5 minutes at room temperature then 3µl of the mix was added to the chemically competent TOP10F' One Shot® *Escherichia coli* (Life Technologies) and left on ice for 30 minutes. The bacterial mixture was then incubated at 42°C for 30 seconds, to heat-shock the bacteria, then placed on ice for 5 minutes. 200µl of super-optimal broth with catabolite repression (S.O.C.) media (Life Technologies) was added to the mix and incubated for one hour in a shaking incubator at 37.5°C (New Brunswick Scientific, Enfield, CT, USA). At the same time Luria broth (LB) agar plates (made in-house) were placed in an incubator cabinet to pre-warm.

After the incubation, the bacterial mixture in the S.O.C. media (Life Technologies) was spread onto the pre-warmed LB agar ampicillin (Sigma Aldrich) (50µg/ml) plates at 37°C overnight. The next day a suitable colony was selected with a pipette tip (Starlab, Milton Keynes, UK) and added to 5ml of LB plus ampicillin (Sigma Aldrich) (50µg/ml) and incubated overnight at 37°C to allow bacteria to proliferate.

2.10.3.1 Purification of DNA from the Bacterial Vector

The following protocol was carried out using reagents from a commercial kit (Life Technologies) and full details can be obtained from product literature. Following on from the section 2.10.3, the *E. coli* culture was centrifuged and pelleted bacteria were resuspended in buffer plus lysis buffer added.

The next buffer that was added precipitates the genomic DNA but leaves the plasmid DNA in suspension, as a result when the suspension and precipitate is centrifuged the supernatant was carried forward and the pellet was discarded. This supernatant is added to a spin column to purify the plasmid DNA. It was spun at 13000 x g and the flow-through was discarded. The column was cleaned, using buffers provided, then spun again to remove any residual wash buffer. The plasmid DNA was harvested using elution buffer and the final product was then ready to be used in QPCR assays.

The above protocols results in a solution of plasmid DNA that, after a set of serial dilutions, gives a series of standards that can be quantified. A 1:100 dilution, 5 μ l of the pure solution was added to 495 μ l of elution buffer (Qiagen), gives a 10^{-2} solution. This process is repeated giving 10^{-4} and then 1:10 dilutions- 50 μ l into 450 μ l were repeated until a concentration as low as 10^{-9} is made. To quantify the absolute copy number in each of the dilutions the optical density (O.D.) was be measured using a nanodrop (Thermo Scientific).

2.10.4 Standard Verification

2.10.4.1 Verification by PCR

Once standards had been produced and cloned the inner primer pair had to be tested. This was done by regular polymerase chain reaction (PCR). The standard, at a concentration of 10^{-5} , was used routinely as a template for this test. The standards were added as template (3 μ l) and the inner primer mix (0.5 μ l of each) was added to a pre-made master-mix (Rovalab). The reaction was carried out using the following programme:

Step	Temperature (°C)	Time
1	95	3 minutes
2	95	15 seconds
3	60	20 seconds
4	72	40 seconds
Repeat 2-4 for 40 cycles		
5	72	7 minutes
6	4	Indefinitely

Table 2.6- End-point PCR Run for Standard Verification. The table above shows the details for the end-point PCR run that was used to confirm the standards designed for the QPCR were appropriate for the final assay.

A 2% agarose (Roche) gel was made up (2g of agarose to 100ml of TAE buffer) with 7 μ l of ethidium bromide (Sigma Aldrich). Once the PCR was complete 20 μ l

of the reaction product was added each well of the gel. A DNA ladder (type IV hyperladder (BioLine)) was also added to determine the size of the PCR product.

The gel was run at 100V (BioRad) for 30-40 minutes. Once the gel had run for a sufficient length of time it was imaged under UV light (Alphamager, East Sussex, UK).

2.10.5 QPCR Assay Proper

Following the verification of the standards and inner primers the formal QPCR assay can go ahead (section 2.10.4). The cDNA preparations were generated as described earlier in the section entitled 'cDNA Synthesis' (2.9). Master mix was made up as described earlier and are listed below:

Ingredient			Volume (μ l/well)
SYBR® Green Fast Mix (VWR)			10
Nuclease	Free	Water	8.2
(Ambion)			
Primer Mix (IDT)			0.4

Table 2.7- Ingredients of QPCR Master Mix- The table above lists the reagents and volumes used to make a master mix for the QPCR assay. The values given are per well as stated.

An example of the quantities and working for the make-up of this master mix are listed below:

26 templates in triplicate= 78 wells

78 x 15% = 89.7

10 μ l/well of SYBR® Green Fast Mix (VWR) x 89.7 = 897 μ l

8.2 μ l/well of Nuclease Free Water x 89.7= 735.54 μ l

0.4 μ l/well of primer mix x 89.7= 35.88 μ l

Total volume of Master Mix made up= 1668.42 μ l

6 μ l of template (cDNA sample) was added to 54.5 μ l of the master mix per well of the set-up plate. The well contents of the set-up plate were mixed thoroughly to ensure consistency between replicates. After mixing, 19.6 μ l of this template and master mix was added to the QPCR plate (Applied Biosystems). In this example the plate was set up with 18 samples, 1 control, 6 standards, and 1 non-template control (NTC) all in triplicate. The plate was covered with a plastic film (Applied Biosystems), spun down in a refrigerated centrifuge, at 400g for 15 seconds and run in the QPCR (Applied Biosystems) machine. The machine was run on the following programme:

Step	Temperature (°C)	Time (s)
1.	95	20
2.	95	3
3.	30	30
Repeat 2-3 40 times		
4.	95	15
5.	60	15
6.	95	15

Table 2.8 Run Details for QPCR Assay. The table above gives the timings and temperatures for each step of the QPCR assays used throughout the work presented in this thesis.

2.10.5.1 QPCR Primers

Primer sequences used for the assays in this work are listed below.

Gene of Interest	Forward Primer (5' > 3')	Reverse Primer (5'>3')
<u>Mouse</u>		
ACKR2	TTCTCCCACTGCTGCTTCAC	TGCCATCTCAACATCACAGA
α-SMA	CCTGAAGAGCATCCGACAC	AGAGGCATAGAGGGACAGCA
Collagen I	GACTGGCAACCTCAAGAAGG	ATGACTGTCTTGCCCCAA
Osteopontin	AGAGAGCGAGGATTCTGTGG	GGACATCGACTGTAGGGACG
TGF-B1	TGGAGCCTGGACACACAGTA	CACGTAGTAGACGATGGGCA
Collagen Iα	TGCTACCCTGCTGTTCTTC	GTGCCCAAATACTCCTCCA
<u>Human</u>		
ACKR2	AGGAAGGATGCAGTGGTGTC	CGGAGCAAGACCATGAGAAG
TATA Binding Protein (TBP)	AGGATAAGAGAGCCACGAACC	GCTGGAAAACCCAACTTCTG

Table 2.9 Primer Details for QPCR. The specific sequences used for QPCR analysis during this thesis are listed above.

2.10.5.2 Analysis of QPCR Data

The data from the QPCR assay were exported into a Microsoft Excel spreadsheet. The data generated from the reference gene assay were analysed first on the spreadsheet. The optical densities of the standard generating wells were used to calculate the number of gene copies in the standards. A worked example is given below:

$$27.8 \text{ ng}/\mu\text{l} = 2.78 \times 10^{-8} \text{ g}$$

$$2.78 \times 10^{-8} \text{ g} \times 6.02 \times 10^{23} \text{ (Avogadro's Constant)} / 68640 \text{ (molecular weight of target product)} = 2.44 \times 10^{11} \text{ copies}/\mu\text{l}$$

2.10.5.3 QPCR Quality Control

In order to ensure the reliability of the results gained through QPCR assays for this thesis a systematic quality control approach was taken. This included thoroughly testing standard curves (as listed in 2.10.4), removing reverse transcriptase from random test samples during cDNA synthesis to ensure no genomic DNA was being amplified, addition of non-template controls (samples with QPCR master mix but with water instead of cDNA) and by running and examining the melt curve generated after the assay.

2.11 Cell Culture

Cell lines were incubated at 37°C in an atmosphere containing 5% CO₂ (Thermo Scientific) unless otherwise stated. All cell lines used for the work done here were tested for mycoplasma infection prior to work being undertaken. This was done with a luminescence assay using a commercial kit (MycoAlert™ (Lonza)) and read using a fluorometer (Fluoroskan Ascent™ FL (Thermo)) cells were verified as being mycoplasma-free using this system.

2.11.1 Culture of Human Embryonic Kidney (HEK) 293 Cells

This adhesive cell line required cell dissociation buffer (Sigma) to remove cells from the flask. The spent medium was removed from the cells and the dissociation buffer was added to the flask. This was incubated for up to 10 minutes at 37°C. Cells were removed from the flask, placed in a 50ml tube (BD) and centrifuged (Thermo Scientific) at 200 x g for 5 minutes.

HEK 293 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) plus sodium pyruvate, L-glutamine, Penicillin/Streptomycin and 10% foetal bovine serum (all Life Technologies). Once the cells had been spun, the supernatant was removed and the pellet was re-suspended in 10 ml of fresh medium. 1ml of this cell suspension was added to 29ml of fresh medium to give a 'split' of 1:10. This new suspension was added to a T75 flask (Corning).

2.11.2 Subculture of ARPE-19 Cells

The ARPE-19 cell-line was grown in a 1:1 mix of Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies) and Hank's F12 medium (Sigma Aldrich). To this mix L-glutamine, Penicillin/Streptomycin and 10% foetal bovine serum (all Life Technologies) was added. The cells were incubated as previously stated and required to be 'split' every 48 hours.

When 'splitting' these cells 3.0mL of (0.05% w/v) trypsin-0.53 mM EDTA solution (Life Technologies) was added to the cells after discarding the growth medium. Cells were rinsed with trypsin-EDTA solution. After this 3.0ml of trypsin-EDTA was added again and the cells were placed in the incubator for 5-10 minutes and monitored for cell dissociation using an inverted microscope (Zeiss). 8.0mL of growth medium (Life Technologies/Sigma) was then added to the cell solution, to remove the cells. This was then harvested and placed in a 50mL centrifuge tube (BD). Cells were spun down (Thermo Scientific) at 125 x g for 10 minutes.

After centrifugation, supernatant was removed and the cell pellet was resuspended in 9mL of fresh growth medium (Life Technologies/Sigma). 3mL of the fresh cell suspension was then added to a 50mL centrifuge tube (BD) containing 27mL of fresh growth medium (Life Technologies/Sigma) to give a 1 in 3 split of the cells.

2.11.3 HDLEC Cell Culture

Human dermal lymphatic endothelial cells (HDLEC) were bought in (Promocell (Heidelberg, Germany)) and grown in a T25 culture flask (Corning). These primary human cells were grown to confluency in endothelial growth medium (Promocell) with added supplements provided (basic Fibroblast Growth Factor, Insulin-like Growth Factor (Long R3 IGF-1), and Vascular Endothelial Growth Factor). The medium was also supplemented with penicillin/streptomycin (Sigma) and gentamycin (Sigma). HDLECs were then transferred into a T75 flask.

2.12 Flow Cytometry

ARPE-19 cells were removed from the tissue culture flask using a non-enzymatic cell dissociation buffer (Life Technologies) and washed in PEF before being

centrifuged and resuspended in PEF. Using a haemocytometer (VWR International) approximately 1×10^7 freshly isolated cells were harvested by centrifugation at $300 \times g$ for 5 minutes at 4°C then washed three times with PEF buffer and re-suspended in chilled PEF. In order to stain intracellular ACKR2 the cells had to be fixed and permeabilised. This was achieved using a commercially available kit (Cytofix/CytopermTM (BD)). Cells were incubated with Cytofix/CytopermTM buffer (BD) for 20 minutes at 4°C then cells were washed twice in 1x Perm/WashTM buffer (BD). The samples used for intracellular analysis were washed in 1x Perm/WashTM buffer (BD) from this point onwards, antibodies were also diluted in the same buffer for these samples.

Primary antibodies (in-house made mouse anti-human ACKR2 (clone 4A5)) or isotype control (mouse anti-human IgG2a (Dako)) were then diluted in chilled PEF and added to the cells and left to incubate for 15 minutes with occasional gentle agitation. Samples were washed twice with 2ml chilled PEF before being incubated with fluorescently-labelled secondary antibodies (anti-mouse FITC (R&D Systems)) in PEF on ice for 15 minutes with occasional gentle agitation. Cells were washed twice more with 2ml chilled PEF and re-suspended in 200 μl PEF. A live/dead cell discriminator-Viaprobe (BD Biosciences, San Jose, CA, USA)- was used to identify viable and non-viable cells, respectively. Samples were analysed on the MACSquant analyser (Miltenyi Biotec).

Both stained cells and cells from the competition assays were analysed using a MACSquantTM analyser. Parameters for acquisition and data collection were established using unstained cells. Data was analysed subsequent to acquisition using MACSquantifyTM software (Miltenyi Biotec), using unstained samples to ascertain a gating strategy.

2.13 Statistical Analyses

Throughout all of the models shown in this thesis the Graphpad (Prism) statistical analysis software was used. The test(s) carried out for each piece of data, along with the relevant statistical information, is listed in the figure legend of each figure in the coming results chapters. In all figures error bars represent standard deviation (SD).

Chapter 3- The Role of ACKR2 in Cutaneous Inflammation

Chapter 3 The Role of ACKR2 in Cutaneous Inflammation

3.1 Introduction

3.1.1 Skin: An Overview

To paraphrase Henderson's Dictionary of Biology, an organ is a part or structure within an organism that serves a specialised function (Lawrence, 2008). This, by definition, makes the skin the largest organ in the human body. Skin is a multi-layered tissue that differs in architecture and structure depending on anatomical location and function.

The constitutive layers of human and murine skin are the epidermis, dermis, and sub/hypodermis. In humans the epidermis is made up of the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, and stratum basale (Martini, Nath and Bartholomew, 2012), however in mice this layer is considerably less complex with only four layers. Another feature that separates mouse skin from human is that the murine hypodermis contains a layer of striated muscular tissue which human skin lacks (Treuting et al., 2012).

Skin carries out many specialised functions including, amongst others: providing a crude barrier to prevent entry of pathogens into an organism; maintaining homeostasis by helping regulate temperature; protecting our organs from harmful UV radiation and from physical damage (Martini, Nath and Bartholomew, 2012). Considering the aforementioned functions of the skin, it becomes clear that any breaches, or trauma, in this tissue require rapid and efficient resolution. A perfect healing process would result in the newly formed skin being identical to the tissue it replaced. In reality, the damage is never resolved to its previous structure and strength, and fibrotic scar tissue forms at the site instead. It is thought that the process of scar formation is the result of an evolutionary push towards rapid repair (Bayat, McGrouther and Ferguson, 2003). It seems logical that, due to the unhygienic conditions we may have lived in many years ago, rapid repair would be highly advantageous. As a result of this,

our wound healing process is more focused on rapid repair over perfect regeneration. As a consequence scars form.

Damage to the skin causes the induction of the inflammatory mechanisms discussed in the introductory chapter to this thesis (section 1.1.1). Chemokines play an indispensable role in the propagation, maintenance and resolution of these processes. During inflammation, immune cells are recruited to areas of trauma and danger. Local cells at the damaged or infected site release chemokines and cytokines to signal trauma and that, in turn, initiates the body's defence mechanisms.

3.1.1.1 The Cutaneous Environment

The skin is an amazingly adaptable organ and, under resting conditions, consists of both resident tissue/stromal and immune sentinel cells (Igyártó et al., 2011). These developmentally distinct cells work together to carry out surveillance of the tissue and react to signs of infection and damage. The skin is the main interface between the host and the outside world and so is constantly exposed to potentially immunogenic mediators. The skin harbours trillions of commensal bacteria and it has been shown that these play a role in tissue homeostasis and shaping the cutaneous immune response (Naik et al., 2012, Sanford and Gallo, 2013). It has been suggested that resident dendritic cells of the skin present the peptides from these commensals to CD8⁺ T cells and in turn this bolsters the barrier function of this tissue. A paper by Naik et al suggested that this process exquisitely shapes the immune response of the skin (Naik et al., 2015). This would suggest that, under physiological conditions, these microorganisms have a protective role to play for the host. However, this may not be the full story.

A study by Canesso et al in 2014 showed evidence from germ-free mice that suggested a detrimental effect of the skin microbiota during the repair of excisional wounds. They showed that in the absence of these commensals the wound repaired more rapidly, with a scar-free phenotype and with a significant reduction in infiltrating immune cells. The main immune cell that they reported to be reduced was neutrophils (Canesso et al., 2014). It has also been shown

that by removing cells that express the transcription factor PU.1, a regulator of myeloid cells, results in a ‘scar-free’ repair phenotype (Martin et al., 2003).

Another unique feature of the skin is the presence of the follicular stem cells that populate it. Hair follicles of the epidermis have been suggested to be immune-specialised as well as being sites of frequent immune cell interaction. Macrophages and mast cells of the skin have been shown to interact with these structures. It has also been suggested that the follicles may act as a ‘portal’ for epidermal entry of effector cells (Chou et al., 2013).

3.1.1.2 Chemokines and Receptors in the Skin

One of the key roles of chemokines is to attract cells to discrete anatomical locations and to retain them there. In this regard the skin is no exception. Stromal cells of the skin release chemokine ligands that recruit cells bearing cognate receptors under both inflammatory and physiological conditions. Three molecules have been shown to help regulate skin homing in both mice and humans, these are CCR4, CCR10 and the cutaneous lymphocyte antigen (CLA) (Reiss et al., 2001). It has been shown that interactions between CLA and E-selectin mediate the exit of lymphocytes from the cutaneous venules in much the same way as described for neutrophil extravasation in the introductory chapter (Santamaria Babi et al., 1995).

Keratinocytes of the basal epidermis secrete the chemokine CCL27 which binds to the receptor CCR10 (Homey et al., 2000) in an axis that is responsible for the recruitment of T cells to the skin (Homey et al., 2002). This is a constitutive process that allows for the positioning of skin-resident memory T cells, also known as peripheral tissue immune surveillance T (T_{PS}) cells (Sigmundsdottir et al., 2007). An interesting process that imprints skin-homing specificity on these T cells involves ultraviolet (UV) light from the sun. As the skin is exposed to UV light, vitamin D_3 is metabolised into its active form $1,25(OH)_2D_3$ by dendritic cells. In a process known as ‘imprinting’ these specialised DCs induce the expression of CCR10 on T cells in the local lymph node (Sigmundsdottir et al., 2007).

The other key chemokine receptor for lymphocyte recruitment to the skin under physiological conditions is CCR4 (Reiss et al., 2001, Gehad et al., 2012). The two ligands for CCR4 are CCL17 and CCL22 and, of these, CCL22 has a higher affinity for the receptor. Interestingly it has been shown that there is an accumulation of these ligands associated with lesions of cutaneous T cell lymphoma (Ferenczi et al., 2002) and that antibody-mediated interference with the receptor is of therapeutic benefit to the patient (Han et al., 2012). This axis is also involved in atopic responses in the skin (Gehad et al., 2012).

3.2 Wound Healing

Since as early as 1500 BC the subject of wound healing has been investigated. Research into the care of wounds can be traced all the way back to ancient Egypt (Sipos et al., 2004, Majno, 1975). This was of interest to ancient civilisations as modern medicine was not available and so efficient healing was essential to prevent infection. It is still an area of active research, thankfully for different reasons, within the scientific and medical communities. The purpose of research in this area is now to optimise the way our bodies heal after trauma so that we can prevent chronic wounds or hypertrophic scar formation.

Wound healing is, after birth, one of the most complex biological processes that the body can go through (Gurtner et al., 2008). The inevitable product of wound healing is scar formation, as mentioned, and it is thought that this may be a product of evolutionary pressure to rapidly close off denuded tissue at the expense of perfect regeneration. Both humans (Colwell, Longaker and Lorenz, 2003) and mice (Adzick and Longaker, 1991) in the foetal stage of development seem to recover from wounds without the formation of scar tissue. Many questions have been raised about the underlying mechanisms of this process.

Some postulated (Block, 1960) that it might be a product of the sterility, local proteins and growth factors in the foetal environment. To address this hypothesis experiments were carried out using marsupials. The thinking behind this was that when these animals are born they are still anatomically and physiologically in a foetal state. The offspring then survive in a maternal pouch until fully developed; this pouch is free of any sort of placental nourishment

and, importantly, is a non-sterile environment. Work in these studies found that wounding the developing opossum in the maternal pouch did not result in a scar until a particular time-point of development. Wounds inflicted six days or more before leaving the pouch healed in a scar-free process, any wounding after this time-point resulted in the formation of a scar (Armstrong and Ferguson, 1995). Interestingly it is at this time in development that the opossum begins to develop an innate immune system (Armstrong and Ferguson, 1995). This work suggested that the placental milieu was not the key mediator of foetal scar-free wound healing and that the developing immune system is more causative of scar production.

The classical model of skin repair involves three or four phases. The first step, according to some, is haemostasis. This is a platelet-driven phase where these vasoactive particles become activated and form a fibrin clot to stop haemorrhage (Coller, 2011). The α -granules of the platelets also release a plethora of mediators such as cytokines, chemokines and growth factors to induce repair processes and recruit immune cells (Hundelshausen et al., 2005). The inflammatory phase follows on from this and involves the influx of cells such as neutrophils and monocytes/macrophages which phagocytose cellular debris and neutralise invading pathogens at the wound site (Gurtner et al., 2008). Following this the proliferation stage commences. The main feature of this phase is the formation of granulation tissue and, during this, angiogenesis and collagen deposition also occur. This is the early-repair portion of the response where fibroblasts produce a provisional extracellular matrix and epithelial cells proliferate and migrate to the surface of the wound to create a covering of new tissue (Gurtner et al., 2008). The final phase of this model is the maturation stage. This is the late-repair phase where the new tissue, laid down in the previous stage, is strengthened and reinforced. The newly synthesised collagen is remodelled and unneeded cells undergo apoptosis. It takes up to two years for this process to be completed in humans (Gurtner et al., 2008).

3.2.1 Healing Processes

Three forms of wound healing have been described and they depend on the form that the original wound takes. Healing by Primary Intention is probably the most

common form of wound healing. This occurs when the two leading edges of the epithelium are in close proximity. This process would occur following a laceration or incisional wound (Standring, n.d.). This healing reduces scarring as less new tissue synthesis is required and is far less traumatic for the local tissue. Healing by Secondary Intention occurs after an excisional wound- i.e. removal of an area of skin leaving a large open wound needing repaired. This process involves the synthesis of granulation tissue- puffy red looking skin that is very well vascularised- and can result in extensive scarring (Standring, n.d.). During this wound healing process, large amounts of new tissue have to be synthesised in order to repair the wound fully. This type of healing would occur in large surgical wounds for example. Finally, healing by Tertiary Intention involves clearing and debridement of the tissue and the wound is left open purposely and would only really occur during procedures like tissue grafting (Standring, n.d.). The work in this thesis focuses mainly on excisional wounding and so healing in these models is by Secondary Intention. That being said many of the process, cells and factors involved are essential in all types of wound healing regardless of healing method and wound type.

The healing process of cutaneous tissue, after a wound, is a complex one and needs to be tightly regulated as ‘under-aggressive healing’ could lead to a chronic wound, or ‘over-aggressive healing’ could lead to a hypertrophic or keloid scar (Yang *et al.*, 2003). The key to wound healing is to replace damaged tissue with new skin that is as structurally similar, high in tensile strength, and elasticity as its predecessor. As mentioned earlier, the inevitable result of this is the formation of scar tissue. A scar will never reach the full tensile strength of normal unaffected skin but it should recover the vast majority of this original strength (Standring, n.d.). At around three-weeks post wounding in humans, collagen deposition peaks in the scar. Tensile strength at this time, however, is a mere 15% of local unaffected skin (Hardy, 1989). Interestingly research has shown that wounds that repair under increased tension repair with scars of a higher tensile strength (Pickett *et al.*, 1996). This has lead to the development of some dressings that apply tension over the healing wound to increase scar tensile strength.

Hypertrophic scars are a result of uncontrolled fibrosis of the skin and follow a similar pathophysiology as other fibrotic disorders in the body (Mori et al., 2001). The cells, growth factors, and processes involved in pathologies such as pulmonary fibrosis and renal fibrosis are very similar to those involved in cutaneous fibrosis (Mori et al., 2001). From wound to fully formed scar is thought to take around one hundred days in humans (Gurtner et al., 2008). For the first three weeks or so the local stromal cells are laying down new collagen and repairing nearby damaged areas under the influence of immune, stromal, and mesenchymal cells (Gurtner et al., 2008). From here until the process is finished the main objective for the skin cells is scar maturation. This is when the scar tissue gains tensile strength (Standring, n.d.). This process is highly dependent on growth factors and cells to achieve these goals.

The result of an over-healed wound is a hypertrophic scar. The result of an under-healed wound is a chronic wound. For one or more of many reasons wounds can fail to heal appropriately and fall into one of these two categories. Some groups are more susceptible to this than others including obese, diabetic (Nguyen et al., 2013), and genetically predisposed patients.

3.2.1.1 The Monocyte/Macrophage Axis and Beyond

Of all the immune cells involved in wound repair the monocyte/macrophage system seems to play one of the most pivotal roles and is involved in all stages of the repair process (Rodero and Khosrotehrani, 2010). Macrophages have a dichotomous role in wound healing both as immune cells and as tissue growth promoting cells (Brancato and Albina, 2011). During the early stages of the wound repair response blood monocytes are recruited to the damaged site and these mature into tissue macrophages (Brancato and Albina, 2011). As discussed in the introduction section of the thesis (1.1.1.4), there are multiple subsets of monocytes and it has become apparent that the macrophage population in wounds reflect this (Daley et al., 2010).

One of the key roles of macrophages as they invade the wound bed is to phagocytose debris and apoptotic neutrophils, a process known as efferocytosis (Brancato and Albina, 2011). The idea of macrophages engulfing neutrophils was

proposed by the man who discovered the cells, Metchnikoff (Metchnikoff, 1893). In a process that aids in the control of inflammation, macrophages can directly induce apoptosis of neutrophils through interactions between CD36 and the β_3 integrin in the presence of membrane-bound TNF- α (Meszaros, Reichner and Albina, 2000). It is proposed that macrophages that are derived from CCR2⁺ monocytes in mice are able to carry out this apoptotic induction however, in a two-part mechanism; it is then that the CX₃CR1⁺ monocyte-derived macrophages phagocytose the dead neutrophil (Brancato and Albina, 2011). The phagocytosis of apoptotic neutrophils induces a profibrotic phenotype in macrophages and stimulates the release of key mediators such as TGF- β 1 and VEGF (Savill and Fadok, 2000).

To add to this, wounding models carried out in CX₃CR1 null mice show reduced wound closure capacity. These wounds contain fewer macrophages and myofibroblasts, a cell key to wound contraction (Hinz et al., 2007). There are also decreased levels of VEGF and TGF- β 1 (Ishida, Gao and Murphy, 2008). More details on these two key mediators in wound repair will be provided later on in this Chapter.

At the subsequent restorative phase more macrophages are recruited, but in this stage they are there to serve a more reparative function (Lucas et al., 2010). In the final stage of repair where the purpose is to aid in the maturation of the developing scar macrophages are also present. It has been shown that macrophage numbers increase during the inflammatory stage, reach a pinnacle during the early repair phase and then decline during the later repair phase (Martin and Leibovich, 2005).

A possible reason for these cells seeming to be of such high importance is that they have an effect on the healing process at every key stage (Brancato and Albina, 2011). In 2010 Lucas et al (Lucas et al., 2010) set-out to define the specific roles played by macrophages at each phase of the wounding response by selectively knocking them out at specific times. This elegant study showed that depletion of macrophages before/during the initial inflammatory response resulted in a delay in the formation of granulation tissue and epithelialisation in the latter early stages of the repair response. They proposed that this was down

to a failure to recruit alternatively activated macrophages that have a key role in repair. The group also showed in this study that macrophages recruited during the mid-stage of the response were important in aiding the stability of newly forming blood vessels and granulation tissue. The macrophage-depleted mice had such unstable new vessels that they suffered haemorrhage. Finally, they suggested that depletion of macrophages in the final maturation phase of the response had a minimal affect (Lucas et al., 2010).

What may not be so intuitive is that, as mentioned above, many of the growth factors that these cells can produce and secrete also play a part in the production of new tissue in the proliferation stage of the healing process. Macrophages secrete factors such as TGF- β 1, fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF) to initiate the process of re-epithelialisation (Brancato and Albina, 2011). This step in the course of healing commences just hours after the trauma and these macrophage-derived growth factors act on tissue cells such as fibroblasts, endothelial cells and keratinocytes to produce new tissue. Keratinocyte migration ceases when the epidermal barrier is restored (Raja et al., 2007).

3.2.1.2 Mast Cells

Mast cells (MC) are involved in multiple stages of the healing response (Noli and Miolo, 2001). They are abundant in barrier tissues and the dermis is a site of fairly high mast cell presence (Noli and Miolo, 2001). It has been shown that mechanical damage is enough to activate these cells and, as a result, they are activated very early in response to a wound (Egozi et al., 2003).

Activation leads to the release of mediators like histamine and vascular endothelial growth factor (VEGF), which cause vasodilation to aid the acute response and the recruitment of effector cells (Canesso et al., 2014). Chymase, an enzyme released by MC, has been shown to play a role in neutrophil recruitment. Histamine can also stimulate the release and production of pro-inflammatory cytokines by keratinocytes. Human keratinocytes express the H1 histamine receptor (Giustizieri et al., 2004) and activation of this results in the release of CCL2, CCL5, CCL20 and CXCL10 from the cells.

MC also aid in the proliferation stage of the healing response (Succar et al., 2014). Mice that lacked some key proteases of murine MC showed much reduced blood vessel formation in the second stage of wound healing (Succar et al., 2014). The stimulation of keratinocytes, mentioned above, also induces the release of growth factors and cytokines that aid the re-epithelialisation process. Through some of the many active mediators that mast cells release they can aid angiogenesis indirectly and they can activate fibroblasts directly. They produce keratinocyte growth factor (KGF) and epidermal growth factor (EGF) both key growth factors in the repair process that will be discussed in more detail in section 3.2.3.

A number of studies in MC null mice have suggested that these cells are dispensable in wound repair. Although subtle differences were noticed, such as collagen remodelling (Iba et al., 2004) differences between the models, the general consensus was that these were not vital cells (Antsiferova et al., 2013, Nauta et al., 2013). Some studies have shown the opposite of this though and suggested a key role for MC in repair (Weller et al., 2006).

Reports have suggested that MC can also affect scar formation in mice and humans. Studies have shown a negative correlation between MC number/activation state and levels of scarring (Canesso et al., 2014). Oral wounds heal with minimal scarring and oral mucosa contains fewer MC during wounding than cutaneous tissue. However the levels of moisture in the oral mucosa may also have a profound effect on the healing process (Costa et al., 2011). Some studies suggest a role for MC in collagen/scar maturation (Iba et al., 2004). The increased presence of MC has also been shown in other fibrotic diseases such as systemic sclerosis where auto-IgE antibodies have been shown to activate these potent cells (Akimoto et al., 1998).

3.2.1.3 Neutrophils

Neutrophils are involved in wound healing arriving earlier than recruited macrophages although their action does not last as long- their role ending at around day five during a normal response (Gurtner et al., 2008). It is now

thought that these cells can carry out almost all of the functions of macrophages including antigen presentation- they have been shown to upregulate class II major histocompatibility complex (MHC-II) in inflamed lesions (Geng et al., 2013). The trauma of the injury suffered during wounding to the local tissue causes CXCL8 production (Roupé et al., 2010). This chemokine is highly chemotactic for neutrophils. Work by Lammermann et al suggested that once neutrophils had left the circulation the signal from chemokines was less important for movement in the interstitium (Lammermann et al., 2013). This paper described an essential role for the lipid mediator leukotriene B4 (LTB4) in the 'swarming' phenomenon attributed to these cells. They also showed that the death of these cells catalyses this response (Lammermann et al., 2013). When the effect of phagocytosis of neutrophils is considered this positive-feedback situation has potential to be an important factor in appropriate wound healing responses.

The key role for these cells during wound healing is probably to quickly and efficiently phagocytose any opportunistic pathogen or foreign debris that may have entered the host at the wound site. It is then the role of the macrophages or other neutrophils (Martin and Leibovich, 2005) to phagocytose the dead or dying neutrophils.

3.2.1.4 Platelets

Platelets are defined as non-nucleated small disc-shaped cell fragments that are involved in blood clotting, which gather at sites of damage and release clotting factors (Gurtner et al., 2008). When skin is wounded, a fibrin clot is produced. This is achieved by the cross-linking of fibrin polymers that act as a temporary barrier to protect the host where the tissue has been breached. Platelets contain granules that are released when the platelet is activated. Of these granules, the alpha (α)-granules are the most abundant (Blair and Flaumenhaft, 2009) and can single-handedly aid the wound healing processes in several tissues (Anitua et al., 2005, Nagai et al., 2005, Moulin et al., 1998).

It has been shown that, from α -granules, platelets are capable of releasing the chemokine CCL5 which is responsible for inflammatory cell recruitment

(Kameyoshi et al., 1992). This suggests that these cell fragments are more than just players in the blood-clotting cascade. Platelets have also been implicated in cutaneous pathologies such as psoriasis with an aggregation of these fragments being seen around psoriatic lesions (Hayashi et al., 1985). It is now widely thought that these cell fragments are key to repair downstream of the coagulation of microvessels in the injured locale. In wound healing the blood clot formed by, and rich in, platelets, acts as a reservoir of chemokines and growth factors that are released to enhance the reparatory process.

The important role of platelets in wound healing is highlighted by platelet-associated treatments that are being increasingly employed in patients with chronic wounds such as diabetic ulcers (Margolis et al., 2001). One treatment involves the topical application of platelet rich plasma (PRP) to the wounded tissue. PRP can increase the levels of local growth factors such as TGF- β 1, platelet-derived growth factor (PDGF), EGF, insulin-like growth factor (IGF) and VEGF. The importance of these growth factors will be discussed later but increasing the levels in the local milieu appears to be advantageous to the patient.

The other treatment is known as platelet-derived wound healing factor (PDWHF) and is the releasate of thrombin-activated platelets (Knighton et al., 1986). Although the mechanism by which this treatment works is not completely understood, in some studies it showed significantly accelerated wound healing when compared to placebo treatments (Steed et al., 1992, Margolis et al., 2001). However other studies have shown no real effect (Senet et al., 2003).

3.2.2 Stromal Cells in Repair

Cells of the immune system are of the highest importance at the early stages of repair. Later on, once any potential infection has been cleared and during the re-epithelialisation and remodelling phases, the stromal cells play the key roles in repair, mainly under the influence of growth factors. Stromal cells are cells that produce new elements of tissue and extracellular matrix. Fibroblasts, myofibroblasts, keratinocytes and endothelial cells are all examples of this cell type that are involved in wound healing (Stappenbeck and Miyoshi, 2009).

Dermal fibroblasts are key cells in the wound repair response (Wong, McGrath and Navsaria, 2007). These cells are important for the synthesis and deposition of extracellular matrix (ECM) and they have been shown to proliferate and migrate in response to cutaneous damage (Wong, McGrath and Navsaria, 2007). Fibroblasts commence migration towards the wounded tissue around five days post-injury and are capable of synthesising new collagen fibres (Musyoka et al., 2013). Growth factors such as fibroblast growth factor (FGF) and transforming growth factor (TGF)- β 1 are responsible for activating this cell type to produce molecules such as collagen (Colwell et al., 2005).

In vitro work on these cells has suggested that under the influence of TGF- β 1, endothelin-1, and IL-1 released from keratinocytes, fibroblasts undergo differentiation into proto-myofibroblasts. From here, with further TGF- β 1 and IL-1 influence, these cells become myofibroblasts (Shephard et al., 2004a, Shephard et al., 2004b). These differentiated cells are equipped with properties of both fibroblasts and of smooth muscle cells, hence the name. They express alpha-smooth muscle actin and, during wound healing, are important in the contraction of the wound edges for closure. As mentioned previously, healing under tension is required to provide the resulting scar with tensile strength; these cells provide much of this. During both incisional wounding and healing by Second Intention, these cells are vital to repair.

Keratinocytes are a key stromal cell of the skin. One of the most essential events in wound healing is re-epithelialisation and the migration and proliferation of these cells is key to this (Werner, Krieg and Smola, 2007). Despite this, the details of the migration of these cells to the site of the wound bed are still poorly defined. Keratinocytes express the LTB₄ receptor BLT2 (Liu et al., 2014a). Agonism of this receptor with another of its ligands, 12(S)-hydroxyheptadeca-5Z,8E,10E-trienoic acid (12-HHT), has been shown to increase the efficiency of wound repair in mice by accelerating the migration of keratinocytes (Liu et al., 2014b). Once they have reached the wound, keratinocytes release factors that can act in both autocrine and paracrine fashions. One such growth factor is TGF- α (Beyeler et al., 2014). *In vitro* work has shown a role for this factor in the repair of wounds by increasing fibroblast

migration to the wound site (Beyeler et al., 2014). Interestingly in the early stages after wounding, it has been demonstrated that these cells release TGF- α (Antoniades et al., 1993) Keratinocytes from keloid scars have been shown to be more effective at inducing fibroblast proliferation (Xia et al., 2004). Another group showed that keloid keratinocytes released increased levels of TGF- β 1, which would in turn cause local fibroblasts to produce more type I collagen (Machesney et al., 1998).

3.2.3 Growth Factors

Growth factors are molecules that cause cell activation and/or cell growth (Lawrence, 2008) and as a result, these molecules are indispensable to the process of wound repair. Growth factors are tightly regulated during the healing process and any dysregulation in this network can lead to the development of a chronic wound or a hypertrophic scar. There are a large number of growth factors involved in the healing of a wound and they all have to strike a delicate balance to ensure appropriate repair.

Epidermal Growth Factor (EGF) family members, such as the already discussed, Transforming Growth Factor (TGF) - α and EGF, are vital to the process. These two factors are secreted by platelets, macrophages and fibroblasts and both have an activating effect on keratinocytes (Schultz et al., 1991, Barrientos et al., 2008). The key function of EGF is re-epithelialisation and this is achieved by accelerating migration and activation of keratinocytes (Jiang et al., 1993).

TGF - β 1 is an important growth factor for wound healing (Roberts, 1995) but has been found to increase the instance of keloid or hypertrophic scars (Niessen et al., 2001)(Wang et al., 2013). It is suggested in the literature that a reduction of TGF- β 1 signalling during the wound repair process results in an accelerated healing phenotype (Flanders et al., 2003, Ashcroft et al., 1999). Work on foetal wounding models has also shown that in the local tissue surrounding the wound there is reduced expression of TGF- β 1 (Bullard et al., 2003).

Platelet derived growth factor (PDGF) is a protein that is important during all phases of the wound healing process. It has been shown to aid angiogenesis and

re-epithelialisation as well as to be chemotactic for innate immune cells in the early stages of the response (Eppley et al., 2004, Hosgood, 1993).

Osteopontin has been shown in many studies to have a significant role to play in wound healing. This highly phosphorylated sialoprotein has been shown to be up-regulated during the inflammatory phase of the wound healing response and is expressed by fibroblasts (Mori, Shaw and Martin, 2008).

3.2.4 Collagen

Members of the collagen family make-up around thirty percent of proteins within the human body and twenty-nine different forms of collagen have been described (Myllyharju and Kivirikko, 2001). These molecules are structural proteins with considerable mechanical strength, properties that are key to a vast array of biological processes including the repair of wounded tissue.

The vast majority of the collagen in the body is of types I-IV. Type I collagen was first discovered in skin, bone and tendon and is the most abundant collagen in the human body being present in almost all tissues (Epstein and Munderloh, 1978). It makes up over 80% of skin collagen (Jinnin, 2010) and exists in a fibrillar form. Type II collagen is a unique molecule that is found in the cartilaginous tissues of the body (Epstein and Munderloh, 1978) and the vitreous body of the eye (Hardy, 1989). Type III collagen is another very abundant collagen that can be found in many different tissues in the body. It is thought to be expressed in the epidermis and surrounding dermal appendages (Epstein, 1974). This makes up about 10% of skin collagen (Jinnin, 2010). Type IV collagen is present in the basal lamina of the skin during normal physiological conditions and, during wound healing, at the leading edge of the epithelial tongue. The importance of this collagen is observed when matrix metalloproteinase (MMP)-9 is over-expressed. MMP-9 is a type IV collagenase and over-expression causes delayed wound healing as it removes this type of collagen (Reiss et al., 2010). Type V collagen is present in the skin but makes up only a small percentage of fibrils (Jinnin, 2010). It is also expressed at low levels during the process of wound repair (Inkinen et al., 1999). Replacing wounded skin with new tissue is a complex and highly regulated process and collagen synthesis is key to

biomechanical strength. Both over, and under, production of collagen during this process can lead to many clinical, pathological and cosmetic complications. The quantity and formation of newly synthesised collagen during wound repair are key areas this research will address.

3.3 Cutaneous Inflammation

Wound healing is just one example of inflammation in cutaneous tissue. As cutaneous tissue is constantly exposed to the outside environment, skin is often exposed to pro-inflammatory agents. The work in this thesis also used chemical reagents to induce models of inflammatory states and investigate the role of ACKR2 in other forms of skin trauma.

3.3.1 Compound 48/80

Burn-type inflammation was simulated using the potent mast-cell activator, compound 48/80 (Paton, 1951). As described above, the granules of mast cells contain many potent immunomodulators such as histamine, heparin, prostaglandins, leukotrienes, cytokines and chemokines. The potency of the contents of these cells is most evident during anaphylaxis; this process is driven by the release of mast-cell contents and causes severe acute symptoms in the patient. Symptoms include widespread vasodilation, rapid heart rate, severe drop in blood pressure, and dyspnoea (breathing difficulty) all of which are caused by a catastrophic release in histamine.

Compound 48/80 causes mast cells to degranulate and, in turn, results in a massive release of the immunomodulators mentioned previously. This then causes local cells to present an active/inflammatory phenotype. During inflammation induced by this reagent, an accumulation of inflammatory cells such as neutrophils and macrophages occurs, and these cells secrete further inflammatory cytokines, chemokines and other factors. If these signals are not regulated a hyper-response will be observed and an increase in local tissue damage will occur. The degranulation process is independent of $Fc\epsilon$ receptor binding in the case of compound 48/80 mediated activation, as it would be in a burn-type wound. Degranulation of mast cells is caused by an opening of the

calcium ion channels on the mast cell surface membranes and results in the release of cell contents (Walsh et al., 2009). As previously mentioned, mast-cells have been shown to express moderate levels of ACKR2 (McKimmie et al., 2008b) and so this work could give a direct insight into the role played by this receptor during the process of mast-cell degranulation.

3.3.2 Allergic Contact Dermatitis

Allergic contact dermatitis reactions are a common form of skin response to materials like nickel in susceptible individuals. People who suffer from nickel sensitivity, for example, present oedema, redness, and itching at the site of contact with the metal. This is a delayed response as it is an adaptive immune driven process. Reagents such as 2,4-dinitrofluorobenzene (DNFB) can be applied in a two stage process involving priming and challenging to mimic a similar response. DNFB is a molecule known as a hapten; these are small immunogenic molecules. The priming stage involves painting the abdomen of the mouse with a mix of the hapten and a vehicle to aid its absorption. This mix of hapten and vehicle causes mild inflammation at the site of contact. The priming agent is picked up by tissue dendritic cells and/or macrophages (both cells are known as APCs) and taken to a local draining lymph node. As mentioned in the introductory chapter this leads to specific T cells that respond to this agent upon any subsequent encounters.

Then after around six days the same animal is challenged on another tissue. The ear is a commonly used challenge site. At this time, the same solution used to prime the animal's immune system is used to challenge it. The effector memory cells, set-up during the priming response, proliferate rapidly and migrate to the site of the challenge. Here they react against the antigen for which they have a specific receptor. They induce inflammation and activate innate immune cells also. The degree of immune reaction is measured in the oedema presented in that tissue. It is the easiest site to measure swelling and as there are two, the untreated ear acts as an internal control specific to each animal.

All of these models are designed to provide us with an insight into the role of ACKR2 in many arms and facets of the immune system. Using the techniques

detailed above we can investigate how ACKR2 affects the innate and adaptive immune systems, and how it affects the response to both chemical and mechanical injury in the cutaneous tissue.

3.4 Results

3.4.1 Wound Healing Model

3.4.1.1 Wound Closure

We began our investigation by looking at wound closure over time in wild type and ACKR2 null mice. This involved making a full skin thickness wound and taking photographs of the wounds at time points until closure had occurred. To ensure a full thickness wound was made without damaging subcutaneous tissue, a skin fold was taken and a biopsy punch made through the entire fold. This method resulted in two wound sites. A representative set of images is shown below, in figure 3.1, to illustrate our findings.

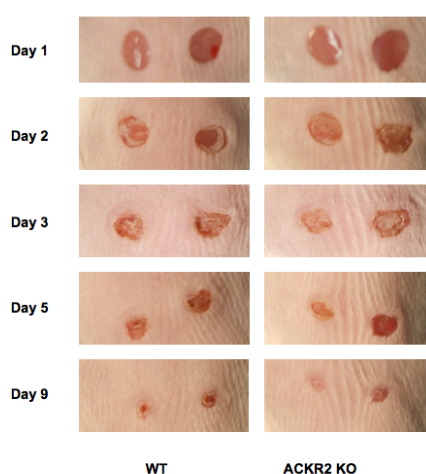


Figure 3.1- The images above depict the rate at which ACKR2 null and WT wounds healed in FVB mice. This is a representative image of many similar models that were also carried out in C57BL/6 mice. This image was selected as it is easier to see closure in FVB mice than C57BL/6 due to lower levels of skin pigment.

The first clear and consistent observation across all of these studies was that the healing kinetics of each genotype were very similar. The images collected in this process are more of an objective observation and they consistently suggested that a lack of ACKR2 resulted in no change to temporal healing kinetics. In order to gain quantifiable data from these images we used computer software to

measure the wound circumference. The graph shown in figure 3.2 below summarises data from multiple studies. The average for each time point in each study is taken to generate the graph below.

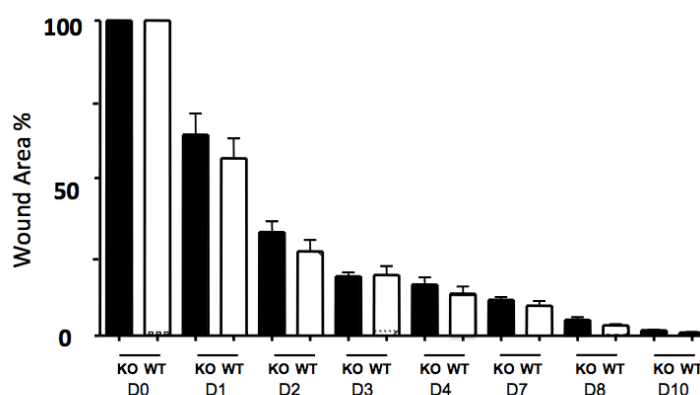


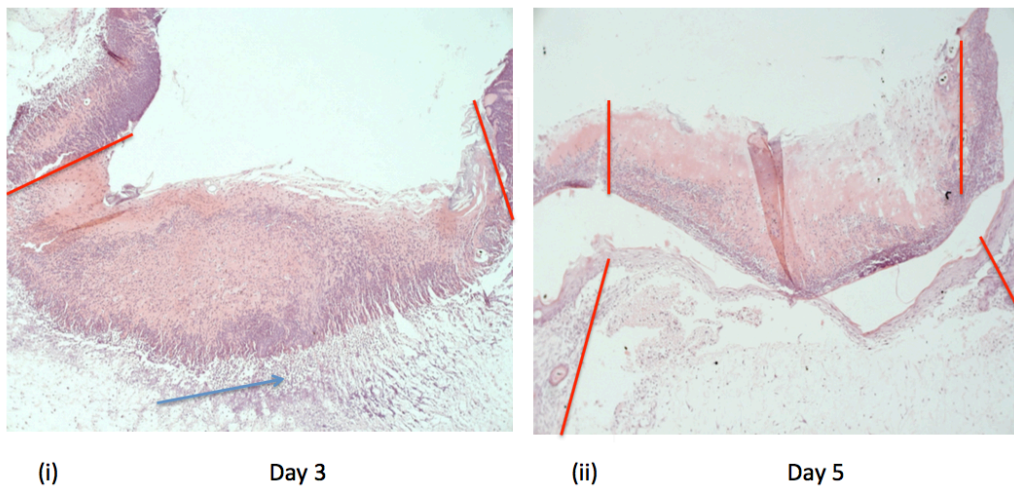
Figure 3.2- The above graph shows quantitative analysis of wound closure. This plot shows wound closure measurements taken from macroscopic images taken over time during the healing process. The data are a representative of multiple studies and suggest minimal differences between the temporal kinetics of wound closure between genotypes. The data above is a representative example of three repeated procedures. Data analysed by ANOVA. n=3

In keeping with the macroscopic images these data show little difference in temporal resolution between wild-type and ACKR2-null mice. Measurements taken to the point of wound closure are comparable despite the presence or absence of ACKR2. This graph in figure 3.2 suggests that the ACKR2 receptor has a minimal role in the closure of wounds at the early stages of healing. Given this we wanted to investigate the wounds further to see if there were any less obvious phenotypic changes in the recovering wound tissues.

3.4.1.2 Wound Histology and Immunohistochemistry

The local and recruited cells at the site of the wound shape the efficiency and ultimate success of the healing process. It was important to investigate any changes in cellular profile of the wounded tissue at different time-points throughout the wound healing process. We did this by quantitative histology, immunocytochemistry and brightfield microscopy. The first stain we carried out was haematoxylin and eosin and this can be seen in figure 3.3.

A



B

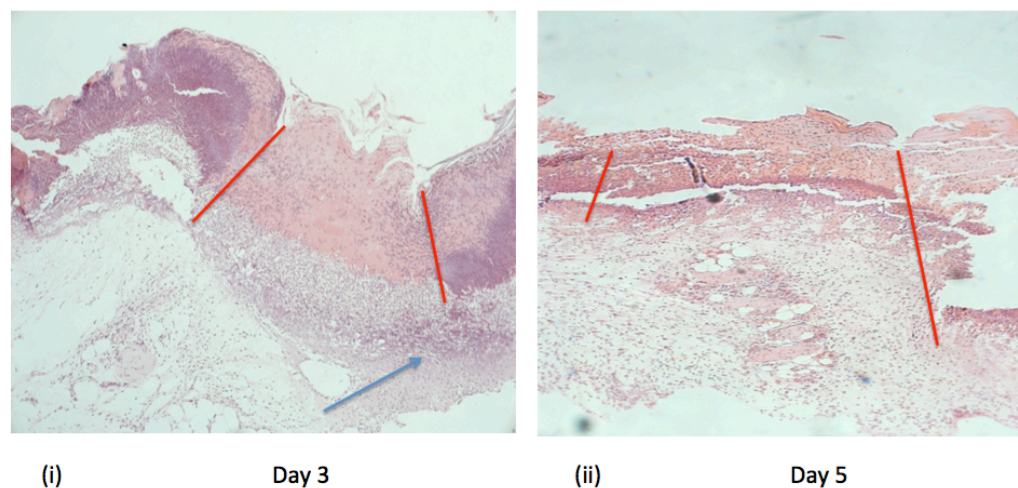


Figure 3.3- Haematoxylin and Eosin staining of early recovering wounds shows little difference- A) Shows representative images of WT mice at day 3 (i) and day 5 (ii) post-wounding. B) Shows the images from the same time-points in ACKR2-null mice. There are more infiltrating leukocytes at the wound margin below the developing scar tissue in the ACKR2-null mice. Red line depicts the wound margin and blue arrow shows area of infiltrating leukocytes. The images above represent findings from three repeated studies. Images taken at 100x magnification.

The images in figure 3.3 indicate that there was an increase in the number of infiltrating cells to the ACKR2-null wounds (fig 3.3B) at day 3 (i) post-wounding compared to that seen in WT mice (fig 3.3A). It is worth noting that this disparity between the genotypes seems to have disappeared by the next time point which was day 5 (ii). Images from WT and ACKR2-null mice at day 5 look similar in terms of cellular recruitment. We wanted to look into this in more detail by investigating more specifically at individual leukocyte populations to see if there were any changes in specific cell recruitment.

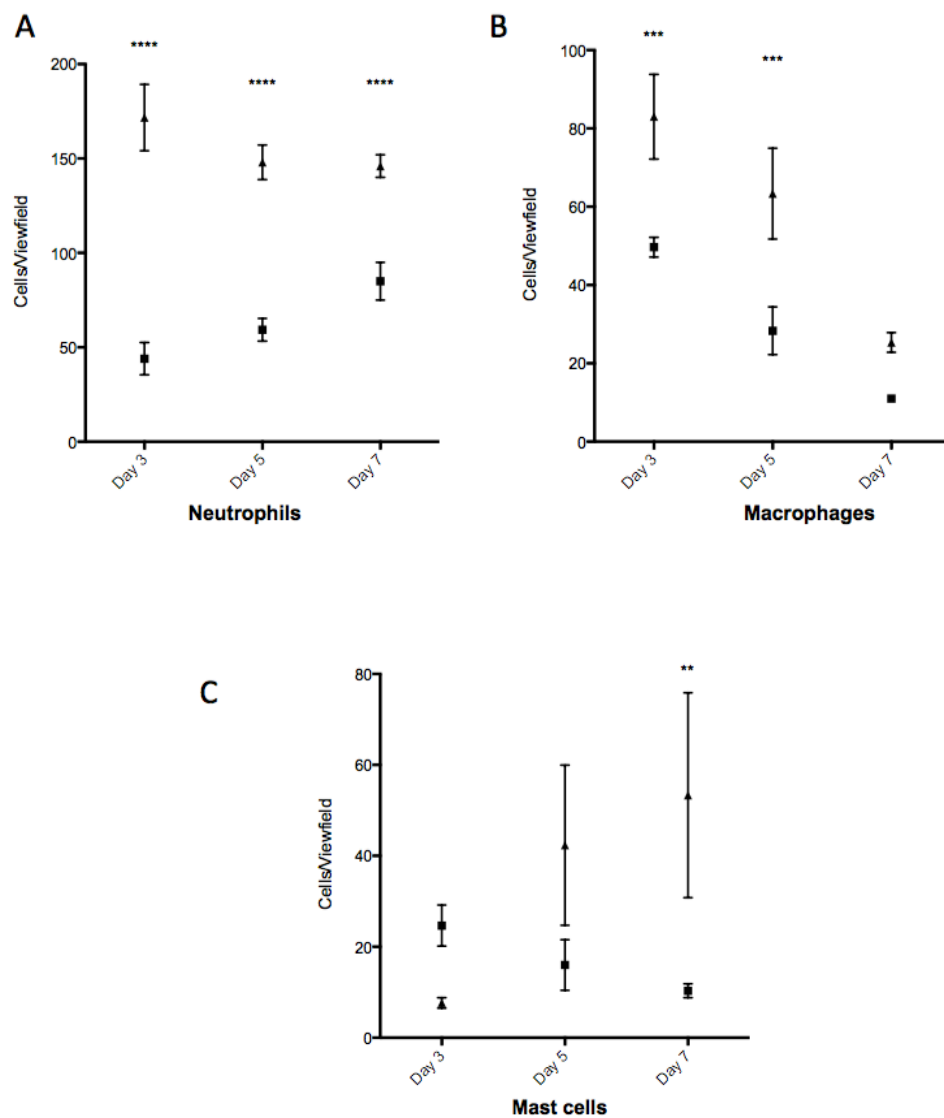


Figure 3.4- ACKR2-null mice (triangles) show increased leukocyte infiltration to wound compared to WT mice (squares). Cells were enumerated by taking an average count of cells over multiple high power fields (400x). (A) shows significantly more neutrophils at day 3, 5 and 7 (B) shows macrophages and again the ACKR2-null mice have an increase in recruitment of this cell type to the wound (C) ACKR2-null mice have increased mast cell infiltration to the wound bed also. Data was analysed using a two-way ANOVA. The data above is representative of three repeats and examples of the staining enumerated can be seen in appendix 3. n=3. (**p<0.01,***p<0.001,****p<0.0001)

Figure 3.4 shows counts of individual leukocyte populations infiltrating the wound site. The specific cells investigated were neutrophils (3.4A), macrophages (3.4B) and mast cells (3.4C). As can be observed in figure 3.4A there is a significantly higher number of neutrophils arriving at the wound site and this appears to remain the case throughout the three time points examined, although this difference lessens with time. Macrophages, shown in figure 3.4B are also

present in higher numbers in ACKR2 null mice when compared to WT mice. Interestingly, the rate at which the presence of these cells declines is very similar between ACKR2 null and WT mice; there is simply a higher number of these cells at every point in the ACKR2 null mice. Finally, mast cells (figure 3.4C) are also present in greater numbers in ACKR2 null wounds throughout the model.

Given the literature surrounding wound healing (Daley et al., 2010), we decided on the basis of these data to further investigate the roles for macrophages (3.4B) in wound healing in wild type and ACKR2 null mice. One of the key roles macrophages play in wound healing is in releasing factors that contribute to collagen synthesis (Rohani et al., 2015). Given the significant increase in macrophage numbers in ACKR2 null mice throughout the model, we decided to assess the collagen content and organisation in the mature and developing scars of ACKR2 null and WT mice.

3.4.1.3 Collagen Staining

During the repair process newly synthesised collagen fibres are laid down in the recovering tissue. This is the beginning of the formation of what will eventually become scar tissue. To assess any possible contributions that ACKR2 was making to this process we stained developing scar tissue with picrosirius red (PR). This histological technique stains type I and type III collagen fibres both of which are vital in wound repair.

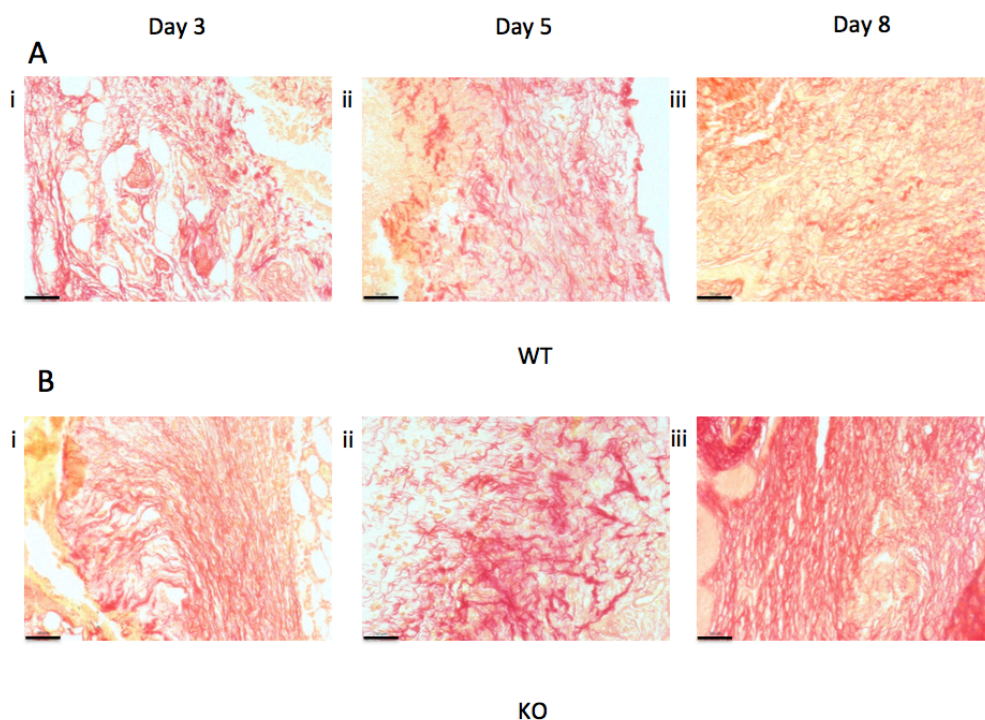


Figure 3.5- Developing collagen fibres of ACKR2-null mice become disorganised at day 8 post-wounding- The images above show picrosirius red stained recovering wound tissue. A) is WT and (B) is ACKR2-null at the time points listed. Images were taken at 200x.

Figure 3.5 shows the developing collagen fibres through time as the wound heals. It can be seen that at day 3 in WT (3.5Ai) and ACKR2 null mice (3.5Bi) there is no discernable difference in collagen profile. As one looks at images from day 5, 3.5Aii and 3.5Bii, there appears to be a phenotype emerging. The ACKR2 null mice appear to have a higher number of larger collagen fibres. By day 8 (iii) it can be observed that the ACKR2 null wound (3.5Biii) clearly has a greater number of collagen fibres than WT (3.5Aiii). These data suggest that a greater number of collagen fibres are being produced in ACKR2 null mice during the wound healing response and that this, at least in part, contributes to the disorganisation phenotype shown in these mice when compared to WT mice.

3.4.2 Scarring Phenotype in ACKR2-Null Mice

We carried out the same wounding protocol as discussed earlier but allowed the wounds to heal until day 21. By this time the wound was healed and the epidermal barrier had been restored. The images below in figure 3.6 show a representation of the picrosirius red stained tissue from this assay.

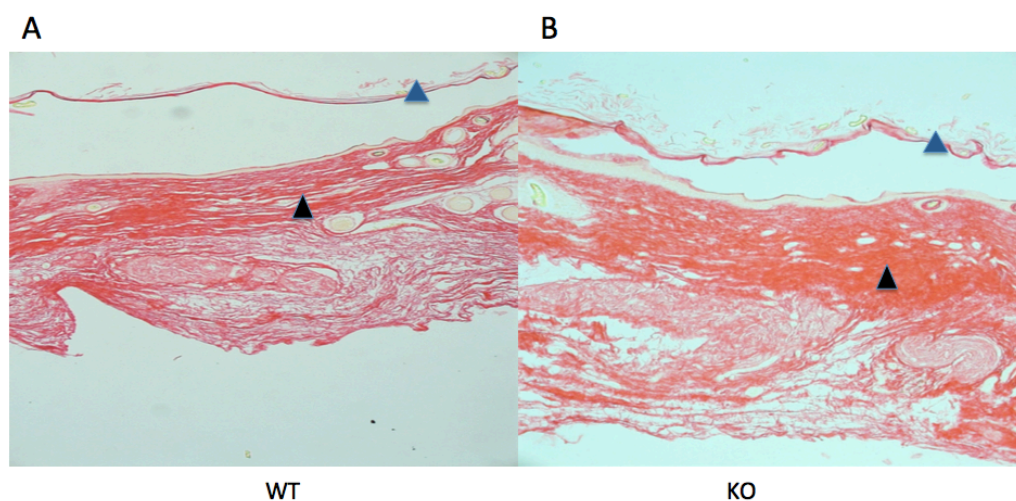


Figure 3.6- Disorganised collagen fibres in the newly-formed scars of ACKR2-null mice at day 21 post-wounding- A) WT and B) ACKR2-null mice. Scars at day 21 in the null mice show a widespread disorganisation in collagen fibrils compared to WT (black triangle). Blue triangle depicts keratinocyte layer. These images represent a phenotype which was repeated across two separate studies. Images taken at 100x magnification.

By looking at the tissue at a later time point a phenotype was apparent in the ACKR2-null mice. At day 21 of repair the collagen fibres in the scar tissue seemed to be less organised in the absence of ACKR2 (highlighted by the black triangle) than in wild type mice. This could be due to a greater number of collagen fibres being synthesised or it could be purely down to lack of organisation as they are synthesised. The data shown here did not allow us to reach any conclusion on this. As a result we wanted to attempt to elucidate the cause of this phenotype. In order to do this we first carried out PR staining of wounds at earlier time-points to observe the development of the collagen fibres over time.

We wanted to look into the possible causes of this hypercollagenous response. We did this using QPCR assays on samples of skin from each of the time points to ascertain if any particular changes in transcription of key genes were contributing to the phenotype.

3.4.2.1 Wounding Model Quantitative-PCR

We analysed expression of five different genes to assess differing facets of the wound healing response. This technique allowed us to address the changes that

ACKR2 was making on the transcription of certain genes that appear to be important to wound healing. The results from these assays are shown below.

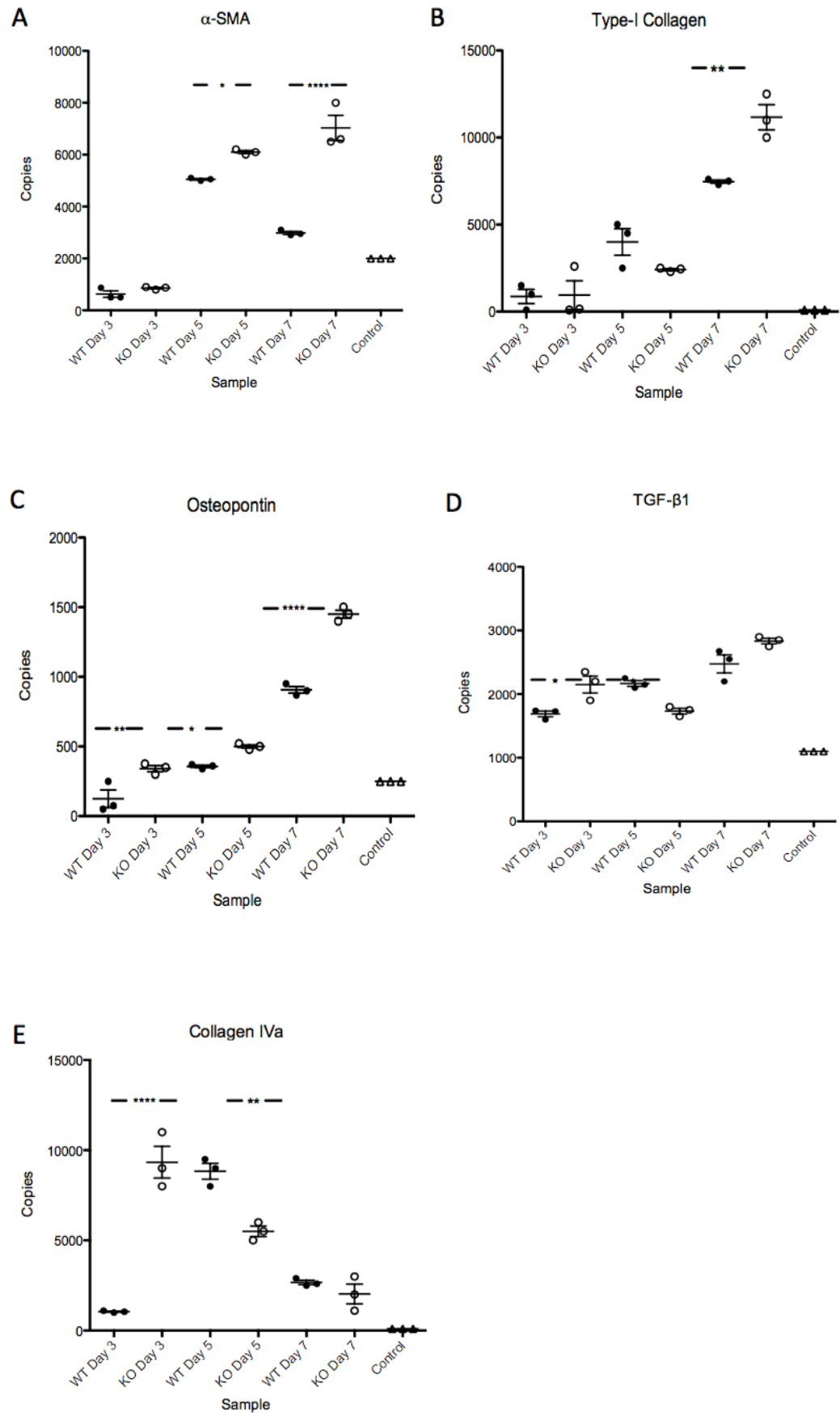


Figure 3.7- QPCR of key genes involved in the wound repair response. (A) α -SMA levels are minimal at day 3 post-wounding. At day 5 there is significantly higher transcription of α -SMA in the ACKR2 null mice than WT and at day 7 post-wounding there is markedly more transcription of α -SMA in the ACKR2 null mice than in the WT mice, this is also significant. (B) There is no significant difference in the transcription of type I collagen between WT and ACKR2-null mice until day 7 post-wounding when there is significantly higher levels in the ACKR2-null mice compared to WT. (C) The levels of osteopontin transcription are significantly higher at all time points in the ACKR2-null mice compared to wild type mice. (D) At day 3 there is significantly more transcription of TGF- β 1 in ACKR2-null mice compared to WT. This is reversed at day 5 and the WT mice have significantly more copies of TGF- β 1 than ACKR2-null mice. By day 7 the difference is not significant. (E) At day 3 post wounding the collagen IV α response in the Ackr2-null mice is significantly increased compared to wild type mice. In Ackr2-null mice the transcription of this gene decreases and there are significantly less copies of osteopontin in ACKR2 null mice compared to wild type mice at day 5. In the WT mice, transcription spikes at day 5 and drops to a similar level as Ackr2-null mice by day 7. Data was analyzed using a two-way ANOVA. n=3. (p<0.05, ***p<0.001, ****p<0.0001)

In the first graph displayed above in figure 3.7A, α -SMA, the copy number seen at day 3 is lower than the untreated control skin; this suggests an active reduction in transcription at this time-point. This phenomenon seems to hold true regardless of the absence of the Ackr2 gene. The transcription of α -SMA is significantly increased in ACKR2 null mice compared to wild type mice at day 5. At day 7 one can see a large divergence in the transcription levels of α -SMA, the presence of Ackr2 is associated with a major drop in transcription but the absence seeing a large rise in transcription, this difference is statistically significant.

Figure 3.7B shows data for the type-I collagen QPCR. In wild-type mice a steady increase in transcription can be observed with time. This holds true for the Ackr2 null mice in that, from day 3 to 5, expression follows a very similar progression to the wild-type counterparts. It is between days 5 and 7 that a dramatic rise in collagen transcription is seen in Ackr2 null mice compared to WT mice. There is around a five-fold increase in Ackr2 null mice transcription between these days and significantly more transcripts in ACKR2 null mice compared to wild type mice.

The osteopontin graph in figure 3.7C reveals a similar pattern to the type-I collagen graph; however at each time point here there is significantly more osteopontin transcription in ACKR2 null mice compared to wild type mice. Again the WT samples show a steady increase in transcription with time, as do the Ackr2 null mice up until day 7 again. In this graph there is a three-fold increase

in gene copy number between day 5 and day 7 in the Ackr2 null mice compared to WT mice.

The penultimate graph (figure 3.7D) shows transcription levels of transforming growth factor (TGF)- β 1. In Ackr2-null mice expression appears erratic. On day 3 there are significantly more TGF- β 1 transcripts in Ackr2 null mice than in WT mice. This phenotype then appears to be reversed as the next time-point of day 5 where there is significantly more TGF- β 1 transcription in the wild type mice compared to ACKR2 counterparts. The transcription levels of TGF- β 1 at day 7 are almost double those observed at day 3. At all time-points there is a relative increase in gene copy number when compared to the control sample.

Finally, figure 3.7E shows the expression of collagen type-IV α . At day 3 the wild type mice show only a small increase in transcription compared to the control sample. The equivalent Ackr2 null sample is showing significantly greater transcription than the wild type sample. At day 5 wild type mice have significantly increased levels of transcription of this gene compared to ACKR2 null mice and have even reversed the transcription levels of day 3. By the seventh day the two groups are transcribing the gene at similar levels.

3.4.3 Wounding Model with Macrophage Depletion

In an attempt to understand the mechanism of action of ACKR2 in the scarring phenotype we decided to target phagocytic cells, namely macrophages. The data presented so far seemed to suggest a role for an increased macrophage response in the absence of ACKR2. We wanted to address if the scarring phenotype observed between ACKR2 null and WT mice was due to the recruitment of these cells as it has been shown that they can affect scar formation (Rohani et al., 2015). We did this by depleting macrophages using clodronated liposomes and carrying out the previously used wounding model.

Our hypothesis here was that a lack of ACKR2 was driving an increase in macrophage recruitment to the wound site. This was resulting in an increase in pro-fibrotic factors such as TGF- β 1, and by reducing the number of these cells the phenotype could be reversed. As described in detail in the 'Materials and

Methods' section we injected the mice with clodronated liposomes to deplete phagocyte numbers.

When this emulsion is injected into the animal, phagocytes engulf the liposome. Once inside the cell this degradation leads to the release of the drug. The drug, clodronate, induces apoptosis in the cell and so depletes the numbers of any cell that phagocytoses the liposomes. For this work the control mice received liposome injections but these liposomes were filled with PBS rather than the drug.

3.4.3.1 Wound Healing Kinetics in Macrophage Depleted Mice

We collected images of the wounds, as before (see figure 3.1), every second day during the healing process to investigate any changes in repair kinetics brought about by the lack of phagocytes. The circumferences of the wounds were then measure, as per figure 3.2, and are shown in figure 3.8 below.

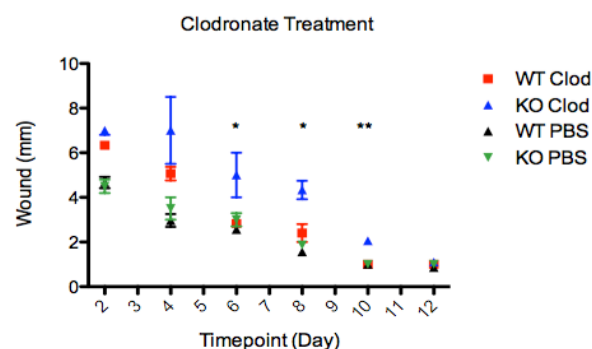


Figure 3.8- The graph above shows measurements of the wound circumference from images taken of the healing wound at the time points displayed. It is clear that the ACKR2 null mice that have been treated with clodronate have a delayed wound closure rate but that after 12 days this difference is no longer apparent. Data analysed by two-way ANOVA and stars shown represent WT clod vs. KO clod measurements at the given time point. $n = 3$. (* $p < 0.05$, ** $p < 0.01$)

The graph shows an interesting phenotype brought about by the lack of phagocytic cells. The data show that, in the absence of macrophages, ACKR2 null mice have significantly delayed wound closure when compared to wild-type mice with and without these cells and ACKR2 null mice with macrophages.

After leaving the wounds for twenty-one days to fully heal and form mature scars the animals were culled and the skin harvested. We then took this skin and stained the scar tissue with picrosirius red. As before we were analysing the collagen fibrils in the scar. The pictures below (figure 3.9) show the resulting collagen formation.

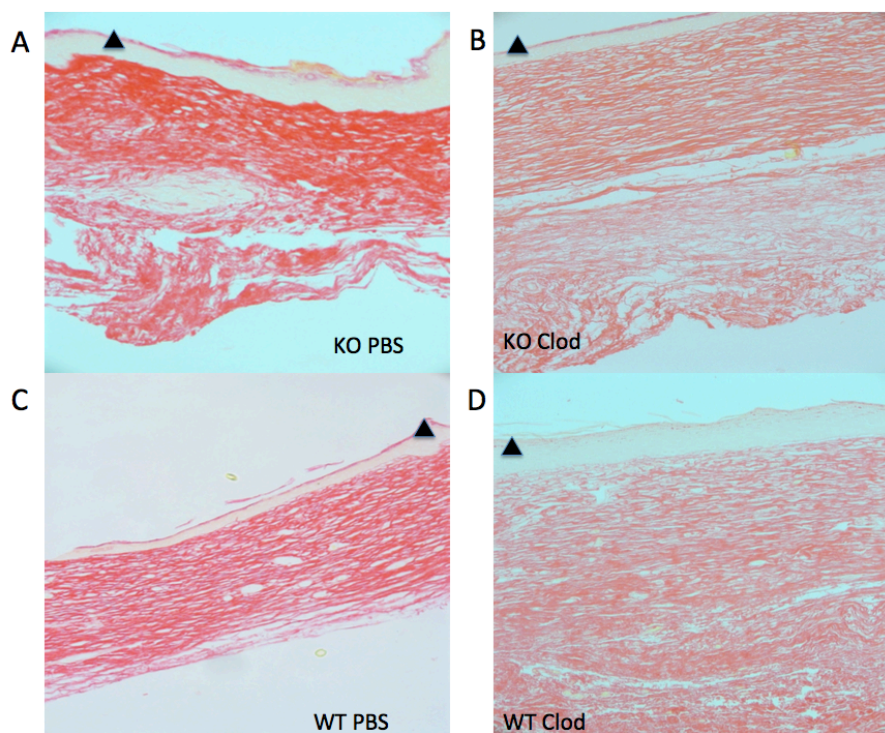


Figure 3.9- The images above are representative examples of scar tissue 21 days post-wounding. The sections are stained with picrosirius red for histological visualisation of collagen fibres. It can be seen that both WT sections have uniform and organised collagen. ACKR2 null mice treated with clodronate have a very similar appearance to WT. The ACKR2 null mice that have been given PBS control have disorganised collagen in the scar tissue with thicker and disorganised fibres. Black triangle depicts the keratinocyte layer. PBS images taken at 100x magnification and clodronate images taken at 200x. These findings were consistent across two repeats. n=3.

The staining shows that whilst in PBS treated mice the ACKR2 null mice display disorganised collagen within the skin this phenotype is reversed by depletion of phagocytic cells. This suggests that by depleting these cells the wound can heal normally and produce a scar of wild type-like quality. The converse of this of course is that during normal wound healing, i.e. in the presence of phagocytic cells, the ACKR2 receptor has an important effect in the mechanism of the phenotype by normalising the magnitude of phagocyte recruitment.

Our results here suggest that the difference observed in the formation of scar tissue between wild type and ACKR2 null mice is down to the inflammatory cells that are accumulating at the damage site. We postulate that in the presence of ACKR2 fewer phagocytes, probably mostly macrophages, are being recruited. This, in turn, results in a more appropriate level of macrophage-derived growth factors in the local area and prevents a hyper-response from stromal cells.

The results above suggest that whilst a lack of ACKR2 seems to play no role in the kinetics of wound closure, it does, through a macrophage-mediated process, have an effect on the eventual scar tissue.

3.4.3.2 Tail Pathology in the Macrophage Depletion Wounding Model

During the frequent injections needed for this work we noticed that the mice displayed inflammation in the tail and that this was more pronounced in the ACKR2 knockout mice. In 3.10A one can see that the tails of clordronate-injected mice both of wild type and ACKR2 null backgrounds were thickened when compared to PBS treated counterparts. It can also be seen that the clordronate treated ACKR2 null tails have an increased diameter compared with clordronate treated wild type tails although this is not significant. Microscopic analysis showed that the pathology resembled psoriasis with lesions of hyperproliferative epidermis including hyperkeratosis in 3.10B (highlighted by the white triangle). Increased leukocyte infiltration could also be seen (black triangle) in ACKR2 null tails treated with clordronate. Neither of these findings were seen in clordronate treated wild type tails. In addition to this the ACKR2 null tails were becoming grossly inflamed towards the end of the time-course of the experiment. Interestingly, these lesions were only present in knockout animals whereas the wild type counterparts showed some oedema but little else.

We next considered the possible cause. Obviously the tails of these mice were subject to mechanical trauma with injections every forty-eight hours but we were unsure how much the drug was contributing to this tissue damage. We wanted to look more closely at this phenomenon.

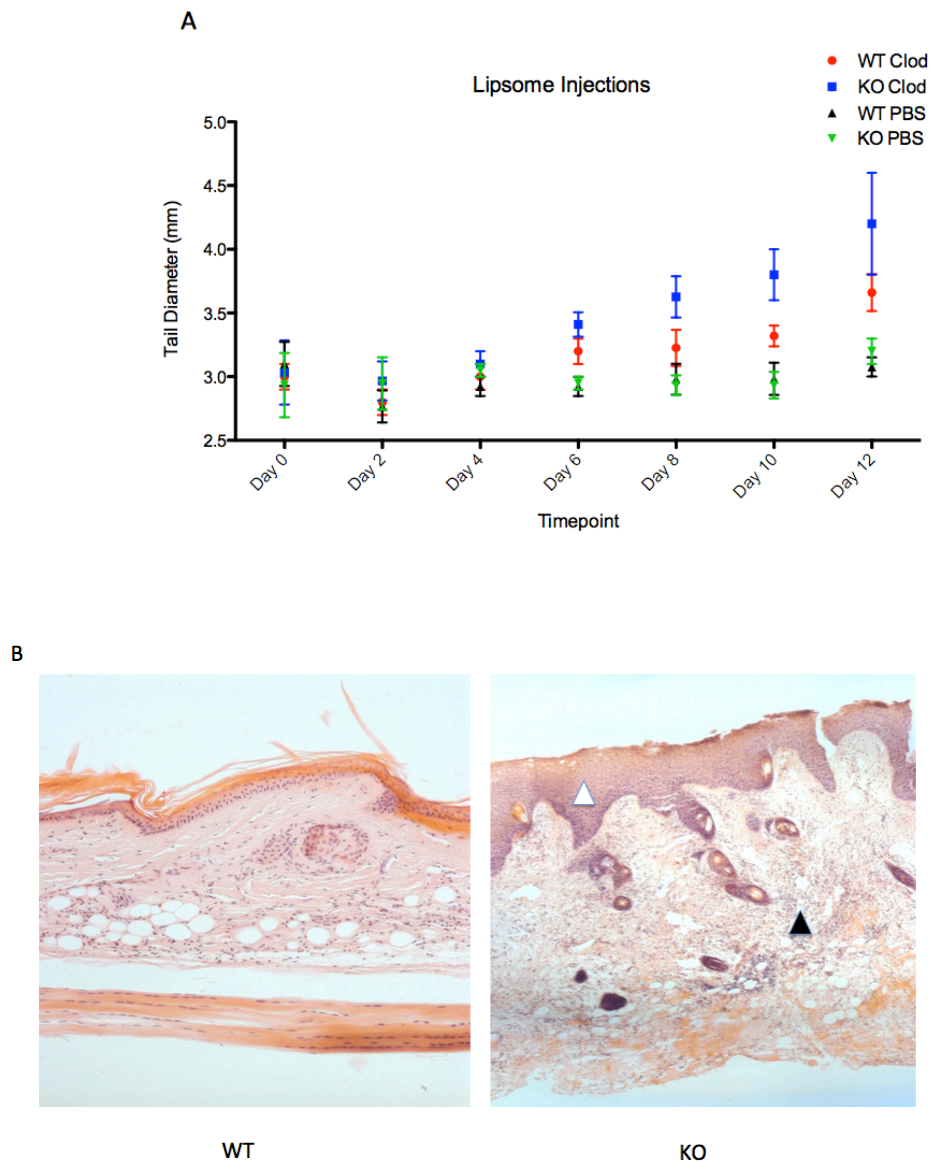


Figure 3.10- Multiple injections during the clodronate model caused inflammation in the tails of the animals. The above images show the quantification and histology of this. A) Tail diameters were measured every 48 hours during this model. The tails of the ACKR2 null mice receiving clodronate became thicker but this did not reach significance. The WT mice receiving clodronate also had inflamed tails. B) Tail tissue was stained with H&E to gain insight into the inflammation. The KO mice had a psoriasisform inflammation in the tail including hyperproliferation of keratinocytes (blue triangle) and leukocyte infiltration (black triangle). Histological Images taken at 100x magnification. Data analysed by two-way ANOVA with a Bonferroni post-test. n=3.

Two groups (one wild type and one ACKR2 null) received the same liposome injections as before, and the other mice received injections of PBS, as discussed. As can be observed in the figure 3.10A, the tail girth of the mice receiving the PBS injections remained at a constant diameter throughout the protocol. Both WT and ACKR2 null mice receiving injections with clodronate had a clear

inflammation of the tail with ACKR2 null mice showing increased oedema when compared to WT mice. This suggests that the inflammatory reaction is related to chemically induced trauma rather than the mechanical damage caused by the injections. However, after analysis, the difference in girth between wild type and ACKR2 null mice was not significant.

3.4.4 Compound 48/80 Experiment

The potent mast cell degranulator compound 48/80 was used to induce a wound similar to that of a burn in the dorsum of the mice. This experiment was designed to give insight into the role of ACKR2 during this form of trauma. Mice were injected intradermally with 20 μ l of the reagent at 10mg/ml at two sites on the dorsal skin using a small volume syringe. The mice were left for four days to allow the action of the compound 48/80 to cause cutaneous pathology. After this time the mice were culled and 5mm biopsy punches of the injection site and surrounding areas were harvested for histological examination.

Macroscopically it was obvious that the ACKR2 null mice were showing increased inflammation as lesions were visible on the dorsal epidermises that were not present in wild type mice. However, by staining the tissue biopsies for H&E it was clear how extensive this increased response was. This is highlighted by the white triangle in figure 3.11Aii showing the marked deposits of invading leukocytes.

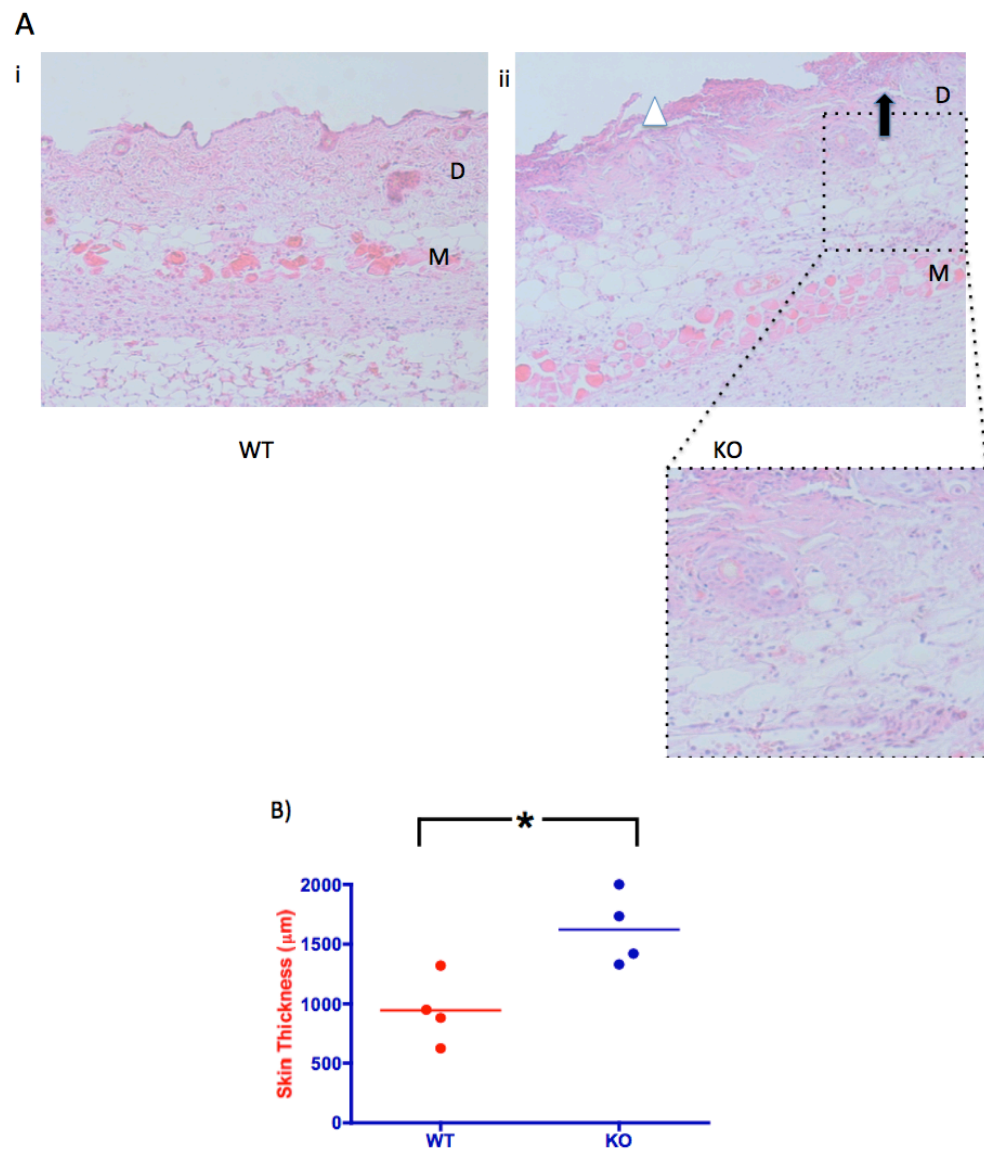


Figure 3.11- Animals were given intradermal injections of 10mg/ml of compound 48/80. A) H&E stained tissue of the injection site. There is a moderate inflammation in the WT tissue (i) but an exaggerated inflammatory phenotype in the ACKR2 null mice (ii) including degradation of the epidermis (white triangle) and leukocyte dermal infiltration (black arrow). B) Slides were blinded and full thickness measurements of the skin were made. The ACKR2 null mice have significantly thicker skin during this model. D- dermis, M- muscle. Histological images taken 100x magnification. Data analysed by Student's t test. n=3. (*p<0.05)

Figure 3.11A depicts the damage caused by the mast cell degranulation in WT and ACKR2 null mice. The figure suggests that the increased accumulation of leukocytes has lead to massive tissue damage at the injection site in the ACKR2 null mice (3.11Aii). There is degradation of the epidermis in both genotypes associated with this degranulation. There is a major accumulation of immune

cells gathering at the dermal-epidermal margin (black arrow) and deeper down in the dermis and the epidermis is disrupted in ACKR2 null mice (white triangle). In the mice lacking ACKR2 it is clear that the degradation of tissue is spreading deeper than the epidermis and starting to affect lower levels of tissue. The tissue architecture in the two pictures is very different. The inflammation in the WT mice is tightly regulated (3.11Ai) whereas, in comparison, in the mice lacking ACKR2 a hyper-inflammation is seen. Figure 3.11B shows that oedema was increased in ACKR2 null mice at the compound 48/80 injection site when compared to wild type mice.

The results above demonstrate that, without ACKR2, mice are unable to clear the inflammatory signals caused by release of histamine. This could imply that ACKR2 could have a protective role in the reaction to a cutaneous burn.

3.4.5 Allergic Contact Dermatitis

The hapten 2,4-dinitrofluorobenzene (DNFB) was used in a model of allergic contact dermatitis. Mice were primed with DNFB in an acetone and olive oil mix (vehicle), which was painted onto the ventral skin. This was followed with a challenge, seven days later, to the right ear. The left ear received vehicle only as an internal control.

The reason for doing this was to determine the role of ACKR2 in the induction of an adaptive response to a cutaneous antigen. All work presented thus far has focused mainly on the innate arm of the immune system. This also allowed us to look at the role ACKR2 is playing in a different anatomical cutaneous location.

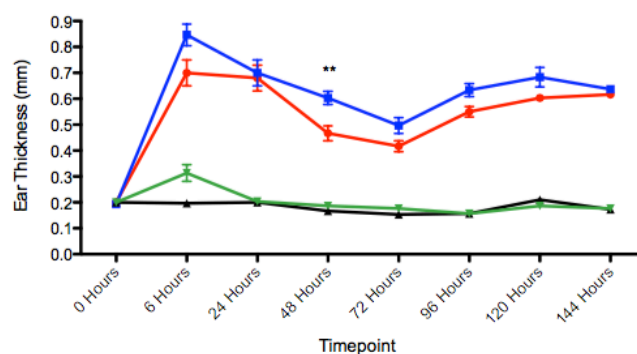


Figure 3.12- WT and ACKR2 null mice underwent a delayed-type hypersensitivity model. The right ear of each animal was inflamed and the left ear was treated with vehicle as an internal control. The graph clearly shows inflammation in the right ear, when compared to the left, but little difference between WT and KO mice. Although there appears to be a statistical difference between groups at 48 hours post-challenge this is not thought to be of biological relevance as it is not consistent across two repeats. Data analysed by two-way ANOVA with Bonferroni post-test. n=3. (**p<0.01)

Red line- WT right ear, Blue line- ACKR2 null right ear, Black line- WT left ear, Green line- ACKR2 null left ear.

The graph of ear thickness vs. time in figure 3.12 shows a clear inflammatory response in the treated (right) ear compared to that of the control (left) ear. The treated ears of the ACKR2 null mice were, in general, thicker than their wild-type counterparts however this only reached statistical significance at the forty-eight-hour time-point. This significance is not thought to be of any biological relevance as it was not a consistent finding across repeated studies. Both genotypic groups showed the same levels of inflammation after challenge, although a pattern of slightly increased inflammation in the ACKR2 null mice can be seen throughout the time points shown in figure 3.12.

3.5 Discussion

3.5.1 Wound Healing

The wound-healing model that was carried out for this thesis gave intriguing results. We initially investigated the differences in the temporal healing kinetics involved in the repair of wounds in wild type and ACKR2 null mice. The results we obtained from this work implied that a lack of ACKR2 does not result in any significant change in wound closure times.

We next decided to look at the key outcome of the wound healing process and investigated scar structure and quality. To do this we looked at the scar tissue three weeks after the wound induction. We noticed that a lack of the ACKR2 receptor resulted in a disorganisation of collagen structure in the newly formed scar. We then tried to identify the mechanism whereby ACKR2 was contributing to this phenotype by ablating phagocytes in the wound model.

Removal of these cells reversed the scarring phenotype in the ACKR2 null mice and resulted in well-structured collagen fibrils in the scar tissue, equivalent to those seen in wild-type animals. These results make sense as an over-expression of macrophage-produced growth factors have been shown to lead to hypertrophic scar production. During the last stage of the wound repair response, remodelling, the collagen fibres laid down at earlier stages are re-aligned and shaped to provide the highest quality scar possible.

A key question that now needs to be answered about this work is how the absence of ACKR2 affects the functionality of this scar tissue. The tensile strength of a scar is a key aspect of its functionality as a new barrier. Skin has to be tough and able to withstand mechanical stresses, the extent of which depends upon anatomical location. As mentioned previously, scar tissue does not achieve the same level of tensile strength of unaffected healthy skin, however it can display over 90% of this strength during optimal healing (Hardy, 1989). Any healing process that reduces this further would be a problem for the host. The disorganisation of the collagen fibrils in the ACKR2 null scar tissue may lend itself to reduced tensile strength but this has not been formally tested.

It also has to be assessed whether these scars are cosmetically different to wild type scars. Hypertrophic or keloid scars can be unsightly and can distress patients that are unfortunate enough to have them. Both of these factors are yet to be assessed.

An excellent next step would be to test the effect of over-expression of the ACKR2 receptor during the wound healing process. An improvement in scar generation would be an important clinical finding. Transfection with an ACKR2

expressing lentivirus, for example, into local cells could be another possible avenue to explore for over-expression studies.

Although the work we have presented so far suggests that an absence of ACKR2 expression in the system may play a role in the resultant scar, we would like to identify the separate contributions of this absence made by immune cells and stromal cells. We could achieve this by setting up bone marrow chimeras. This would involve transplanting ACKR2 null mice with wild type bone marrow- giving ACKR2 null stroma and ACKR2 positive immune cells- and a wild type mouse with ACKR2 null bone marrow- giving ACKR2 positive stroma and ACKR2 null immune cells. This would allow us to assess the individual contributions of ACKR2 from these two cell-types.

Macrophages are key to the process and a lack of ACKR2 could be causing an increased recruitment of these cells to the wounded tissue, then downstream of this there are more growth factors being released by these cells. This is just a hypothesis but it is, however, logical when taking current literature into account.

3.5.2 Compound 48/80

It would seem from our results that mast cell degranulation in ACKR2 null mice is causing local inflammation that is not being controlled when compared to wild type mice. The animals lacking ACKR2 are unable to scavenge, to the same extent, the inflammatory chemokines being released by local tissue cells subsequently increasing the extent of cellular influx. In a positively regulated loop type mechanism this is then causing an increase in inflammatory markers in the local area and therefore an increase in tissue damage. The extent of this damage was so great that the epidermis had almost been completely lost and lesions had formed on the dorsal skin of the mice. This was not the case with the wild type mice.

3.5.3 Allergic Contact Dermatitis

The allergic contact dermatitis model carried out on wild type and ACKR2 null mice gave us inconclusive results. They did, however, show that there was a

possible trend towards increased inflammation in the ACKR2 null mice although, apart from 48-hours post challenge, this was not significant. It would be worth repeating this model with larger groups of mice in order to confirm if this was a biologically relevant trend or not.

One could also repeat this model and add histological analysis to the data as well as the ear thickness measurements. It would be interesting to see if there were any differences in the amount of leukocytes being recruited to the challenged ear. Finally, given the work done by Lee et al, looking at the cellularity of the local draining lymph node would be of interest (Lee et al., 2011). This paper suggested that a lack of ACKR2 results in leukocytes binding to the lymphatic vessels and preventing the efficient transit of fluid and cells out of the tissue and into the lymphatics (Lee et al., 2011). There is a chance that this is having an effect on the phenotype shown here and so would be worth investigating.

Chapter 4- The Role of ACKR2 in Ocular Inflammation

Chapter 4 Experimental Autoimmune Uveitis

4.1 Introduction

4.1.1 The Eye

The eye is a sensory organ and during embryonic development the optic nerve and the retina are outgrowths of the brain. For this reason the eye is considered part of the central nervous system (Standring, n.d.). The anatomy of the anterior (the part of the eye that faces the outside world) of the eye has evolved to both control and manipulate light to optimise the amount that ultimately hits the retina, the site at which light signals are converted into nerve impulses. The cornea (the exposed tissue of the eye) has both a protective and lens-like role as light first hits the eye at this site. After this the components of the eye that are most recognisable such as the iris, the pupil and the sclera (the white of the eye) play their parts. The pigmented epithelium of the iris (which gives colour to eyes), and the pupil sizes, vary to control the amount of light the eye is letting in. When the light passes through the pupil and into the eye itself it passes through the lens. Like any lens the purpose of this tissue is to help focus the incoming light onto the retina. The only thing between the lens and the retina is the vitreous humour. The word vitreous is Latin for 'glass-like' which accurately describes the gelatinous mass that aids the transit of the light from the lens to the back wall of the eye. The retina is located at the posterior of the eye and is neural tissue. The location of these tissues can be seen in figure 4.1.

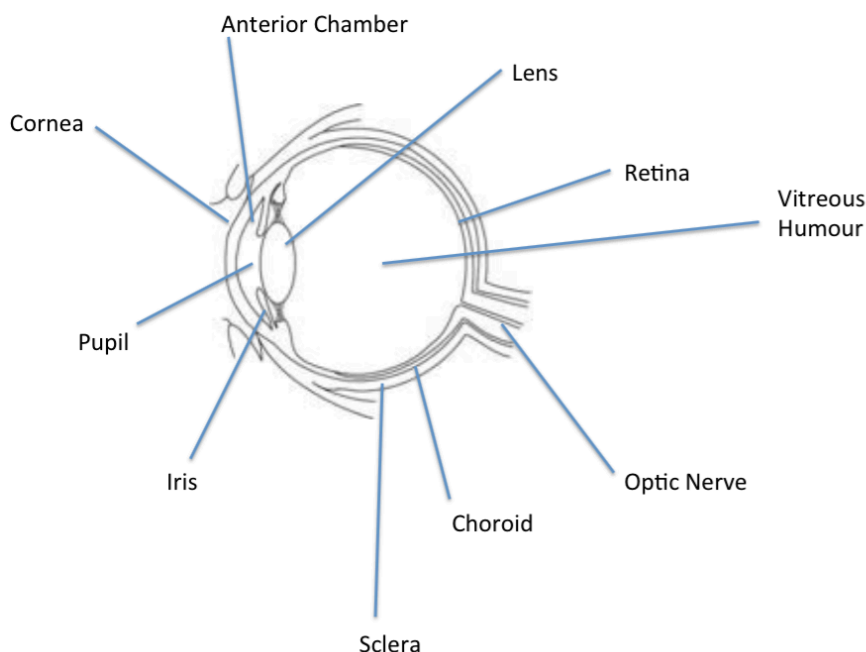


Figure 4.1- The Anatomy of the Human Eye- The figure above shows the key anatomical sites and tissues of the human eye.

The retina is a complex and multi-layered tissue that contains photoreceptor cells (Lens, Nemeth and Ledford, 2008). It is, in fact made up of ten discrete layers including the retinal pigmented epithelium which will be discussed in more detail later in this chapter. The two most abundant of the photoreceptor cells are the rods and the cones. Rods contain rhodopsin and are responsible for collecting information in low light; in fact they are almost entirely responsible for night vision (Remington, 2012). They are expressed more in the peripheral retina than the central retina and also have a significant role to play in peripheral vision. The cells responsible for distinguishing colour are the cones. In bright light the rods quickly become saturated by light signals and this is when the cone cells begin to work. There are three different types of cone in the human eye. One can receive long wavelength light (red), one medium (green) and one short (blue). The medium wavelength cones are the most abundant, a fact that is illustrated by the human eye's ability to distinguish more shades of green than any other colour (Remington, 2012). Photons that hit the retina are converted into neural signals by the resident ganglion cells and relayed to the

brain via the optic nerve. The retina has a high metabolic demand and as such receives a blood supply from both the central retinal artery and choroidal blood vessels (Remington, 2012).

4.1.2 Immunology of the Eye

The importance of sight needs no explanation and it is due to this that the eye has a unique immunological environment. It is said that the eye is an immunomodulating tissue, with particular activity in the pigmented cells of the iris and the retina (Caspi, 2010). It was Peter Medawar, the pioneer of transplantation, who first described the unique properties of corneal transplant and the fact that rejection was very rare in this tissue. Medawar placed skin explants into the anterior chamber of the eye and noticed this phenomenon, furthermore when he transplanted tumours into the same place he observed that they were able to grow almost unperturbed (Medawar, 1948). With further research the mechanism behind this became clearer and was named anterior chamber associated immune deviation (ACAID) by Streilein (Streilein, 2003). Continued work done by Kaplan and Streilein was key in defining this phenomenon. It was once thought that ocular immune privilege was due to an inability of the immune system to come into contact with retinal antigens. This is now known to be untrue as ocular peptides, including those explanted into the anterior chamber are presented to the immune system. In fact the peptides are presented by resident F4/80⁺ macrophages to B cells, NKT cells, CD4 and CD8 cells in the spleen (Caspi, 2010). This leads to peripheral tolerance to these peptides.

The uvea lies beneath the sclera (the white of the eye) in the middle of three layers that make up the outer portion of the eye. Its name comes from the Latin word for grape and is one of the main sites for resident ocular immune cells such as macrophages, dendritic cells and mast cells. This layer provides much of the blood supply to the eye and helps in the formation of the image on the retina by reducing reflection of light within the eye (Lens, Nemeth and Ledford, 2008). As previously discussed, the cornea is a highly specialised tissue. It is a so-called barrier tissue as it faces the outside world and will very frequently experience potential inflammatory situations but due to its functional role needs to be

completely transparent. The cornea has a fixed lens function and refracts light into the eye so any opacity will reduce the efficiency of this. The transparency of the cornea is so important that it has no direct blood supply. This tissue becomes oxygenated through its direct contact with the atmosphere (Lens, Nemeth and Ledford, 2008).

4.1.2.1 Pigmented Epithelium

The pigmented epithelium of the iris uses contact dependent interactions with inflammatory cells to modulate their action whereas the more posterior, retinal pigmented epithelium uses soluble factors (Gregerson *et al.*, 2007). In the iris pigmented epithelium molecules such as CTLA-4 bind active T cells and prevent activation (Gregerson *et al.*, 2007). The intraocular environment is highly immunosuppressive under physiological conditions. TGF- β 1 is expressed at relatively high concentrations in the fluid of the eye (Streilein, 2003). This cytokine also has a role to play in the other pigmented epithelium of the eye, namely that of the retina. Retina pigmented epithelium (RPE) serves many functions in the retina. The main role of RPE is to absorb scattered light and improve the function of the eye, it also provides nutrients to photoreceptor cells as well as phagocytosing them when damaged or dying. Finally, and most relevant to this work, the RPE uses soluble factors, like TGF to aid in the immunomodulation of the eye (Vega *et al.*, 2010).

This cytokine has multiple roles and has been shown to enhance the conversion of conventional T cells into regulatory T (T_{REG}) cells (Jonuleit *et al.*, 2002). Animal models of human uveitis have provided important insights into the molecular and cellular mechanisms that are in place to protect the eye from damage and what ultimately overcomes these processes during inflammation.

A small population of natural T_{REG} (nT_{REG}) cells has been shown to reside in the eye and it is thought that these aid the resistance to inflammation in this tissue (Grajewski *et al.*, 2006). nT_{REG} cells are formed and exposed to retinal antigens in the thymus in a process known as central tolerance (Avichezer *et al.*, 2003). During this process developing thymocytes, the precursors of T cells, are exposed to self-antigens expressed by medullary epithelial cells of the thymus

under the control of the transcription factor AIRE (Gotter *et al.*, 2004). Deficiencies in this gene have been shown to result in multi-organ autoimmunity (Mathis and Benoist, 2009). It has also been shown that it is during this process that these developing cells encounter retinal peptides for the first time (Avichezer *et al.*, 2003). If the antigen receptor (TcR) of a thymocyte reacts too strongly to a peptide it is deleted from the developing cellular pool in a process known as negative selection (Zhang *et al.*, 2003). It is thought that in the case of nT_{REG} cells their TcR responds strongly to a given peptide but not strongly enough to bring about deletion (Zhang *et al.*, 2003). These cells have been shown to play important roles in the control of inflammation and autoimmunity (Sakaguchi and Sakaguchi, 2005).

The nT_{REG} cells account for some of the immunomodulatory capability of the eye but when ‘committed’ effector T cells enter the tissue under inflammatory signals they are able to overwhelm the quelling action of these cells. It has been shown that within hours of the induction of antigen-induced uveitis, retinal-antigen specific lymphocytes are present in the eye. In these models, utilising adoptive transfer of antigen-specific T cells, the remaining injected cells tend to migrate to the spleen. The cells then proliferate in the spleen before migrating to the eye along with inflammatory leukocytes slightly later in the model. It is at this point that the pathology of these models start to become observable. It has also been shown that if uveitis is induced and allowed to take its course it is a self-limiting pathology in wild-type animals (Klaska and Forrester, 2015). Also, once the pathology has subsided, there is an increase in the number of resident T_{REG} cells in the eye compared to pre-inflammation levels (Silver *et al.*, 2015). This makes the tissue even more anti-inflammatory and able to convert active T cells into a regulatory phenotype (Silver *et al.*, 2015).

4.1.3 Experimental Autoimmune Uveoretinitis

4.1.3.1 Cellular response to the model

One of the most interesting findings from work on rodent models of uveitis has been the subtlety of the pathology that can result depending on the way in which the model is induced and the strain of mouse used (Klaska and Forrester, 2015). The inflammatory pathology of uveitis models has, in the past, been

attributed to a T_H1 response. However, more recently, the T_H17 cell has become implicated (Amadi-Obi *et al.*, 2007). Specifically the cytokine, IL-17, that these cells produce has been implicated in the pathology (Sun *et al.*, 2015). In fact what has been shown is that how the inflammation is induced skews the T cell response and is a defining factor in what the uveitogenic T cell population is. In models where dendritic cells are pulsed with retinal antigens then injected into a recipient mouse the resulting pathology appears to be of a T_H1 phenotype (Caspi, 2010). In immunisation-based models, such as the injection of IRBP with CFA, the resulting damage is attributed to T_H17 cells (Caspi, 2010). Another counter-intuitive finding from work in these models is that, in the eye, the highly inflammatory cytokine interferon- γ may have anti-inflammatory and immunomodulatory properties. This conclusion has been reached as neutralizing this cytokine exacerbates disease (Caspi *et al.*, 1994).

4.1.3.2 Translational value of the model

Experimental autoimmune uveoretinitis (EAU) is an animal model of posterior uveoretinitis in humans (Klaska and Forrester, 2015). The purpose of this model is to simulate the pathology and mechanisms involved in human uveitis in order to gain insight into the aetiology of the damage. Humans with this pathology can present symptoms ranging from redness of the eye through to loss of vision and pain. It is a pathology that presents as a comorbidity in a variety of conditions and can be observed in systemic disorders like multiple sclerosis, Behçet's syndrome, or infectious diseases like Lyme's disease (Forrester, 1991). Uveoretinitis is, by definition, inflammation affecting the uveal and retinal tissue which consists of the choroid, the ciliary body, the iris, the sclera, and the retina. This inflammatory pathology can be anterior, intermediate, or posterior depending on what section of the uvea the inflammation is affecting. As has already been discussed, the eye has multiple mechanisms in place to prevent inflammation in the tissue. During the induction of this pathology the blood retina barrier (BRB) is seemingly broken down allowing for the accumulation of inflammatory leukocytes in the eye (Forrester, 1991).

Autoimmune uveitis is a chronic inflammatory disease and the treatment regimen is similar to other diseases of this type such as rheumatoid arthritis (RA) and psoriasis. One of the pathological contributors in uveitis is overproduction of

the potent cell activating cytokine $\text{TNF}\alpha$ which, when blocked, can provide symptomatic relief to patients (Gardner *et al.*, 2015). Some of the key cells which produce and release this cytokine are CD4^+ cells which have been shown to drive the inflammation during this pathology along with neutrophils, macrophages and NK cells (Grivennikov *et al.*, 2005, Gardner *et al.*, 2015). Another treatment strategy used by clinicians in the treatment of uveitis that has commonality with RA is that of glucocorticoids (LeHoang, 2012). Drugs from this family, such as dexamethasone, have been shown to limit the $\text{T}_\text{H}1$ response and limit the damage induced by pro-inflammatory cytokines released by these cells (Guo *et al.*, 2007). To add to this, drugs such as dexamethasone significantly reduce the amount of $\text{TNF}\alpha$ produced in the retinal tissue (Yossuck *et al.*, 2001).

4.1.3.3 Methods of EAU induction and associated-pathology

EAU can be induced by various methods, as already mentioned, and this has an effect on the overall pathology. For the work presented here a retinal peptide, inter-photoreceptor retinoid binding peptide (IRBP) 1-20, was injected with an immunological danger signal (Complete Freund's Adjuvant (CFA)) to induce an immune response. CFA contains inactive *Mycobacterium tuberculosis* emulsified in mineral oil and is used to boost the immune system. In the literature other retinal peptides can be used to immunise the mouse and each is effective. Our model was carried out over twenty-eight days before samples were harvested, more details are provided in section 2.2.1.

Given that this is an immunisation-based inflammatory induction model it can be assumed that the pathology here is a $\text{T}_\text{H}17$ and macrophage/monocyte driven inflammatory process. Eyes are known as immuno-specialised organs, this means that retinal peptides are treated differently during development but when given with an immunological danger signal can induce inflammation. This means that during the 'priming' phase of an EAU model an eye peptide specific to the uvea is injected subcutaneously into the dorsal skin of the mouse along with a 'danger signal' and an adjuvant that will both alert and enhance the immune system respectively (Klaska and Forrester, 2015).

4.1.4 Chemokines and the Eye

Literature in this field supports an essential role for chemokines and their receptors in the initiation, induction and maintenance of the inflammation associated with uveitis (Adamus, Manczak and Machnicki, 2001, Crane et al., 2006, Zhao, Chen and Xu, 2014). Two pathways for tissue damage have been described in the clinic; patients can be diagnosed as having granulomatous or non-granulomatous uveitis. The former is associated with an influx of the monocyte/macrophage cell type whereas the latter is a neutrophil driven process. Interestingly the extent of tissue damage appears to be similar in both diagnoses.

A study by Forrester et al has shown a need for CCR5 to enable the transition of T_H1 cells across the blood retinal barrier. In this study uveitogenic T cells were isolated from the spleens of mice and treated with CCR5-blocking antibodies. This was shown to significantly reduce the number of cells able to breach the barrier. The study suggested that CCR5 was required for crossing the blood retina barrier, and not rolling or adhesion in the local endothelium as previously thought (Crane et al., 2006).

The CCR5 Δ 32 mutation, discussed in the main introduction (section 1.3.2.1), has been studied in cohorts of patients with Behçets Disease (BD). As already discussed, this disease includes retinitis and almost all studies involving the CCR5 Δ 32 mutation show that it lessens inflammatory pathologies. One study, in fact, found that this mutation was non-protective in a cohort of Italian BD patients (Atzeni *et al.*, 2012) whereas another study found that in Portuguese patients it has no significant effect on pathology (Bettencourt *et al.*, 2014). Finally, a meta-study found that the mutation brought about increased risk of BD if the patient was of the HLA-B51 antigen-type (Song, Kim and Lee, 2014). One study of single nucleotide polymorphisms (SNP) in the genes of ACKR2-related ligands and receptors found that these mutations had altering effects on the development and progression of idiopathic immune-mediated posterior segment uveitis (Ahad *et al.*, 2007)

Another study of patients with uveitis found expression of chemokines in the conjunctival epithelium. Almost paradoxically, there was a significant increase in the expression of the T_H2-associated chemokine receptor CCR4 in patients with uveitis and just weak expression of the T_H1-associated chemokine receptor CCR5 (Trinh et al., 2007). The pathophysiological relevance of this is unknown but it may be reasonable to postulate that the eye is attempting to quell damage brought about by the inflammatory state caused by incoming cells.

Work on CCR2 knockout mice showed an absence of infiltrating macrophages resulting in a more neutrophil driven inflammation (Sonoda et al., 2011). The classical leukocyte response in this model is dominated by macrophages and a small number of neutrophils. The lack of CCR2 brought about an almost complete reversal of this. Interestingly, there was no significant difference in the number of recruited T cells between the two genotypes. There was also no significant difference in the eventual pathology resulting in either strain. These studies, and others, provide good evidence to suggest a possible involvement of ACKR2-related classical chemokine receptors in the chemotaxis of inflammatory cells to the eye (Diedrichs-Möhrling et al., 2005, Crane et al., 2006). Other studies provide evidence of the role of ACKR2 ligands accelerating the pathology of EAU (Crane et al., 2001) (Zhao, Chen and Xu, 2014).

Another recent study has shown that, in mice lacking CCL2 and CX₃CR1, the tissue damage caused in the EAU model is reduced. The findings of this work showed a reduction in the number of CD45⁺ cells infiltrating the eyes of the knockout mice at 25 days post-immunisation. Again, in this model there were fewer F4/80⁺ macrophages but an increase in the number of neutrophils infiltrating the eye. Zhao et al went on to show that at 60 days post-immunisation there was a reduction in macrophages and myeloid-derived suppressor cells in the knockout mice but a significant increase in recruited lymphocytes (Zhao, Chen and Xu, 2014). The findings in this more recent study correlate nicely with those in the Sonoda paper which showed that a lack of CCR2 resulted in a neutrophil-dominant EAU pathology (Sonoda et al., 2011).

Taken together, the findings in these publications provide a reasonable basis for a possible role of ACKR2 in regulating the inflammatory pathways causing the

pathology associated with EAU. Our aim was to test the hypothesis that a lack of ACKR2 would ameliorate/alter the pathology associated with the IRBP 1-20 immunisation model of EAU. We also ultimately wanted to draw some relevance from this to the human disease of uveoretinitis.

4.2 Results

4.2.1 The Role of ACKR2 in EAU

4.2.1.1 Histology

Increased inflammation in ACKR2-null mice during EAU model

After immunisation (section 2.2.1) to induce inflammation we checked for differences in pathology between wild-type (WT) and ACKR2 null mice using standard histological methods. As mentioned in the materials and methods, the retinal peptide IRBP 1-20, Complete Freund's Adjuvant and Pertussis toxin were injected into WT and ACKR2-KO C57BL/6 mice. Figure 4.2 shows the gross pathology of the eyes. It can be seen in figure 4.2B that there is mild inflammation in treated wild type mice including leukocyte infiltration (1), granuloma formation (2), and folding of the retina due to oedema (3). The histology in 4.2C showed a marked increase in cellular influx to the inflamed eye in the mice lacking the ACKR2 compared to WT mice. In 4.2C one can see thickening of the retina and choroid layers (4 and 5, respectively), leukocyte infiltration into the photoreceptor rod outer segment layer (6) and retinal detachment (7).

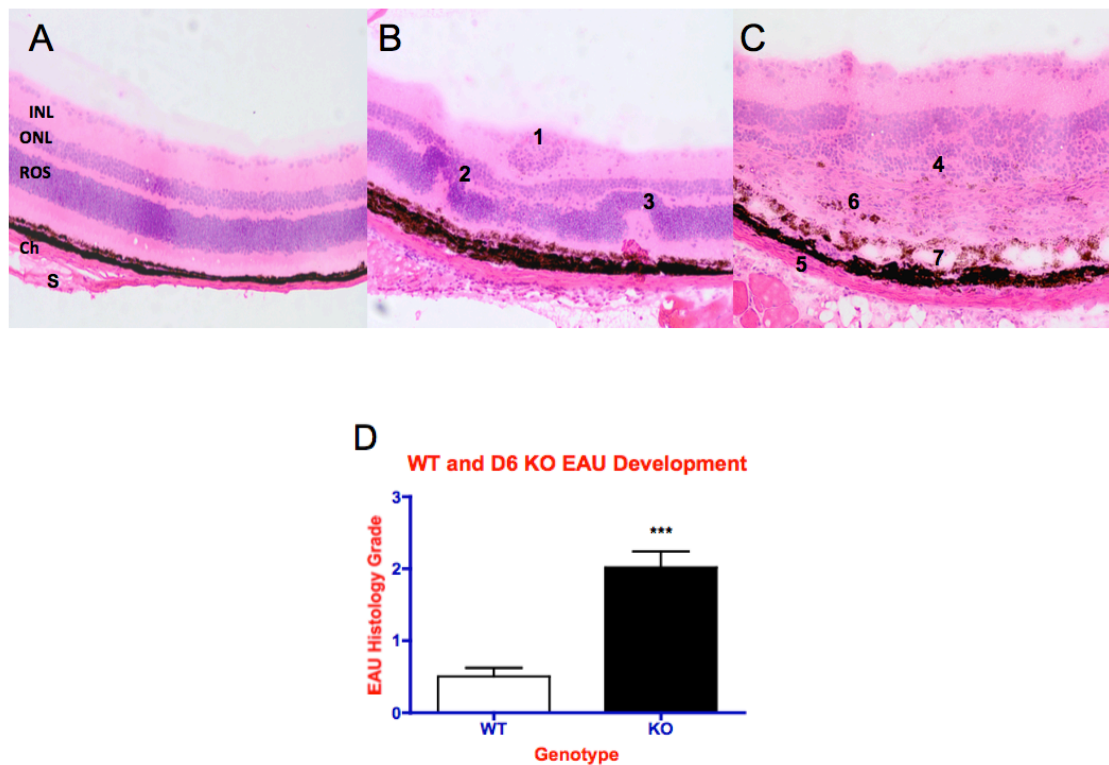


Figure 4.2. ACKR2 null mice developed more severe EAU compare to WT mice as shown by the histological examination (H&E stain) (A) Naive WT eye; (B) WT mild EAU. 1-perivascular inflammatory cell accumulation, 2-granuloma formation and 3-retinal folding; (C) ACKR2 null mice with severe EAU. 4- Thickening of retina and 5- choroid layers, 6- cell infiltration in ROS with 7- obvious retinal detachment. (D) Eyes were graded using a customised histopathological grading system. The results show significantly increased pathology in ACKR2KO mice. Student's t test was carried out on this work and the data was consistent across two separate studies. Images taken at x100 magnification. n= 7. (***) $p < 0.001$.

INL- Inner Nuclear Layer ONL- Outer Nuclear Layer ROS- Photoreceptor Rod Outer Segment Layer Ch- Choroid S- Sclera. Grading system from Xu et al. 2008.

Using a customised histopathological scale (see Materials and Methods section 2.2.2) we were able to quantify the tissue damage caused. H&E stained slides were blinded before being analysed according to the scale. It is a complex system that takes into account information on a range of pathological contributors such as discrete sites of inflammation, cellular influx and tissue degradation in order to get an objective score for the pathology. From the examples above, using the system the scores would be as follows: A would score a 0 as the architecture of the tissue look to be normal and healthy. B would be given a score of 0.5, as although there are signs of inflammation, all layers of the retina are discernable and intact. The example in C is highly inflamed with a great deal of tissue damage. The retina is detached/detaching, the layers of the retina are both difficult to determine and hyperplastic too. This would give this

sample a score of 2.0. All eye samples were quantified using brightfield microscopy.

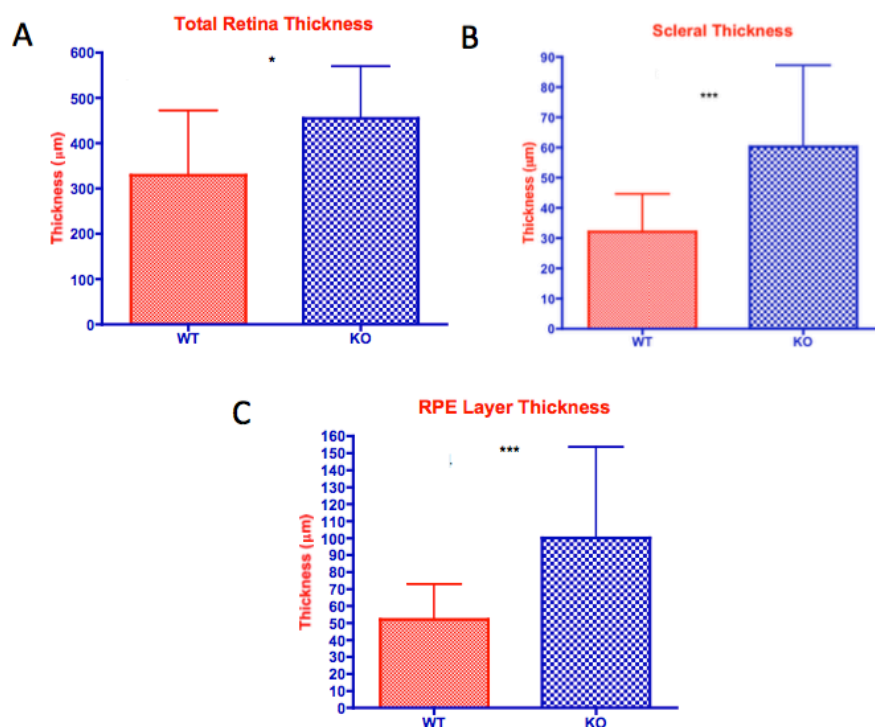


Figure 4.3 - Retinal inflammation is significantly increased in RPE layer and sclera of ACKR2 null mice. The above figures show the average of six high-power views. Blinded measurements made for each tissue in each genotype. (A) Shows increased total retinal thickness between the WT mice (red) and ACKR2 null mice (blue). (B) Shows a marked increase in thickness of the sclera of ACKR2 null mice over WT. (C) A similar pattern to the sclera with significant difference between genotypes. n= 7. (*p<0.05, **p<0.001)

The above figure suggests that across the whole retina, and in different tissue layers of the eye, there was inflammation in the ACKR2-null mice. It is unclear if wild-type mice had already gone through this inflammatory process and recovered or whether this would be apparent throughout the model. The histology images in figure 4.2 B (WT) and C (KO) illustrate this finding. One can clearly see swelling in all layers of the eye in the ACKR2 null mice but figure 4.3 summarises the findings across the whole model as well as showing statistical analysis of the data.

Here we show that ACKR2 provides a protective effect during the EAU model. We show that in the absence of this receptor there is an increase in leukocyte recruitment to the eye and an increase in tissue damage.

Increased collagen accumulation in ACKR2-null mice during EAU model

When we did our work on inflammation of the skin, presented in chapter 3, we noticed an increase in collagen formation in the ACKR2 null mice (section 3.5.1.2). Within our group this is a finding that has been present across numerous ACKR2 null models (data not shown). With this in mind we stained the eyes with picrosirius red to investigate any changes in collagen distribution during the EAU model. These findings are shown in figure 4.4.

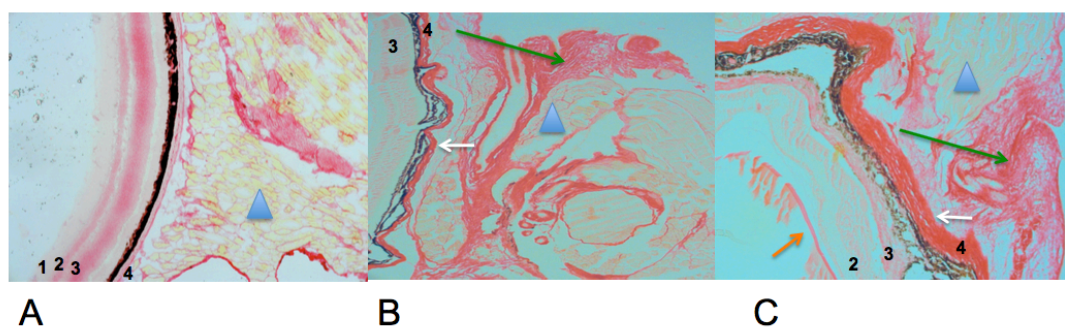


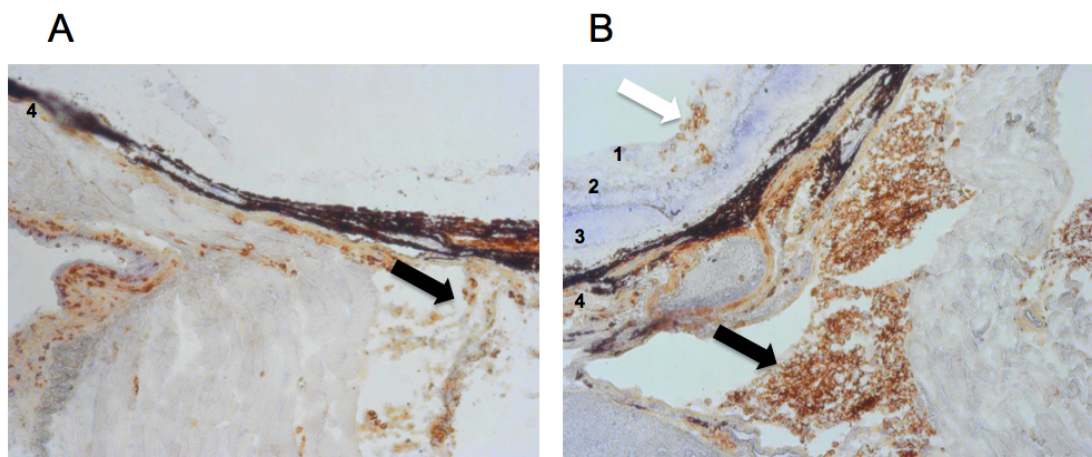
Figure 4.4. Picrosirius red stain suggests collagen accumulation. (A) Naïve WT eye. (B) WT mouse with mild EAU. (C) KO mouse with severe EAU. Pericocular fibrosis involving sclera and sub-conjunctiva along with loss of photoreceptors (orange arrow). Also subretinal fibrosis (green arrow) and thickening of the choroidal uveal tract layer (white arrow). Triangle denotes the optic nerve. 1/6 WT Mice present this accumulation, 5/6 KO. Images taken at x100 magnification. 1- Inner Nuclear Layer, 2- Outer Nuclear Layer, 3- Photoreceptor Rod Outer Segment Layer 4- Choroid

Figure 4.4 shows the optic nerve region of the eye (denoted by the triangle) and it can be seen that there is an accumulation of collagen fibres around this area of the eye. The most obvious region of increased collagen accumulation is the choroidal uveal tract layer (denoted by the white arrow). As shown in A, this layer is barely visible in resting mice, in B it is a distinct and obvious layer, whilst in C there is profound thickening of this layer. As leukocytes arrive at the inflamed eye they may be accumulating and causing this fibrosis. We wanted to investigate the possible cause of both the increased inflammation and the collagen accumulation in the ACKR2 null mice so carried out immunostain for more specific cell-types.

4.2.1.2 Immunocytochemistry

Increased CD45⁺ cell accumulation in ACKR2-null eyes during the EAU model

Having shown that ACKR2-null mice were presenting increased uveal pathology we wanted to investigate the cellular profile of the immune infiltrates to the eye. Specifically we investigated if the disparity in tissue damage was as a result of a larger leukocyte influx or if there was an altered cellular profile. To achieve this we performed immunohistochemistry with CD45 and MAC-2 as targets. CD45 is also known as the leukocyte common antigen and is expressed by all haematopoietic cells. MAC-2 is a lectin which is expressed by macrophages and some dendritic cells. The conclusion from the work done for Chapter 3 was that in ACKR2-deficient mice there was a change in the influx of macrophages to sites of cutaneous inflammation. Therefore we wanted to investigate if this was also having a role in the pathology of the ACKR2 null mice in the EAU model.



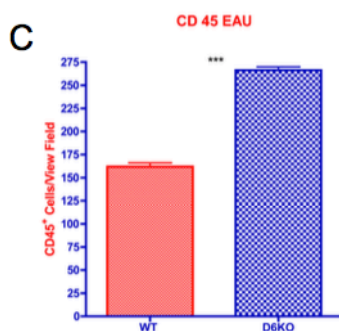


Figure 4.5. Anti-CD45⁺ immunocytochemistry and cell counting showed accumulation of leukocytes around the outer layers of the retina with increased presence and penetration of the retina in ACKR2 null eyes.. (A) EAU day 28 WT showing CD45⁺ cell infiltration (black arrow). (B) Increased infiltration of leukocytes posterior of the retina (black arrow). Inflammatory cells are visible in the retina also (white arrow). (C) Analysis of high-powered fields of view confirmed increased leukocyte infiltration in ACKR2KO mice during EAU. Images taken at x200 magnification. n=7. (**p<0.001)

1- Inner Nuclear Layer, 2- Outer Nuclear Layer, 3- Photoreceptor Rod Outer Segment Layer 4- Choroid

As can be seen in figure 4.5, there is a clear infiltration of leukocytes to the eyes of the mice during the EAU model. These representative images show that in WT mice (A) there is a mild influx of CD45⁺ cells to the subretinal tissue with some leukocytes infiltrating further into the retina itself. In (B), the ACKR2-null mice, one can see a greater number of CD45⁺ cells including increased retinal infiltration and deeper retinal infiltration. Part C of the figure shows the enumeration of these positive cells across the cohort and demonstrates that the data is statistically significant.

Having observed the significantly increased influx of CD45⁺ cells to the inflamed eye of the ACKR2 null mice we wanted to gain further information as to the possible cell-types responsible for this disparity. To do this we studied macrophages as, from previous work in this thesis, we had observed a marked difference in the recruitment of these cells in the absence of ACKR2. We used the MAC-2 stain described previously along with brightfield microscopy in order to quantify the number of cells arriving and to gain information as to where they were localising.

Increased MAC-2⁺ staining in WT mice during EAU model

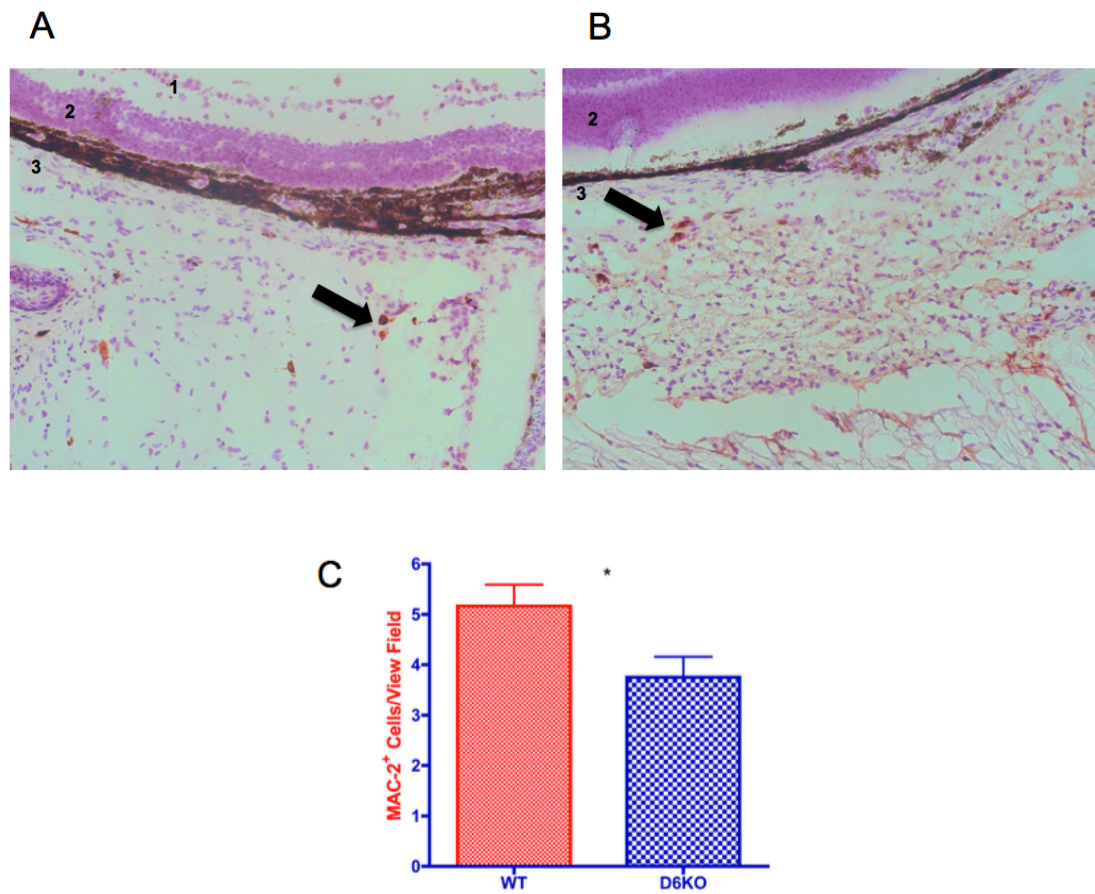


Fig. 4.6 Anti-MAC-2 immunocytochemistry suggested a significant increase of macrophage migration to the eye in WT mice. (A) WT eye with increased macrophage infiltration compared with (B) the ACKR2KO eye. (C) The graph is an accumulation of counts in all sections showing significantly more macrophage infiltration in WT mice. Images taken at 200x magnification. n= 7. (*p<0.05)
1- Outer Nuclear Layer, 2- Photoreceptor Rod Outer Segment Layer 3- Choroid. Black arrow denotes macrophage.

The results shown in the above figure suggest that although there is a greater influx of total leukocytes arriving into the eyes of ACKR2 null mice (4.6B) there is a decrease in the recruitment of MAC-2⁺ cells. Figure 4.6C suggests a reduction in the accumulation of macrophages to the ACKR2-null eyes. The MAC-2⁺ cells that are recruited in this process are probably macrophages and due to the resulting pathology of the knockout mice it is reasonable to suggest that WT mice may be preferentially recruiting alternatively activated macrophages. Due to the colour of the retinal pigmented epithelial (RPE) cell layer and the fact that pigment picked-up by cells phagocytosing RPE cells can look like positive

DAB staining, red alkaline phosphatase was used as a substrate for this work to give a red colour (see section 2.6.4).

4.2.1.3 ‘Glasgow Model’ vs. ‘Aberdeen Model’ of EAU

This work was performed in collaboration with the Forrester group from the University of Aberdeen. We provided them with ACKR2 null mice allowing them to parallel our analysis at their facility. Surprisingly the results from their model suggested no significant difference in inflammation between the genotypes (personal communication). As a result of this we compared the protocols used during the immunisation of the mice. Table 4.1 shows the concentrations of the reagents used for induction of inflammation.

Glasgow	Aberdeen
600µg IRBP 1-20	500µg IRBP 1-20
100µl CFA	100µl CFA
500ng Pertussis toxin	1µg Pertussis toxin
	Extra 25mg Mycobacterium tb to CFA

Table 4.1- The above table shows the reagents used to make up the immunisations that each mouse received during the EAU models. On the left is the protocol we used and on the left is the protocol that the University of Aberdeen use for the same model.

As can be seen, there are a few discrepancies between the protocols. The Aberdeen protocol uses less of the retinal peptide but is, in general, more inflammatory. It contains double the pertussis toxin, and the literature suggests that this aids in the breakdown of the blood-retina-barrier. Although the same volume of complete Freund’s adjuvant was used they added extra *Mycobacterium tuberculosis* which would provide more of a ‘danger signal’ to the immune system. As a result of the discrepancies between our results and our protocols we decided to swap protocols and see if protocol differences explain the discrepant results or the animal units housing the mice were effecting the phenotypes. The results from the two models we ran are shown below in figure 4.6. As can be observed, we were able to recapitulate the results that the Aberdeen group had when we used their protocol. It is also worth noting that the

group in Aberdeen also found the same phenotype as we did when they used our method (personal communication).

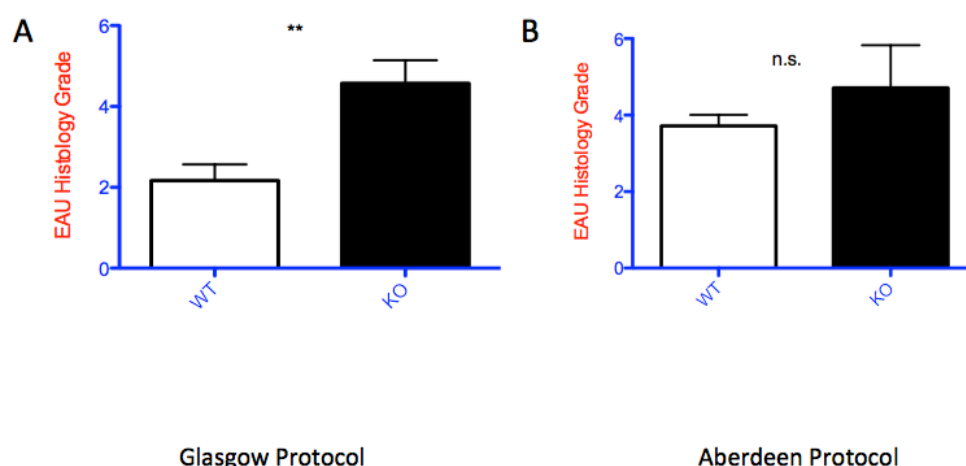


Figure 4.7- The figure above shows the histopathology scores from the two EAU models we carried out. When the model was run using our original protocol (A) the ACKR2 null mice had significantly worse inflammation in the ocular tissue. When the same model was carried out on the same strain of mice using a protocol provided by our collaborators (B) there was no significant difference in the histopathology score. Although ACKR2 null mice show the same levels of inflammation across both protocols it is worth noting that the WT pathology score appears to be almost twice as high using the 'Aberdeen protocol'. This is thought to be due to the more inflammatory nature of the immunisation inoculum. In both cases a Student's t test was used. n=7. (**p<0.01)

It is clear from these results that in both protocols there is increased pathology in the eyes of ACKR2-null mice. Interestingly, using the 'Aberdeen protocol' seems to cause an increase in inflammation in the WT mice (as shown by the increase in histology grading) but this is not as pronounced in the knock-out mice although not significant. This may suggest that using the more inflammatory protocol of our collaborators is 'overwhelming' the protective action of the ACKR2 present in the WT mice and contributing to this increased damage.

We wanted to quantify the level of ACKR2 expression in naïve and EAU treated WT mice to see if any transcriptional changes were being induced by the model. We removed the vitreous humour from the eye as this is largely acellular and may have had a diluting effect on the transcriptional signal of ACKR2.

4.2.1.4 Transcriptional Analysis of *Ackr2* in EAU

We wanted to look in more detail at expression in the two tissues that contain pigmented epithelium namely the iris and the retina along with whole eye samples. The pigmented epithelium of the eye, present in both the iris and the retina, is a highly immunomodulatory tissue and as such may be a site of ACKR2

activity (discussed in more detail in section 4.1.2). Laser-capture microdissected tissue from the eyes of mice was harvested by our collaborators at the University of Aberdeen. They had immunised B10.BR/III mice, using the same peptide as we used but with a slightly altered inoculum (as per table 4.1), and allowed the EAU model to progress to seventy days. At the termination of the model the eyes were harvested and tissue laser-captured to yield RNA from the whole eye, the retina and the iris. We had been given naïve and inflamed eyes so we could also investigate the ACKR2 transcriptional profile of these tissues before, and after, the induction of inflammation. With these data we could now identify any change in regulation of transcription of the receptor during the EAU pathology and at separate anatomical locations.

As discussed in the Materials and Methods section (2.2.1), this particular experiment was carried out on a different strain of mouse, the B10.BR/III as opposed to the C57BL/6J used previously.

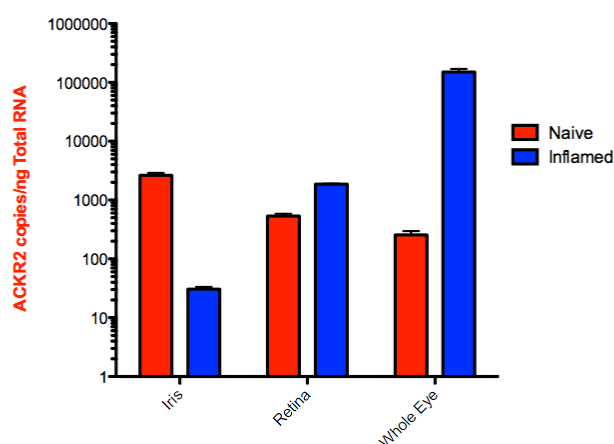


Figure 4.8- The graph above shows the levels of transcription of *Ackr2* in naïve mice (red) and inflamed mice (blue). Laser capture microdissection was used to harvest the tissue for this assay. This model was carried out on B10.BR/III mice and eyes were harvested at day 70 post-immunisation. n=1 (error bars represent 3 technical replicates).

The results in figure 4.8 show an increased expression of *Ackr2* transcription in the whole eye compared to iris and retinal tissue at seventy days post-immunisation. Interestingly there seems to be higher *Ackr2* transcriptional activity in the inflamed retina than in the naïve retina the functional significance of this was not tested and is unknown. One possible reason for this is that EAU is a model of posterior uveoretinitis and, by definition, affects more tissues than just the retina. This model should induce a minimal response in the

iris and the data above even suggest a downregulation of Ackr2 transcription in this tissue. This result is in-keeping with previous findings from other models that ACKR2 is upregulated during inflammation (Singh et al., 2012).

The results presented above suggest that ACKR2 expression may be increased slightly at the site of pathology but that there could be a more widespread response to inflammation in the eye. These data also suggest that ACKR2 may play a protective role in the mouse eye during the EAU model. Mice lacking the receptor had a significant increase in ocular tissue damage and a markedly larger leukocyte infiltration to the eye. As this work was a model of human disease the next step for this was to investigate the possible relevance of these findings for the human eye.

4.2.2 ARPE-19 Cell Line Characterisation

4.2.2.1 Transcriptional Analysis

As discussed in the introduction to this chapter (section 4.1.2.1), it is the retinal-pigmented epithelium of the eye that has some of the greatest immunomodulatory capacity. In order to ascertain if ACKR2 was expressed in the human eye we purchased a human retinal pigmented epithelial cell line. This seemed like a likely place for ACKR2 to be expressed in the retina and contribute to the phenotype observed *in vivo*. We investigated a human retinal-pigmented epithelium cell line (ARPE-19). This allowed us to characterise ACKR2 expression in this cell line and further investigate how the levels of this receptor change with inflammatory stimulation. We carried out protein and transcriptional analysis of Ackr2 on these cells. We started the characterisation of this cell line with QPCR to assess the levels of transcription of this gene. We used another human cell line, human embryonic kidney (HEK) 293 cells, that do not express Ackr2, as a negative control. As a positive control in this assay we used HEK293 cells that had been transfected with human Ackr2.

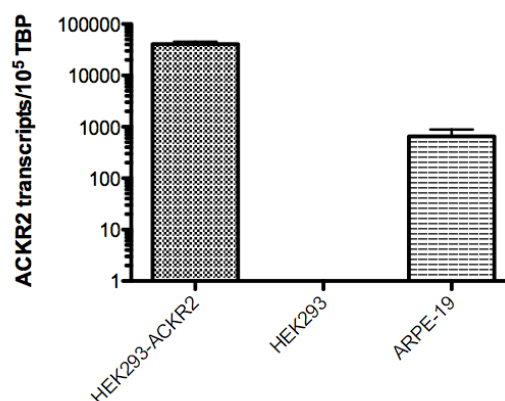


Figure 4.9- The graph above shows the transcription of *Ackr2* in the ARPE-19 cell line under resting conditions. HEK293-ACKR2 cells were used as a positive control whilst HEK293 were used as a negative control. The data suggests that ARPE-19 cells are able to transcribe *Ackr2*. TATA binding protein (TBP) was used as a reference gene for this assay. $n=3$.

Data from the PCR analysis are shown in figure 4.9. The above data suggest that, in a resting state, the ARPE-19 cell line is able to transcribe *Ackr2* at a detectable but low level. Once this had been shown we went on to investigate, and quantify, protein expression levels using various techniques. Much like starting with transcription, we utilised the more sensitive techniques first. ACKR2 molecules, as mentioned in the introduction to this thesis, are much more abundant in cytoplasmic vesicles of the cell with a low level of expression on the cell surface. As a result of this a total protein analysis was required to assess the level of, ACKR2 protein expression in the ARPE-19 line.

4.2.2.2 Western Blot

Results were controlled using both positive (HEK293 cells transfected with ACKR2) and negative (HEK293 cells) controls. Monolayers of cells were grown and equal numbers of cells were lysed before analysis by western blotting. The results from this assay are shown in figure 4.10.

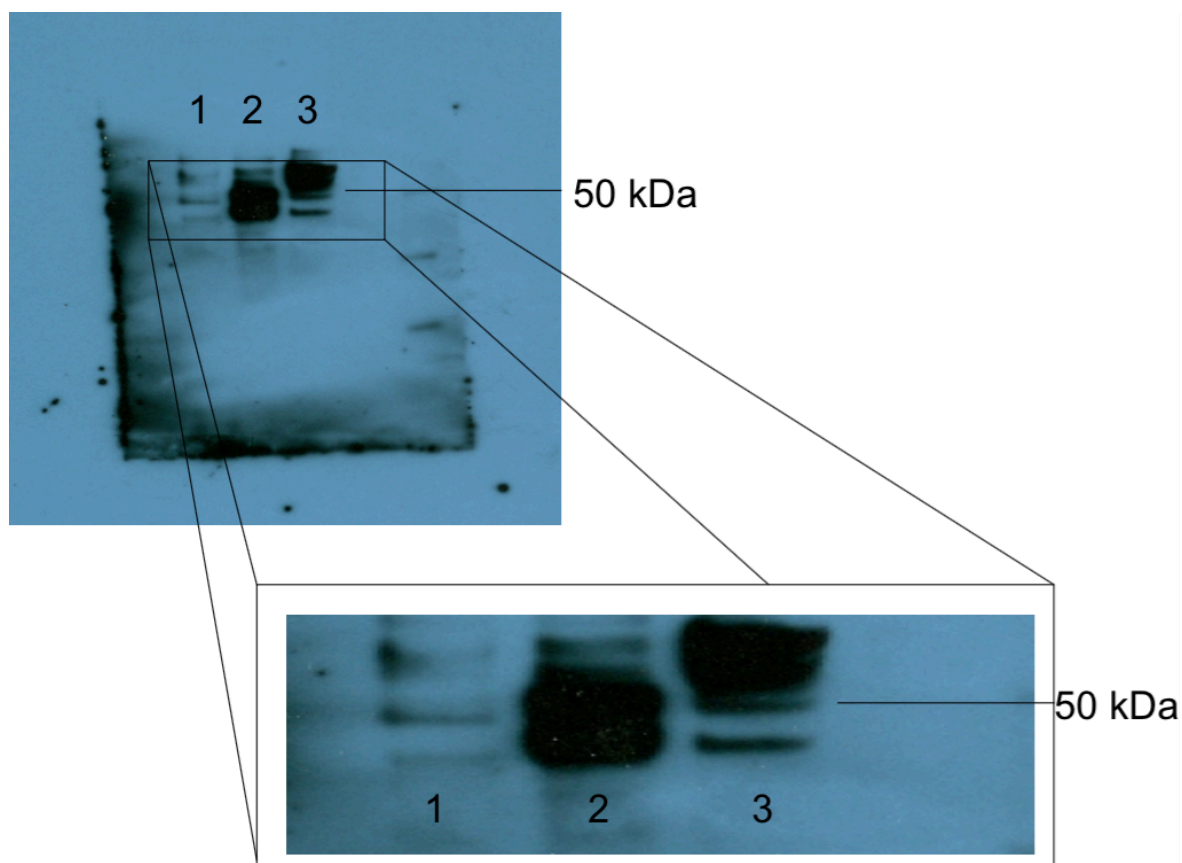


Figure 4.10- The figure above is a western blot from a lysate of ARPE-19 cells. On the blot from left to right lane 1 is HEK cells (negative control), lane 2 is ARPE-19 cells and lane 3 is HEK-ACKR2 (positive control). A protein ladder is in lane 4 and 50kDa is marked (ACKR2 is 49kDa).

The results from the western blot suggested that this cell line does express ACKR2 at the protein level and at higher levels than the transcriptional data in figure 4.9 would suggest. One of the possible reasons for this is that ACKR2 protein is very stable and has a long half-life (Weber *et al.*, 2004). As a result it can have a low level of transcription but relatively high levels of protein expression. However, it can be observed that there is a size difference between the bands from the ARPE-19 cells and the bands from the HEK-293-ACKR2 cells. This is probably a result of a post-translational modification like, for example, glycosylation. We did not test this formally however so this remains speculation.

We went on to use flow cytometry and immunocytochemistry to see if this expression displayed the same intracellular predominance as seen in other cells. By permeablising the cell membranes flow cytometry allowed us to quantify the expression on the cell surface against the expression within the cell and

compare it to other cells of known expression levels. The immunocytochemistry allowed us to visualise the expression on the cells more precisely.

4.2.2.3 Flow Cytometry

In order to truly quantify the levels of ACKR2 in the cytosol, versus that on the cell surface, we utilised a flow cytometry-based analysis method. Having shown that the cells express ACKR2 protein the next step was to accurately quantify the levels of surface versus intracellular expression. This assay provided us with a baseline reading for ACKR2 expression in these cells. The idea here was to create a surface vs. intracellular ACKR2 level ratio for cells at a resting state.

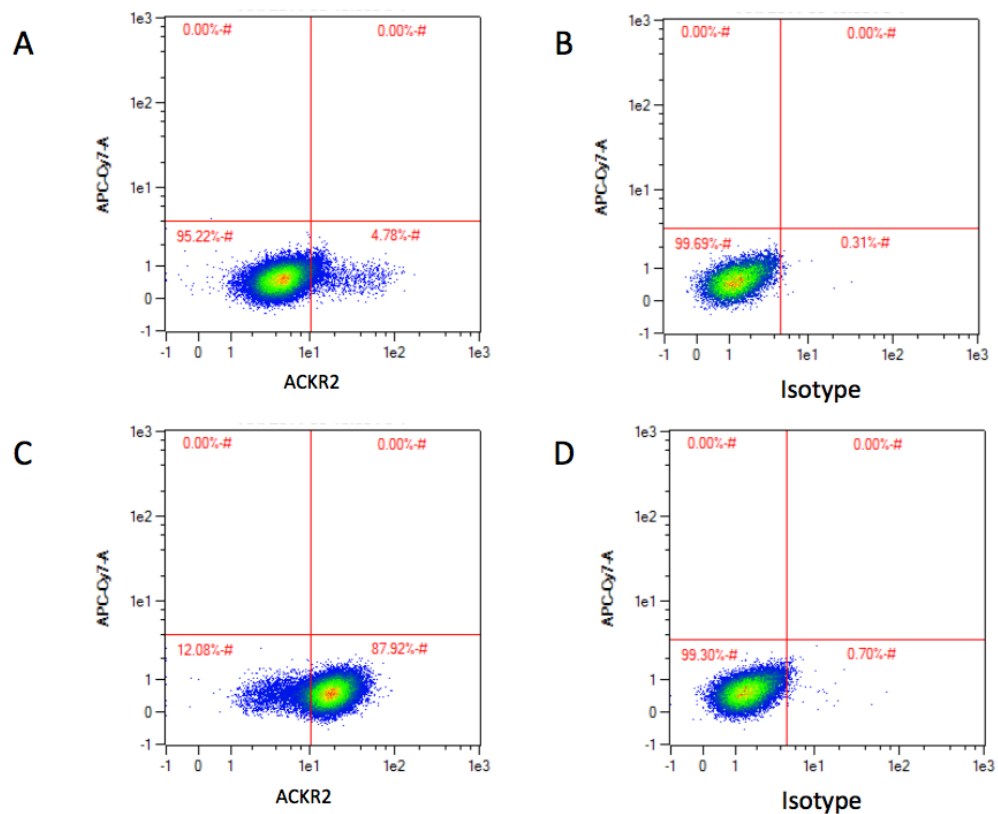


Figure 4.11- The plots above are the results from flow cytometry carried out on the ARPE-19 cell line. (A) Shows the surface expression of ACKR2. (B) Shows isotype control for surface expression stain. (C) Shows the intracellular expression of ACKR2. (D) Shows isotype control for intracellular stain.

The data in figure 4.11 above show that the ARPE-19 cells express high levels of ACKR2 in the cytosol, however levels of the protein on the cell surface were low in comparison. This is a normal expression pattern for ACKR2. These results,

taken along with the data from the western blot assay, suggests that the ARPE-19 expresses the ACKR2 receptor in a manner not unlike many other cells our group have characterised in the past with most ACKR2 in intracellular stores. Having shown that these cells can transcribe the gene and express the ACKR2 protein we wanted to show the functionality of the protein.

4.2.2.4 ARPE-19 Cell Chemokine Uptake Assay

Again this was achieved using a flow cytometry based approach. We used a competitive uptake assay to determine this. This was a technique developed in our group by Dr Chris Hansell (Hansell *et al.*, 2011) and one that can be used for multiple chemokine receptors. By taking a fluorescent ligand for ACKR2 (CCL22 in this case), and incubating the cells with it, binding is allowed to take place and is assessed by flow cytometry. CCL22 can bind more than just ACKR2 though. In order to determine the chemokine binding that is ACKR2-specific a non-fluorescent competitor (CCL2 in this case) is added in excess. Only ACKR2 binds both CCL2 and CCL22. As a result the mean fluorescent intensity (MFI) of the sample will drop in relation to the amount of ACKR2 present on the cells. The results below (figure 4.13) show the data obtained from this assay.

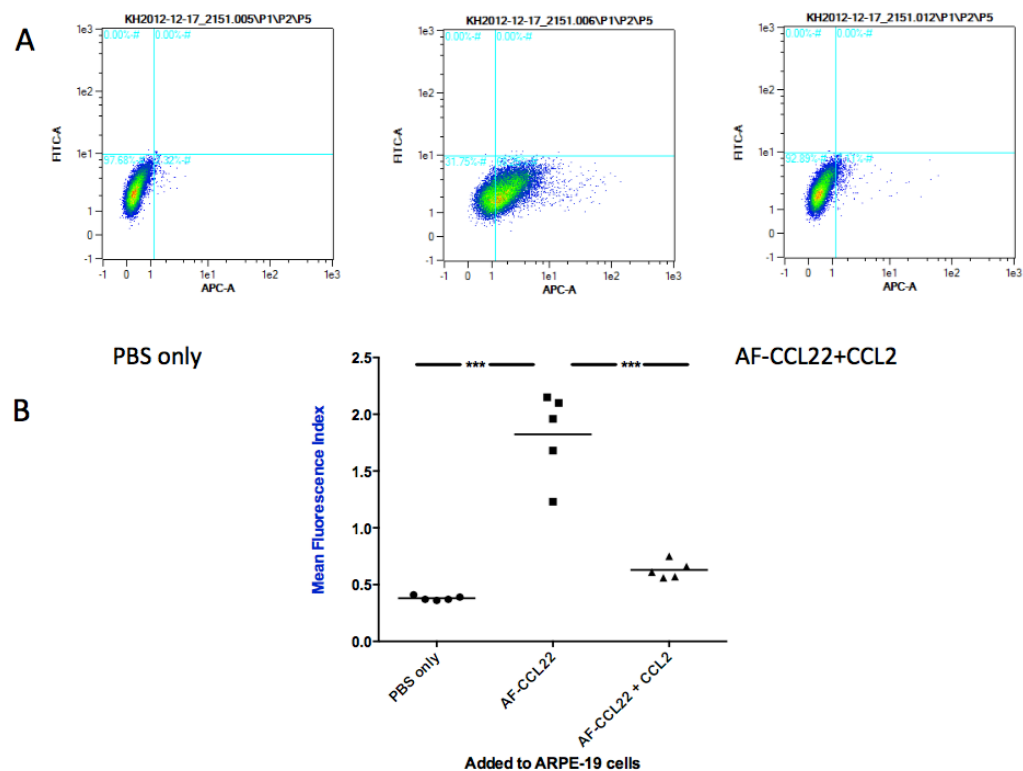


Figure 4.12- The plots in A above are representative samples showing the results of a competition assay carried out on ARPE-19 cells. From left to right, the first plot is a control sample of the cells with PBS added to them. The middle plot is the cells plus fluorescently-labelled ACKR2 ligand CCL22, the panel on the right shows the fluorescence level once unlabelled CCL2 was added in excess. B shows the culmination of many samples in the same assay. Student's t tests were carried out on these data. n=5. (***) $p < 0.0001$

It is clear from figure 4.12, that as the non-fluorescent chemokine is added to the ARPE-19 cell line the MFI of the sample drops considerably. Using the relevant controls, as shown, we can take from these results that the ARPE-19 cells express ACKR2 and it is functional. Although this is an experimental model using a cell-line, it does suggest that the equivalent cell-type in the human body may express ACKR2.

4.2.2.5 Immunocytochemistry

Once we had established that these cells expressed ACKR2 at the total protein level we wanted to use immunocytochemistry to visualise the expression of the protein within the cell. The images below were taken using an epifluorescent microscope. It is important to point out that the cells were permeabilised before staining to detect both surface and intracellular protein as described above.

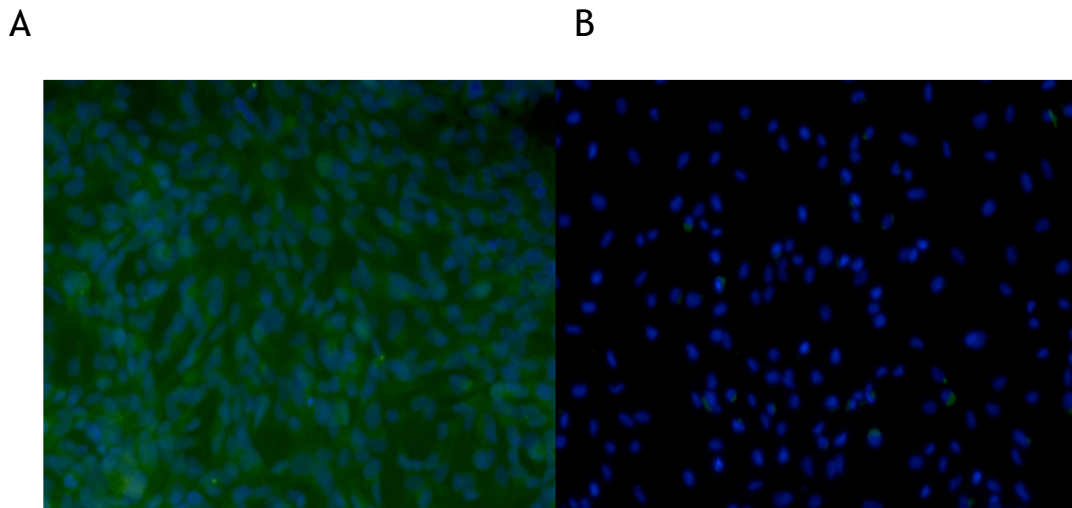


Figure 4.13- The images above show the results from fluorescent staining of the ARPE-19 cell line with Alexafluor 488-conjugated anti-ACKR2 (A) and isotype control (B) Images shown were taken at 100x magnification with the same exposure times for each image.

The images show a high level of ACKR2 positive cells within the ARPE-19 monolayer. These results further confirm ACKR2 protein expression by ARPE-19 cells. Taken together our results suggest that ACKR2 may play a considerable role in the anti-inflammatory response in the eye. They also suggest that the RPE cells may be an area of particularly high activity of this molecule.

4.3 Discussion

Our findings show that ACKR2 has a protective role to play in the IRBP 1-20 immunisation EAU model in C57BL/6 mice. The results we have obtained from this model are in agreement with current literature surrounding EAU and chemokines. Published work (Crane et al., 2001) on this model has shown that ACKR2 ligands and ACKR2-related receptors can alter, and drive, the inflammation associated with EAU. The data presented in our models show that the presence of the ACKR2 receptor confers a protective advantage.

The EAU model we used is one of a number of possibilities for studying uveoretinitis and it would be very interesting to see if this phenotype was repeated if other models were used. Like C57BL/6J mice, the B10.BR/III background is also susceptible to EAU and breeding ACKR2 null mice onto this

background would be useful to test the pathology they present. Different strains of mice mount different immune responses to models of inflammation and it would be interesting to see if there is any change in the pathology in B10.BR/III-A^{CKR2}-null mice compared to C57BL/6/J-A^{CKR2}-null mice. It has also been discussed, in section 4.1.3, that the way in which the model is induced can affect the resulting pathology. In all of the models presented in this section the pathology was induced by immunising the mice. It would be worth investigating the pathology that results from pulsing DCs with retinal antigens before transferring them back into the animal. This would drive a more T_H1-type model and so may alter the pathology slightly; it would be intriguing to see if A^{CKR2} conferred more or less protection in this setting.

The models carried out for this work went to an end-point of 28 days after immunisation and the results were obtained from the eyes at this point. What our results have not been able to show is the progression of the pathology with time and this would be of great importance to investigate. There is a reasonable chance that the A^{CKR2}-null mice have a delayed recovery from the inflammation in the model and that the peak pathology in the two genotypes are similar. As mentioned in the introduction to this section, the EAU model is self-limiting (Caspi, 2010) in wild-type mice so there is a chance that these mice may have had a similar level of inflammation at one point and that this has resolved. Another possibility is that the pathology could continue to develop and worsen. To look into this one could use the fundus imaging technology that many labs working in ocular research employ (Crane et al., 2006). This allows the investigator to look at the inflammation in the eye without culling the mouse and so progression of pathology can be quantified throughout the development of the model rather than at an end-point as per our work.

In addition to the general histopathology of the condition we also investigated the individual cell types that could be contributing to the inflammation. The cell-based work presented revealed a stronger inflammatory profile during EAU in mice lacking the A^{CKR2} receptor. Interestingly, the WT animals show a more extensive influx of macrophages, assessed by MAC-2 staining, to the eye during the model and we would like to investigate whether these are alternatively activated macrophages. As macrophages are often thought of as inflammatory

cells this does not seem to fit with the pathological phenotype. This is why it would be reasonable to suggest that this increase in macrophage recruitment could, in fact, be the anti-inflammatory alternatively activated macrophages. It would also be worthwhile investigating other leukocyte populations such as neutrophils and monocytes to see what cells account for the increased CD45⁺ cell accumulation in the ACKR2-null eyes. Studies on chemokines in EAU have shown that alterations in chemokine ligand and receptor levels have significant effects on the incoming cellular infiltrate (Crane et al., 2001, 2006). We would postulate that the population of recruited cells in the ACKR2 null mice during EAU is different to that of the wild-type. This is something that should be looked into in more detail possibly through a CD45 separation of inflamed eye lysates by flow cytometry to add to the immunohistochemistry shown in figure 4.5. This would allow us to see exact percentages of key leukocyte subtypes arriving at the site of pathology during this model.

One of the most interesting findings of our study was the differing results from our model compared to those of our collaborators. Upon swapping protocols and emulating each other's results this raised questions as to the possible mechanisms behind the discrepancies. The data suggested that when mice are immunised along with a more potent adjuvant and immunological danger signal the protective effects of ACKR2 are lost or reduced. It would seem reasonable to postulate that, like the immunomodulatory capacity of the eye, the protective effects of the atypical chemokine receptor are lost when the inflammatory signal becomes too great. Again further research into the kinetics of these findings would be welcome and fundus imaging would be key to this. It would also be interesting to investigate if the profile of the recruited cells was changed in any way using this more potent immunisation protocol. A paper from Lee et al in 2011 showed that ACKR2 plays an essential role in the process of lymphatic drainage. This paper showed that without ACKR2 expression on LECs inflammatory leukocytes interact with non-cleared inflammatory chemokines. This results in a disruption to the drainage of fluids and cells to local lymph nodes (Lee et al., 2011). This is, of course, something that should be considered during the EAU model in ACKR2-null mice.

Having shown the effect of a lack of ACKR2 on the pathology of EAU we wanted to look into the transcription of the gene under resting conditions and during the development of the pathology. QPCR of RNA from normal eye tissues, versus EAU day 70 eye tissues, revealed a shift of ACKR2 transcription from the iris, under naïve conditions, to the retina. This could be explained by the fact that ACKR2 is found, for the most part, on barrier tissues. The iris would be more likely to encounter inflammatory insult under normal conditions than the retina would. However, as the model of EAU used here targets the posterior segment of the eye it seems reasonable that transcriptional activity of this gene would be slightly increased in the retina. In a paper by Singh et al investigating ACKR2 in psoriasis an interesting finding was noted which might be of relevance to this. In this work it was suggested that in psoriatic lesions there is an increase in the transcription of ACKR2 in non-lesional skin (Singh et al., 2012). This, it has been suggested, may be a response to limit the spread of lesions in the skin by local cells and that trauma caused a drop in this activity of ACKR2 which allowed 'opportunistic inflammation' (Singh et al., 2012). It is reasonable to postulate that a similar mechanism may be at play here.

Reliable anti-human ACKR2 antibodies are available so we wanted to look at human cells to see if they expressed ACKR2. Our work on the human cell line, ARPE-19, has given a great deal of hope that our findings in the murine model can be extrapolated to human uveitis. Upon receiving the cells we rigorously tested them for expression of ACKR2. Our findings were promising and it appeared that they are able to transcribe the gene and express the functional protein. Unfortunately we did not get round to adding inflammatory stimuli to these cells to investigate any effect that had on ACKR2 expression but this would be important to investigate.

As the ARPE-19 cells express ACKR2 it suggests that the receptor may be expressed on primary human retinal-pigmented epithelial cells. We are currently working to gain access to human eye tissue as this would provide more validity to our findings on the human cell line. With this we would be able to stain for ACKR2 using immunohistochemistry and, if the receptor were expressed in human tissue, it would be logical to predict that our findings in the mouse models may translate to human pathophysiology and that ACKR2 may help lessen

inflammatory processes in the eye. Importantly having this tissue, and the expression data to match it, would allow us to visualise where in the eye ACKR2 is expressed and any possible functional significance of this. If it were the case that ACKR2 is expressed at these sites in the human eye then ACKR2-based therapeutic interventions could be designed to improve human disease.

To summarise, the findings presented in this chapter provide evidence of a role for the atypical chemokine receptor ACKR2 in the maintenance of the anti-inflammatory state of the eye. We also show that our findings may translate into human tissue.

Chapter 5- The Role of ACKR2 in Rheumatoid Arthritis

Chapter 5 Rheumatoid Arthritis

5.1 Introduction

5.1.1 Symptoms and Pathology

Rheumatoid arthritis (RA) is a progressive chronic inflammatory disorder that affects the synovial joints. It is the most prevalent of the inflammatory arthritides (Scott et al 2010). According to the World Health Organisation (WHO) around 1% of the Caucasian populations of America and Europe are affected by RA (Symmons, Mathers and Pflieger, 2003). This is a disease which can be incredibly debilitating and as such comes with a high socioeconomic cost (Firestein, 2003). It has long been established that women have a greater risk of developing RA than men and there is also evidence that the onset of symptoms can be associated with negative life events (McInnes and Schett, 2011).

The primary symptom of RA is symmetrical inflammatory polyarthritis that affects the synovium. The synovial membrane is made up of connective tissue that lines the movable part of a joint and secretes proteins to lubricate movement (Lawrence, 2008). Other clinical manifestations include synovial hyperplasia, neoangiogenesis and local immune cell infiltration (McInnes and Schett, 2011). Local symptoms include excruciating joint pain, synovitis (inflammation of the lining of the joint), loss of cartilage and bone remodelling/erosion (Majithia and Geraci, 2007). The synovium also becomes hyperplastic with an infiltration of inflammatory leukocytes and proliferation of local fibroblast-like synoviocytes (FLS) (Sweeney and Firestein, 2004). A new mass of tissue, known as the pannus, forms as the synovium invades the cartilage and this plays a key role in bone erosion (Ainola et al., 2008). The formation of this pathological tissue happens so rapidly that it out-paces the neoangiogenic processes in the local area. The result of this is a hypoxic environment in the arthritic joint (Strehl et al., 2014).

RA is associated with a large array of co-morbidities. Due to the patient's increased systemic inflammatory state, the person can become far more susceptible to myocardial infarction, stroke, and even depression (Dougados et al., 2014, Kawada, 2014, Solomon et al., 2015). Cardiovascular disease is a

frequent comorbidity in RA and conditions such as atherosclerosis are far more common in RA patients than in the general population (Dougados et al., 2014). Patients with RA also have a reduced life expectancy compared to the average population (Pincus, Sokka and Wolfe, 2001).

Great lengths have been taken by clinicians to work out the stages that patients progress through during this disease. The European League of Associations for Rheumatology (formerly the European League Against Rheumatism)(EULAR) recently published a set of criteria for the phases of the disease path (Gerlag et al., 2012). What has become clear is that this described clinical 'pathway' is far from a linear progression. Patients can go both back and forth through the phases of the disease (Jutley, Raza and Buckley, 2015).

The cytokine TNF- α plays a key role in the inflammation of RA and beyond. It has been shown that TNF- α can have neuropsychiatric effects on patients (Wolfe and Michaud, 2004). The cytokine can bind the serotonin receptor and affect the hypothalamo-pituitary-adrenocortical (HPA) axis leading to conditions such as depression (Tillmann et al., 2013). This axis has emerged as an important player in the onset, maintenance and co-morbidity of RA (Capellino et al., 2010). The onset of RA can be associated with stressful life events and it is thought that this is due to involvement of the HPA axis (Tillmann et al., 2013). It has also been shown that the degree of stress positively correlates with the severity of the resulting pathology (Capellino et al., 2010). RA patients who are positive for rheumatoid factor (RF) tend to be diagnosed faster and have lower levels of depression than RF-negative patients (Tillmann et al., 2013).

For some time now rheumatologists have suggested that RA has such variable pathology that it is more than likely a number of different conditions that have been crudely gathered under one name. RA can be thought of clinically as a syndrome that involves the activation of multiple inflammatory pathways of the body. These pathways eventually converge and cooperate to cause the joint pathology seen in the disease (van der Helm-van Mil and Huizinga, 2008, Scott, Wolfe and Huizinga, 2010). The full pathogenesis of the disease remains to be completely elucidated, but this disease is a sizeable contributor to disability and absence from work in the industrialised world (Majithia and Geraci, 2007).

Due to chronic nature of RA patients tend to have to live with the condition for many years. This can have profound effects on the productivity of patients in the workplace along with increased absenteeism leading to cessation of paid work (Filipovic et al., 2011). Patients suffer from fatigue and malaise during RA (Wolfe and Michaud, 2004).

What is becoming apparent in the literature is that the early classification and initiation of treatment is key to patient outcome (Raza et al., 2006, van der Linden et al., 2010). There are three factors that have reached widespread agreement in creating increased susceptibility to disease and these are being female, smoking and certain genetic subtypes (Aho and Heliövaara, 2004). Other factors such as oral inflammation and the microbiome of the gut have been implicated, along with smoking, in creating epigenetic modification which leads to a breakdown in immunological tolerance to self (McInnes and Schett, 2011).

5.1.2 Epidemiology

In the United Kingdom RA affects about 1% of the population and women are three times more likely to be diagnosed with this condition than men (Symmons et al., 2002). Although not impossible, the occurrence of being diagnosed with RA before the age of 15 is very uncommon but the chance of a positive diagnosis increases year on year from then until the age of 80 (Majithia and Geraci, 2007). Interestingly, it has been shown that autoantibodies such as rheumatoid factor (RF) and anti-citrullinated peptide antibody (ACPA) can sometimes be detected in patients many years before the positive diagnosis of arthritis (Rantapää-Dahlqvist et al., 2003).

There have been many advances in the epidemiology of RA and it is now apparent that certain major histocompatibility complex (MHC) haplotypes make patients susceptible to the development of disease (Klareskog et al., 2006). Certain HLA alleles leave patients more susceptible to developing RA. HLA-DRB01 has been shown to be particularly susceptible to production of auto-antibodies following post-translational modification of self-peptide (Klareskog et al., 2006). Citrullination of self-peptides can trigger the cascade towards pathology. Citrullination is a process where the amino acid arginine is modified to citrulline.

Smoking can cause the citrullination of self-peptides and that this, in turn, can lead to the development and production of ACPA (Willemze et al., 2011). Studies on twins further implicate genetic factors in the susceptibility of RA. There is a 15-30% chance of monozygotic twins both being affected by the disease whilst dizygotic twins have a 5% concordance rate (MacGregor et al., 2000).

5.1.3 Diagnosis and Progression

The diagnosis of RA can be complex and there is no single-test that confirms a patient is suffering from this condition. As a result of this patients are classified by many pathological criteria in order to stratify them. These criteria include the levels of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and autoantibodies. The American College of Rheumatology (ACR) and the EULAR jointly devised this system (Aletaha et al 2010). A summary of this system can be seen in Table 5.1.

	Score
A. Joint involvement *	
1 large joint	0
2-10 large joints	1
1-3 small joints (with or without involvement of large joints)	2
4-10 small joints (with or without involvement of large joints)	3
>10 joints (at least 1 small joint)	5
B. Serology (at least one test result is needed for classification)	
Negative RF and negative ACPA	0
Low-positive RF or low-positive ACPA	2
High-positive RF or high-positive ACPA	3
C. Acute-phase reactants (at least one test is needed for classification)	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1
D. Duration of symptoms	
<6 weeks	0
≥6 weeks	1

Table 5.1- The above table summarises the symptomatic factors that are used by clinicians to stratify the patient in terms of what form the RA is taking. (Aletaha et al. 2010).

When it comes to monitoring the progression of a patient's disease state, and their response to treatment, the Disease Activity Score of 28 joints (DAS28) system is used. This system looks at joints of the hands, feet, shoulders, knees and wrists and attempts to gain information on tenderness and swollenness (van Gestel et al., 1996). To this score details are added from other clinical markers of inflammation such as ESR and the patient is asked for a self-assessment of their symptoms over the seven days before the interview with the clinician (Prevoo et al., 1995). This allows the clinician to make informed decisions as to whether the patient should have a change in treatment strategy or continue on the clinical path that they are currently on. If a patient scores above 5.1 on the DAS28 scale they would be judged to have 'very active' disease and a change to their treatment would be required.

5.1.4 Therapy

The goal of therapy is to achieve a state of clinical remission and, upon a positive diagnosis of RA, patients are rapidly put onto an aggressive treatment regimen. Current therapy for RA patients is often centred on drugs known as DMARDs (disease modifying anti-rheumatic drugs). The most commonly prescribed DMARD is methotrexate (MTX), which is used as a first-line therapy (van der Linden et al., 2010). The drug was originally used as a chemotherapeutic agent in high doses but at low doses had efficacy in the treatment of rheumatoid arthritis (CRESS and DEEVER, 1964). The complete mechanistic action of MTX is not fully known. *In vivo* studies show little effect of MTX on cytokine production but do show reduces RF production and neovascularization (Hansen et al., 2006).

Biological therapy has been heralded as one of the most significant advances in treatment of RA patients in recent years (McInnes and Schett, 2011). These therapies are given to patients, when conventional DMARDs are no longer effective, and provide both symptomatic and pathological relief. The most common type of biological agents, used in the clinic, target the potent inflammatory cytokine TNF- α . Drugs are either neutralising monoclonal antibodies against the cytokine (such as Infliximab or Adalimumab) or soluble TNF receptors (such as Etanercept).

Another biological therapeutic agent that has been shown to have great efficacy as a treatment in RA is Abatacept (ABA) (trade name Orencia)(Conaghan et al., 2013). This molecule targets the immune system by exploiting regulatory mechanisms that are in place for the control of activation of T cells. ABA is a fusion protein with the F_C portion of human IgG1 and the F_{AB} region of cytotoxic T lymphocyte-associated antigen (CTLA)-4. CTLA-4 (inhibitory of T cell activation) binds to the costimulatory molecules CD80/86 with a higher affinity than CD28 (key in the costimulation and activation of T cells). The result of a lack of costimulation in T cell activation is T cell anergy and this also reduces the amount of IL-2 that T cells produce which is essential for their proliferation (Brunner et al., 1999). The mechanism of action goes beyond this primary function of T cell regulation and plays a role in B cell biology also (Ballesteros-Tato et al., 2012). $CD4^+$ T-helper (T_H) cells are required to activate B cells in order to induce antibody production (Calvo et al., 1986). It has been shown that a secondary effect of ABA treatment is the reduction of B cell activation which can lead to a reduction in titres of autoantibodies in seropositive patients (Scarsi et al., 2014).

The pathogenic contribution of B cell activation in RA can be targeted more directly by treatment with another biologic agent known as Rituximab (RTX) (trade name mAbThera). This molecule is a monoclonal anti-CD20 antibody that causes death in both activated, and memory, B cells and is accompanied by a reduction in autoantibody production (Shaw, Quan and Totoritis, 2003). It is worth noting that upon the withdrawal of treatment this reduction is lost (Lopez-Olivo et al., 2015).

Interestingly the state of clinical remission is very rarely achieved during therapy and, if achieved, is lost upon the removal of therapeutic intervention (Jutley, Raza and Buckley, 2015). This suggests that there is something else at play here that could be targeted to bring about a more permanent and less drug-dependent remission in patients.

5.1.5 Immune System Involvement

Although this is an inflammatory autoimmune disease and there is clearly hyperactivation of multiple immune pathways, there is a breakdown in regulatory mechanisms of the immune system too. The new wave of biologic therapies, discussed above, illustrate some of the ways in which the immune system is involved in the pathogenesis of RA. Genome-wide association studies have implicated the breakdown of immune system regulatory factors as having a key role to play in the pathology of RA (Wellcome Trust Case Control Consortium, 2007). So it seems that there is a hyperactivation of the immune system that is complimented by a reduction in regulation creating a multiplicative effect.

5.1.6 Cells and Cytokines

RA is an autoimmune condition with involvement from both the innate and adaptive arms of the immune system. Synovitis, one of key pathological features, is caused by an influx of leukocytes to the synovial tissue (Sweeney and Firestein, 2004). Cellular involvement includes recruitment of monocytes/macrophages, dendritic cells, T and B cells, NK cells, and neutrophils (McInnes and Schett, 2011). There is also involvement from activated resident stromal cells in the inflamed joint milieu (Jutley, Raza and Buckley, 2015). Some of the main contributors to the pro-inflammatory state are fibroblast-like synoviocytes and macrophage-like synoviocytes (Jutley, Raza and Buckley, 2015). Studies suggest that the increase in cell number in the synovium is due to cellular influx as opposed to proliferation at the site (Talbot, et al., 2015).

It is worth noting though that local tissue cells also perpetuate the inflammation in this pathology. Chondrocytes are cells that synthesise, release and maintain the cartilaginous extracellular matrix (ECM) factors that make up the joint (Standring, n.d.). During RA inflammatory cytokines such as IL-17, IL-1 β , and TNF act on chondrocytes and cause them to release damaging matrix metalloproteinases (MMPs) (Moran et al., 2009). These enzymes are responsible for building and degrading elements of ECM and in the case of activated chondrocytes the profile of the released MMPs tend towards the more damaging side of the spectrum and contribute to the pathology.

The overexpression of TNF- α , as already discussed, is a key target for therapeutic intervention by clinicians. It has long been established that there is an increase in production of TNF- α in the synovial lining of the joint, in the synovial fluid (Di Giovine, Nuki and Duff, 1988) and increased expression of TNF- α receptors locally too (Sennikov et al., 2015). Another finding relating to this cytokine is that it appears to colocalise with areas of high bone erosion activity (Redlich et al., 2002).

It is important to mention that this is not a one-sided process and that anti-inflammatory cytokines are present in the synovium of RA patients. The T_H2-related cytokines IL-4 and IL-10 have been shown to attenuate the production of T_H1-related cytokines like the aforementioned IL-1 β , IL-6 and TNF- α which subsequently brings about a reduction in tissue damage (Sugiyama et al., 1995, van Roon et al., 1995). The quelling actions of these cytokines appear to be overwhelmed by their inflammatory counterparts.

Bone cells are also implicated in the process here. The two main bone cells are osteoblasts and osteoclasts that synthesise and degrade bone tissue, respectively. Inflammatory cytokines like IL-1 β , and TNF bring about hyperactivation in the degradative osteoclastic cells and this results in the release of more MMPs and the breakdown of bone tissue (Shingu et al., 1993). This bone erosion is a key pathological symptom of RA and one of the more debilitating features of the disease.

Fibroblast-like synoviocytes (FLS) are key mesenchymal cells of synovial joints that synthesise, amongst other things, collagen which is required for optimal joint function (Dasuri et al., 2004). These cells differ from the canonical fibroblast cell-type in that they secrete lubricating factors into the joint which other fibroblastic cells are incapable of doing (Dasuri et al., 2004). One of the key examples of this is the protein lubricin which aids in joint movement (Al-Sharif et al., 2015). During RA these cells lose their contact-inhibition (a proliferation regulatory mechanism) as well as adhesive properties both of which result in a marked increase in the number of these cells (Dasuri et al., 2004). On top of this it has been shown that FLS can secrete pro-inflammatory cytokines

like IL-6 and TNF which add to the pathological milieu of the joint by activating local cells (Smeets et al., 2003). They also secrete MMPs upon activation and so contribute to local tissue destruction (Sekine, Nanki and Yagita, 2014).

5.1.7 Cells of the Innate Immune System

Some of the main cellular drivers of local inflammation are those of the innate immune system. Cells of the innate immune system use pattern recognition receptors to sense potential problems such as infectious organisms, or damage, and during the inflammation in RA this is no different. This is discussed in more detail in the introduction to the thesis (section 1.1.1) but damage-associated molecular patterns (DAMPs) can activate leukocytes and many of them are expressed in the rheumatic joint (Foell, Wittkowski and Roth, 2007). Heat-shock proteins (HSP) are an example of this and they bind to receptors on leukocytes and cause activation (Tukaj et al., 2010).

Although neutrophils appear not to be present within the synovial tissue they are the most abundant leukocyte in synovial fluid (Talbot et al., 2015). It has been shown in experimental models that blocking the infiltration of these cells contributes to a reduction in pathology (Eyles et al., 2008). These cells release potent immunomodulators that contribute to pathology such as prostaglandins and studies suggest that the cells may arrive, in this situation, under the guidance of CCR2 (Talbot et al., 2015). Of course, CXCL8 is the classic neutrophil chemoattractant in humans and this is still important in the recruitment of these cells during RA. One of the cell-types that is responsible for the secretion of CXCL8 are the FLS (Zhu et al., 2013).

One of the main cell-types that contribute to inflammation in synovitis are macrophages (Cornish et al., 2009). The local milieu both attracts and activates these innate effector cells being high in cytokines such as macrophage colony stimulating factor (M-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF) (Cornish et al., 2009). As macrophages are recruited into the hypoxic rheumatic joint they have to adapt to the low oxygen availability in order to survive (Strehl et al., 2014). One of the responses to hypoxia from these cells is to release pro-angiogenic factors

in order to improve the oxygen supply in the local area and lessen the metabolic pressure on nearby cells (Strehl et al., 2014).

Mast cells become activated in the hypoxic joint and can release the potent mediators that are stored in the cytoplasmic granules of these cells (Suurmond et al., 2014). Among these are vasoactive amines which help promote the activation of local endothelial cells to further recruit inflammatory leukocytes, they also release potent cytokines into the local milieu (Lee et al., 2013). Mast cells secrete TNF, which has already been discussed in this introduction, but is a potent activator of inflammatory pathways (Gordon and Galli, 1990). Mast cells also release proteases which contribute to tissue breakdown in the joint (Di Girolamo et al., 2006).

Although a non-cellular component of the immune system, platelets have also been shown to play a role in the inflammatory state of the RA-affected joint (Yeo et al., 2015). These anuclear fragments that come from cellular precursors, megakaryocytes, are highly active modulators of the immune system (Janeway, 2001). One of the primary roles attributed to these fragments are to trigger and aid the clotting cascade but in RA they seem to have other roles in inflammation including the release of chemokines (Yeo et al., 2015).

5.1.8 Cells of the Adaptive Immune System

5.1.8.1 T cells

Patients with RA have many T cells in their synovial tissue and these are thought to contribute to pathology largely through the release of pro-inflammatory cytokines (van Roon et al., 1995). Interestingly, when broad-spectrum T-cell depleting agents were trialled as potential therapeutics in RA, they were shown to be relatively ineffective (Panayi, 2006).

CD4⁺ T cells arrive in abundance especially those of the T_H1 and T_H17 background. During a normal acute immune response T_H1 cells secrete pro-inflammatory cytokines such as IL-1, IL-6 and TNF- α whilst T_H17 cells secrete the potent cytokine IL-17 (Aggarwal et al., 2003). These cytokines activate cells such as monocytes/macrophages, neutrophils and FLS to produce a similar cytokine

signal which perpetuates the system (Aggarwal et al., 2003). Recruited effector T cells arrive at the synovial tissue and require IL-15 as a growth factor but this cytokine has no relevance to effector function, purely survival (McInnes et al., 1997).

In RA there is not only an increase in activity of pro-inflammatory factors, but also a decrease in the anti-inflammatory processes that are in place to regulate inflammation (Choy and Panayi 2001). One of the best illustrations of this is the decreased activity of T_{REG} cells in RA patients. The mechanisms of this defectiveness in these key cells are not fully understood but it has been proposed that the potent T cell modulating molecule CTLA-4 is defective on these cells due to over-expression of TNF (Flores-Borja et al., 2008). One of the classic effector cytokines of T_H1 cells is interferon (IFN)- γ , during active RA these defective T_{REG} cells cannot control the secretion of this inflammatory mediator (Ehrenstein et al., 2004). The paper by Muri et al. suggested that by treating the patient with anti-TNF molecules, Infliximab in this study, the effector functions of T_{REG} cells is restored and may help reduce pathological markers (Ehrenstein et al., 2004).

5.1.8.2 B Cells and Autoantibody Involvement

B cells are responsible for producing antibodies and, as mentioned previously, autoantibodies can play a role in the pathology of RA. It has been shown that the presence of these autoantibodies can precede the onset of the arthritis by many years and the presence or absence of them has an effect on the resulting pathophysiology and disease course (Verheul et al., 2015).

RF was first described around half of a century ago and, although its presence is not specific to RA patients, around 70% of RA patients express titres of this antibody at some level. The autoantibody exists mainly as an IgM molecule, although other isotypes can be present, and the titre tends to correlate with disease severity (Song and Kang, 2010). ACPA is another autoantibody implicated in RA pathogenesis and is a more recently discovered pathological player in comparison to RF. Like RF the titre of this autoantibody correlates with disease severity and with both RF and ACPA, the titres negatively correlate with disease remission rates (Gupta et al., 2015). It is important to mention that some RA

patients are seronegative and do not have the presence of detectable RA-associated autoantibodies.

It has now been shown that RF can have a synergistic affect on ACPA to further enhance pathogenesis. The study showed that patients who were positive for both RF and ACPA tended to have a higher DAS28 score when compared to single positive or double negative RA patients. They also suggested that the presence of both antibodies resulted in a higher peripheral inflammatory burden. They went on to suggest that this synergy brings about an increase in macrophage-activating cytokines in patients (Sokolove et al., 2014).

The involvement of B cells can be best observed with the efficacy of some of the drugs mentioned in the 'Therapy' section (5.1.4). RTX targets and depletes the system of all B cells, except plasma cells, and so ameliorates pathology by reducing the production of autoantibodies by these cells. The production of antibodies is one of the primary immunophysiological roles of B cells. They also secrete cytokines, such as IL-6 and IL-10, which can activate local cells and contribute to pathology.

One of the pathological contributors to RA is the formation of ectopic germinal centres (GC) in the joints (Timmer et al., 2007). GC normally form in lymphoid tissues and serve to increase the efficiency of T cell-B cell interactions and activation. B cells mature and develop in GC and are important in the production of antibodies. The formation of ectopic GC in RA further promotes tissue damage and inflammation. RTX reduces the formation of these structures and this is one of the ways in which they contribute to aiding the reduction of pathology.

5.1.9 Complement

A contribution of the inflammatory milieu in the joint comes from activation of the complement system, there are strong correlations between complement activation and RA pathogenesis (Okroj et al., 2007). This is a key arm of the innate immune system that consists of a cascade of around 30 proteolytic proteins. There are three types of complement activation known as classical, alternative and lectin which involve sequential events which culminate in the lysis of target cells/organisms (Walport, 2001a, 2001b). It has been shown that

there is an increase in complement activation fragments in the peripheral blood of patients (Morgan, Daniels and Williams, 1988).

5.1.10 Involvement of the Chemokine Network

Chemokines play a fundamental role in the pathogenesis and maintenance of RA. The chemokine receptor expression of cells within the synovium and peripheral blood are altered in disease compared to healthy controls. There is a large body of evidence that suggests key roles for cytokines and chemokines in the pathology of RA (McInnes and Schett, 2007). It is worth noting that chemokine ligands and receptors have been the target of many clinical studies by the pharmaceutical industry and have achieved minimal efficacy in treatment. It is thought that this may be due to the proposed redundancy associated with the chemokine network (Zlotnik and Yoshie, 2000) although other theories exist. An intriguing, yet complicating, factor that has recently been described is the citrullination of chemokines in RA. Yoshida et al found that CCL2 and CXCL5 can become citrullinated and that this has an altering affect on their biological function (Yoshida et al., 2014).

RA is a condition involving the influx of immune effector cells to joints along with marked angiogenesis. By definition the main role for chemokines in the body is to bring about the influx of leukocytes to sites of damage and they are also heavily implicated in angiogenesis. It is of no surprise then that chemokines play a substantial role in the orchestration and maintenance of the pathology. Given this it could also be suggested that chemokine and receptor interactions could play a vital role in the remission of the disease. Inflammatory chemokines, and their cognate receptors, are abundantly expressed in the rheumatoid joint.

5.1.10.1 CC Chemokines

Due to the inflammatory nature of RA it is unsurprising that inflammatory CC chemokines are implicated in the pathogenesis of the disease. CCL2, CCL3 and CCL5 have all been shown to induce the recruitment of cells such as T cells, monocytes and NK cells to the rheumatic joint (Koch et al., 1992, Villiger, Terkeltaub and Lotz, 1992, Volin et al., 1998). As mentioned above CCL2 has been shown to be a target of citrullination during RA and this, in turn, affects its

biological function (Yoshida et al., 2014). The CCL2/CCR2 axis is one of the main drivers of inflammatory monocyte recruitment to the joint (Smith et al., 2008) but this modified ligand has been shown to be markedly less effective at this task (Yoshida et al., 2014).

CCL20 is implicated in the pathology of RA due to its receptor CCR6 and its expression on T_H17 cells. These potent producers of IL-17, hence their name, are highly pathological and are recruited to the joint via the CCL20/CCR6 axis (Hirota et al., 2007).

It has also been shown more recently that the ligand-receptor pair of CCL28 and CCR10 have a role in angiogenesis in the joint (Chen et al., 2014). Another chemokine axis that has been implicated in the pathogenesis of RA is the CCR7-CCL19/21. This axis is indispensable in the efficient transit of APCs to the draining lymph node and setting-up the adaptive immune response. In RA patients increased levels of CCL19 has been shown in synovial fluid and is secreted by fibroblasts and macrophages. CCL19 can induce the secretion of VEGF from fibroblasts (Pickens et al., 2011) and promote angiogenesis and the concentration of this in patient plasma is a predictor of a patient's response to rituximab therapy (Sellam et al., 2013).

5.1.10.2 CXC Chemokines

Chemokines have been shown to aid the process of angiogenesis which helps perpetuate the rheumatoid pathology. The ELR motif that was mentioned earlier (section 1.2.2) is key to the angiogenicity of the CXC family of chemokines. ELR-positive chemokines such as CXCL-1, 5, 7, and 8 promote the synthesis of new vessels in the joint. Like most biological processes there are negative regulators of angiogenesis in the joint. The ELR-negative chemokines are angiostatic (i.e. block angiogenesis) these include ligands such as CXCL4, 10, 12, and 13. The rheumatic joint has been shown to be an area of high angiogenic activity, in fact it has actually been compared to a neoplastic tumour. The ELR-positive CXC chemokines play an important role in driving the formation of new vessels which help perpetuate the pathology of the joint.

CXCL-8 acts on local vascular endothelial cells in a mitogenic manner and can induce chemotaxis of distal endothelial cells. This chemokine is produced by synovial macrophages (Koch et al., 1991). Another key role of CXCL8 is in the recruitment of neutrophils to sites of inflammation (Detmers et al., 1990). CXCL12 is expressed by synovial endothelial cells (Buckley, 2003) and is thought to contribute to lymphangiogenesis and T cell recruitment in RA (Bradfield et al., 2003).

The formation of ectopic GC in RA increases the production of autoantibodies and, in turn, the formation of immune complexes. Chemokines have indispensable roles in lymphoid organisation. CCL21 and CXCL13 have been shown to drive the organisation of the T cell compartment and B cell follicles, respectively. This is no different in RA and these chemokines have been shown to function in this manner in the organisation of ectopic GC. It has been shown that serum levels of this key B cell-related chemokine CXCL13 is increased in patients of RA and that the levels of this chemokine correlate with the levels of RF (Jones et al., 2014).

One of the earliest symptoms of RA is synovitis. A study by Scheel-Toeliner et al in 2015 set out to disseminate the differences between joint-recruited macrophages in very early RA versus resolving synovitis and long established disease. The finding of this work was that there was a significant increase in the synovial levels of CXCL4 and CXCL7 in the patients of very early RA. The expression of these chemokines reportedly colocalised with blood vessels, platelets and macrophages (Yeo et al., 2015). CXCL7 is produced by platelets and has been found in the sera and synovial tissue of RA patients (Castor et al., 1992). This is an angiogenic chemokine that also induces proliferation of synovial fibroblasts and subsequent synovial fibrosis (Castor et al., 1992, 1993). In contrast CXCL4 is an ELR-negative, and hence, anti-angiogenic chemokine (Strieter et al., 1995). CXCL4 is produced by synovial macrophages and fibroblasts (Patel, Zachariah and Whichard, 2001) and recruits inflammatory leukocytes such as monocytes and neutrophils (Korniejewska et al., 2011).

5.1.10.3 C and CX₃C Chemokines

XCL1 has been shown to play an indirect role in the reduction in synthesis of MMP-2 which has a protective effect in RA. XCL1 also plays a role in facilitating the recruitment of CD4⁺ and CD8⁺ T cells in RA (Blaschke et al., 2003) which contribute to the reduction in MMP-2. CX₃CL1, the only member of this family aids the recruitment of anti-inflammatory monocytes (Bazan et al., 1997). It also acts as a T cell adhesion molecule and may contribute to the cardiac comorbidities seen in RA patients (Volin et al., 2001, McDermott et al., 2003, Pingiotti et al., 2007).

5.1.10.4 Chemokine Receptors

It has been shown that expression of CCR2 on neutrophils plays an essential role in the tracking of these cells to the rheumatoid joint (Talbot et al., 2015). CCL2 is upregulated in the joints of RA patients and the same finding has also been shown in experimental models of arthritis. The CCR2/CCL2 axis is associated with inflammatory accumulation of leukocytes during normal immune responses. CCR5 is another key inflammatory chemokine receptor and is expressed by many cells recruited into the rheumatic synovium (Haas et al., 2005, Norii et al., 2006, Chou et al., 2010). Cells such as synovial macrophages and T cells both express CCR5 and single nucleotide polymorphisms (SNPs) in this gene have been shown to alter the severity in RA (Zapico et al., 2000, Pokorny et al., 2005).

It has been shown that the T_H17 cell-type has a key role in the pathogenesis of RA. The recruitment of T_H17 is facilitated through interactions between CCL20 and, its cognate receptor, CCR6. A polymorphism of CCR6, known as CCR6DNP, has been shown to increase the expression of CCR6 in humans and increase their susceptibility to a multitude of autoimmune diseases including RA. This polymorphism was also shown to be associated with an increase in serum IL-17 levels, a key effector and inflammatory cytokine released by these cells (Kochi et al., 2010).

FLS secrete increased levels of the CXC chemokine CXCL10 in experimental models of RA. The CXCR3 ligand has been shown to aid the infiltration of these cells to the joint which, in turn, increases the pathology thanks to the

inflammatory cytokines that FLS are known to secrete. In one study it was shown that in a rat model of RA, blocking CXCR3 reduced tissue damage and this was associated with a smaller number of these cells arriving to the joint. Animals that were given increased CXCL10 also showed increased infiltration of FLS and increased tissue damage (Laragione et al., 2011).

Chemokine receptors are key targets for the pharmaceutical industry with regards to potential therapeutics in RA. It is mainly the inflammatory CC chemokine receptors that have been pursued as they are essential to the recruitment of inflammatory leukocytes that are so critical in driving the inflammatory state within the rheumatic joint. This, however, has been largely unsuccessful and in many ways illustrates the possible redundancy in the inflammatory chemokine response. One of the best examples of this is the CCR5 antagonist Maraviroc, as discussed earlier. Unfortunately the clinical efficacy of this drug has been disappointing, providing little clinical efficacy and possibly worsening symptoms in some cases (Fleishaker et al., 2012).

Interestingly, *in vitro* studies have shown that chemotaxis of human monocytes induced by human RA synovial fluid can be inhibited by neutralising antibodies to CCR1, CCR2 and CCR5 (Lebre et al., 2011). This is not recapitulated *in vivo*. The reasons for this remain a mystery but it would be reasonable to suggest that in the rheumatic joint there is just too great a proinflammatory signal that the neutralising antibodies are overwhelmed and unable to deliver any improvement. CCR1 neutralising antibodies have been shown to be efficacious in RA and at least two interventional molecules that target CCR1 are in clinical trials and showing promise (Tak et al., 2013, Santella et al., 2014).

CCR1 ligands CCL15 and CCL23 have been found in high concentrations in the synovial fluid of the RA joint (Berahovich et al., 2005, Chou et al., 2010). There is also a marked influx of CCR1 expressing leukocytes in the joint that are thought to play a key role in driving inflammation. Cells such as neutrophils, macrophages and osteoclasts all express high levels of CCR1 and, as mentioned in the previous paragraph, blocking this receptor seems to provide some promise in reducing inflammation.

5.1.10.5 Possible role for ACKR2

More details on the structure, function, and normal expression of ACKR2 can be found in the introductory chapter to this thesis (section 1.4.4). However, from the studies mentioned above, it can be observed that many of the key chemokines involved in this pathology are the inflammatory CC chemokines. This would suggest that the atypical chemokine receptor, ACKR2, could have a role in the regulation of this condition.

It has been observed that in the joint there is an up-regulation of ACKR2 at a transcriptional level (unpublished observations by Dr Helen Baldwin see appendix III). This data can be coupled with similar protein based data to intimate that ACKR2 is performing some function in this context. As described in the introduction to this thesis it has been shown that ACKR2 has a role in targeting and degrading the inflammatory CC chemokines. The theory then is that ACKR2 is limiting the influx of cells to the joint by reducing the chemotactic signal that is causing the immigration of these cells.

Previous work in our group has shown the presence of ACKR2 in the inflamed joint (McKimmie et al., 2008b). Immunostaining of human rheumatic samples have shown the presence of ACKR2. It has also been shown, in the same study, to be upregulated in the peripheral blood of patients. It was the purpose of the work presented in this chapter to attempt to elucidate possible causative factors for the upregulation in transcription of this gene.

The hypothesis of the work done in this chapter was that the levels of transcription of the *Ackr2* receptor could be used as a prognostic biomarker for patients with rheumatoid arthritis.

5.2 Results

5.2.1 *Ackr2* in Peripheral Blood Cells of RA Patients

This work was carried out with one of the aims of investigating if the transcription levels of *Ackr2* in peripheral blood mononuclear cells (PBMCs) could be used as a biomarker for disease state and/or progression in RA patients. For

the work done in this study patients were split into three separate clinical groupings depending on the state of their condition. Patients were either:

Clinic 1- Escalating RA: Patients whose current therapy regimen is no longer effective with disease diagnosed over 12 months before sample collection

Clinic 2- Established RA: Well-managed disease diagnosed over 12 months before sample collection

Clinic 3- Early RA: Diagnosed within 12 months of sample collection

By splitting the patient samples into three distinct states of the disease this allowed us to look at Akr2 transcription in these separate pathological contexts. We made this split depending on the clinic that the patient was attending at the time of the study. As patients go through their clinical pathway they change the clinics that they attend. It was using the clinics that enabled us to categorise the patients into these groupings defined above.

Previous findings in our lab by Dr Helen Baldwin had suggested an increase in the levels of Akr2 transcription in the peripheral blood of RA patients (see appendix III). As a result of this finding we decided to further investigate this. Samples of peripheral blood were taken from these patients and analysed for levels of Akr2 transcription in PBMCs. Below is a figure showing all of the samples from RA patients (samples from all groups pooled) and compared to healthy control blood samples in terms of Akr2 transcription.

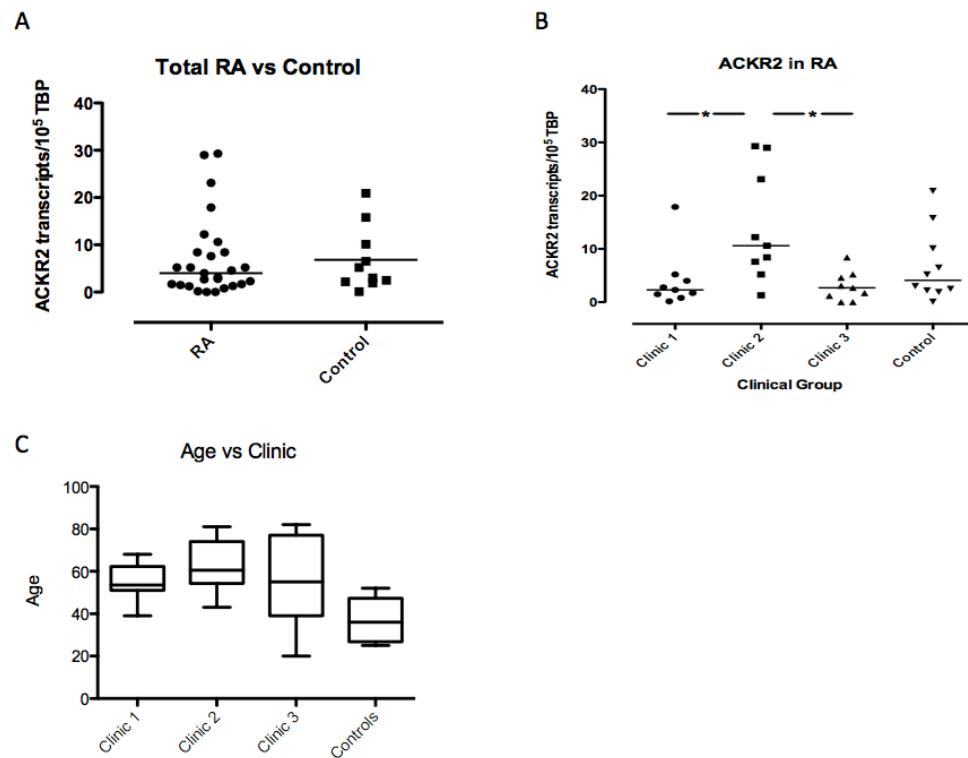


Figure 5.1- The above figure shows the level of ACKR2 expression of patient samples (A) all clinics pooled versus healthy controls. No significant difference was observed, line represents the median value of each group. Mann-Whitney test showed no significance. (B) Patient samples were divided into the clinics they were collected from and healthy controls against ACKR2 expression. One-way ANOVA was carried out on this data. (* $p < 0.05$). (C) Shows the ages of the patients that donated samples to the study per clinic.

It can be seen in figure 5.1A that there is no significant difference between the transcription levels of *Ackr2* in the peripheral blood samples of RA patients when compared to that of healthy controls. Although there are two RA patients that have a higher level of *Ackr2* transcription the means of both groups were almost identical at about 8 copies of *Ackr2*/ 10^5 copies of the reference gene TBP which is a low level of expression.

In figure 5.1B it can be seen that patients in clinic 2 have a significantly higher level of ACKR2 transcription in peripheral blood compared to clinic 1 and clinic 3 patients. Figure 5.1C shows the age spread of the patients involved in each of the clinics along with the healthy controls.

When patient blood was taken the clinician would interview them, as per the normal clinical setting, and a disease activity score (DAS) would be taken. This

score was then correlated with the levels of Ackr2 transcription in the patients. To test correlation between Ackr2 transcription and disease severity, the data were split into the clinical groupings described at the top of this section. The results from this analysis can be observed in figure 5.2.

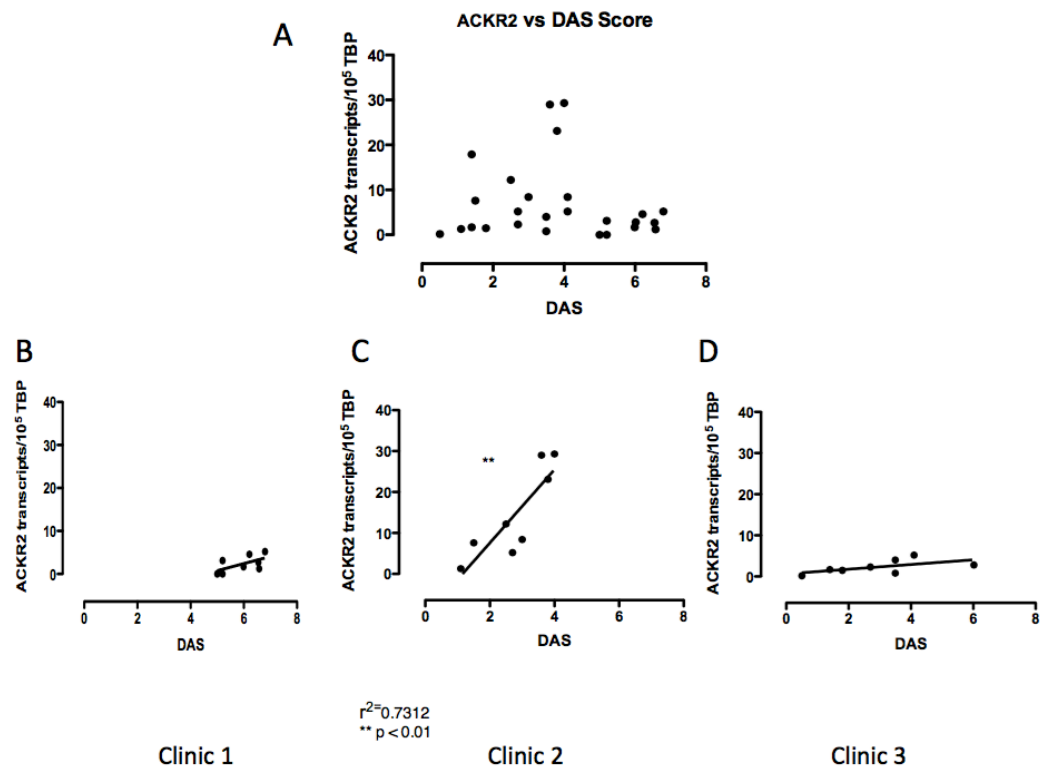


Figure 5.2- The figure above shows the correlations between Ackr2 transcription versus the disease activity score (DAS) from each clinic. It can be seen that there is a positive correlation between the DAS score of patients and Ackr2 transcription in the samples from clinic 2 ($r^2=0.7312$). A- All patient samples pooled, B- Clinic 1 (Escalating RA), C- Clinic 2 (Established RA), D- Clinic 3 (Early RA) this labeling will be used for the coming figures also. (** $p<0.01$)

The data above suggest that in the patients whose treatment regimen is working effectively that the level of Ackr2 transcription correlates positively with the DAS. The other two clinical groupings didn't have the same significant correlation. Along with the DAS scores taken during the clinical meeting with the patient, a number of other pathological measurements were made. These included the erythrocyte sedimentation rate, the levels of C-reactive protein, rheumatoid factor levels, and age.

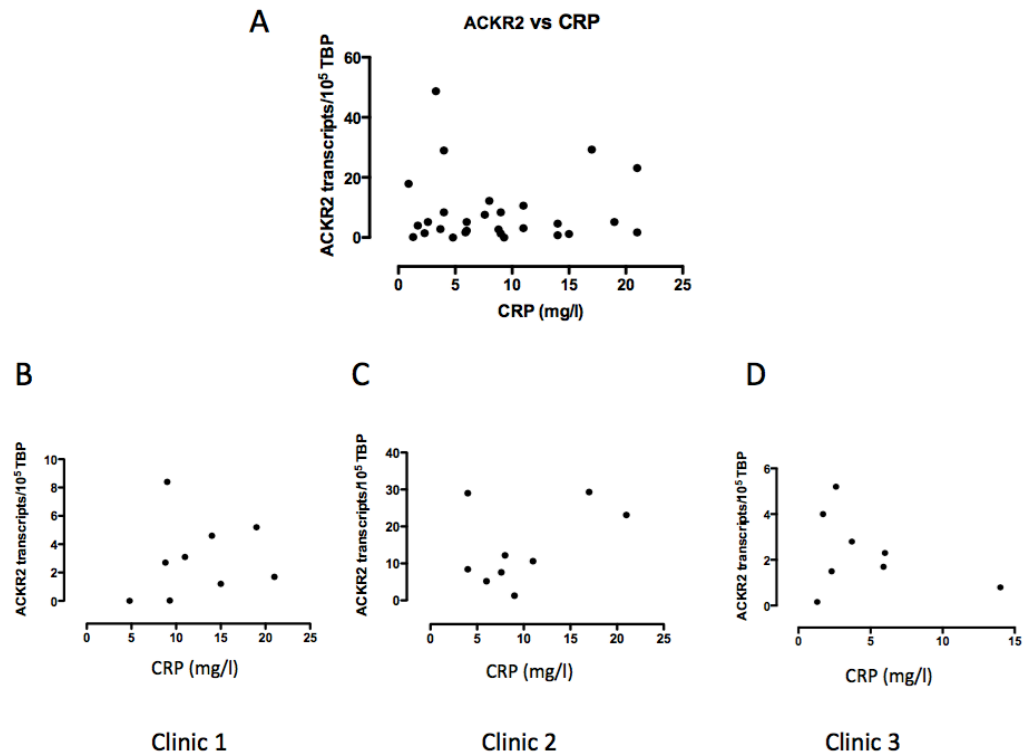


Figure 5.3- The above figure shows the correlative analysis of the C-reactive protein (CRP) levels vs Ackr2 transcription. It can be seen in all three clinical groupings that the levels of transcription do not correlate with CRP levels. A- All patient samples pooled, B- Clinic 1 (Escalating RA), C- Clinic 2 (Established RA), D- Clinic 3 (Early RA).

C-reactive protein is known as an acute phase protein and is one of the key pathogenic markers in RA. Although trends may be visible in patients from clinic one and clinic two there is no significant correlation between the transcription of Ackr2 and CRP levels in patient peripheral blood samples. It may be of note that patients from clinic three show less of a pattern towards a correlation between CRP and Ackr2. These patients probably have the highest inflammatory burden and this may be contributing to this lack of a trend.

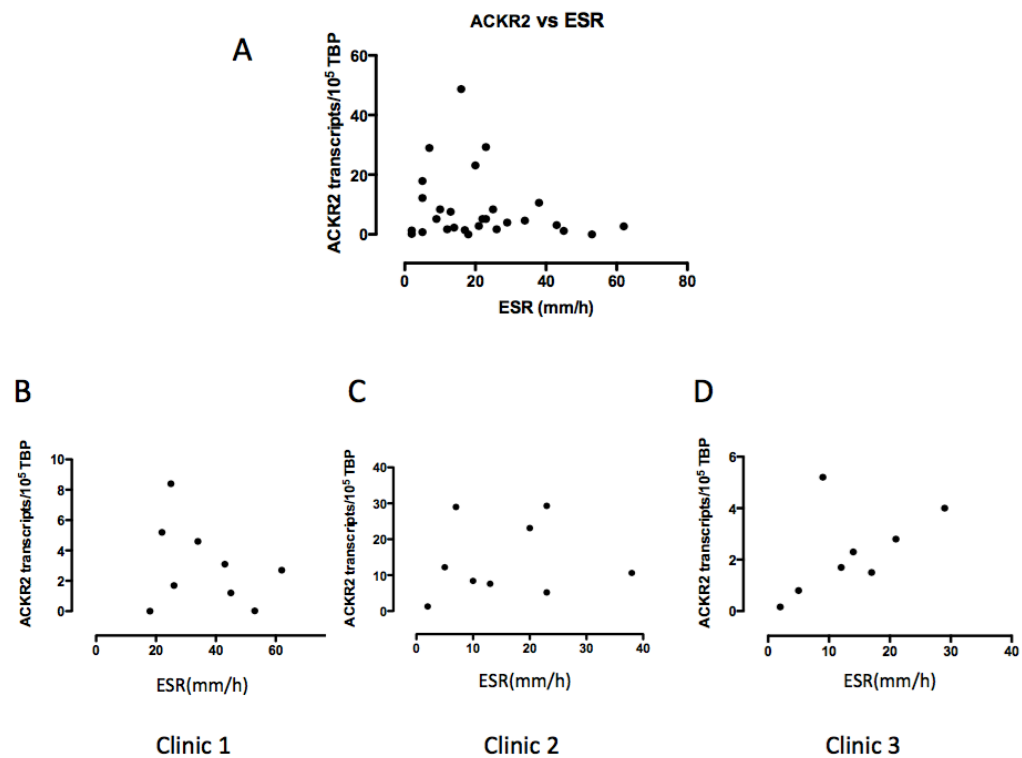


Figure 5.4- The above scatterplots show the correlations between erythrocyte sedimentation rate (ESR) and Ackr2 transcription. There is no significant correlation between these two factors. A- All patient samples pooled, B- Clinic 1 (Escalating RA), C- Clinic 2 (Established RA), D- Clinic 3 (Early RA).

Erythrocyte sedimentation rate is another clinical score that is used to make up the DAS28 score mentioned in section 5.1.3. In the graphs above it can be observed that in clinics 1 and 2 there is no correlation between ESR and Ackr2. Clinic 3 looks as though there is a trend although this is not significant, with a higher sample number this may reach significance.

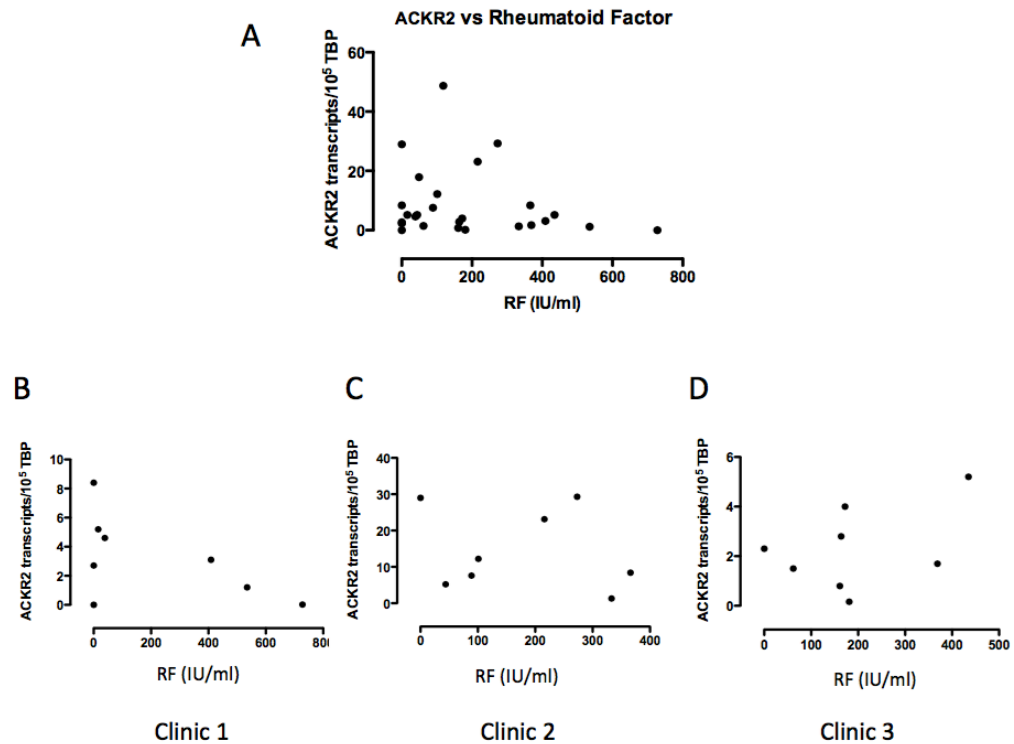


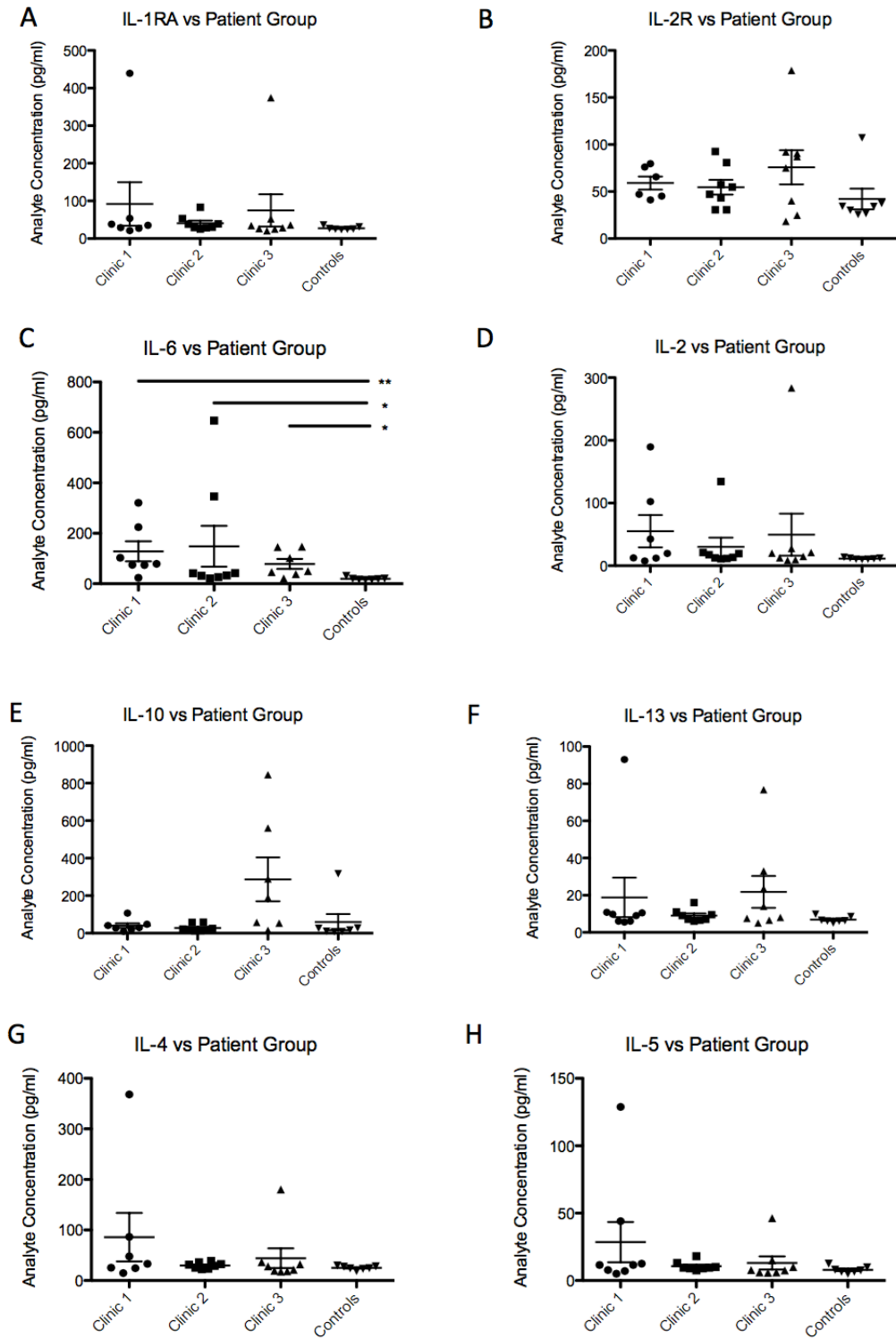
Figure 5.5- The graphs above are the correlations of the autoantibody rheumatoid factor (RF) and Ackr2 transcription. There is no positive correlation between RF and Ackr2 in this cohort. It is worth noting that not every patient is positive for Ackr2 and in clinic 1 you can see 5 patients that are RF negative. A- All patient samples pooled, B- Clinic 1 (Escalating RA), C- Clinic 2 (Established RA), D- Clinic 3 (Early RA).

The above figures show all of the other clinical scores that were taken from patients as the blood was taken from them for this study. The data shows no other significant correlation of clinical scores and Ackr2 transcription in peripheral blood. One result that did stand out was that when patient samples are divided into their individual clinics the patients of clinic 2 have significantly higher levels of CCL2 than other groups. This is an interesting finding and is intriguing as clinic 2 patients also had significantly higher transcriptional activity of Ackr2 in their PBMCs.

5.2.2 RA Luminex

Having examined correlations between Ackr2 and clinical scores of RA patients, the next step was to investigate the blood plasma for protein levels of key cytokines, chemokines and growth factors that are involved in the pathogenesis of RA. This allowed us to see if there was any significant correlation between

the levels of Akr2 transcription in peripheral blood mononuclear cells and the cytokine/chemokine profile in the plasma.



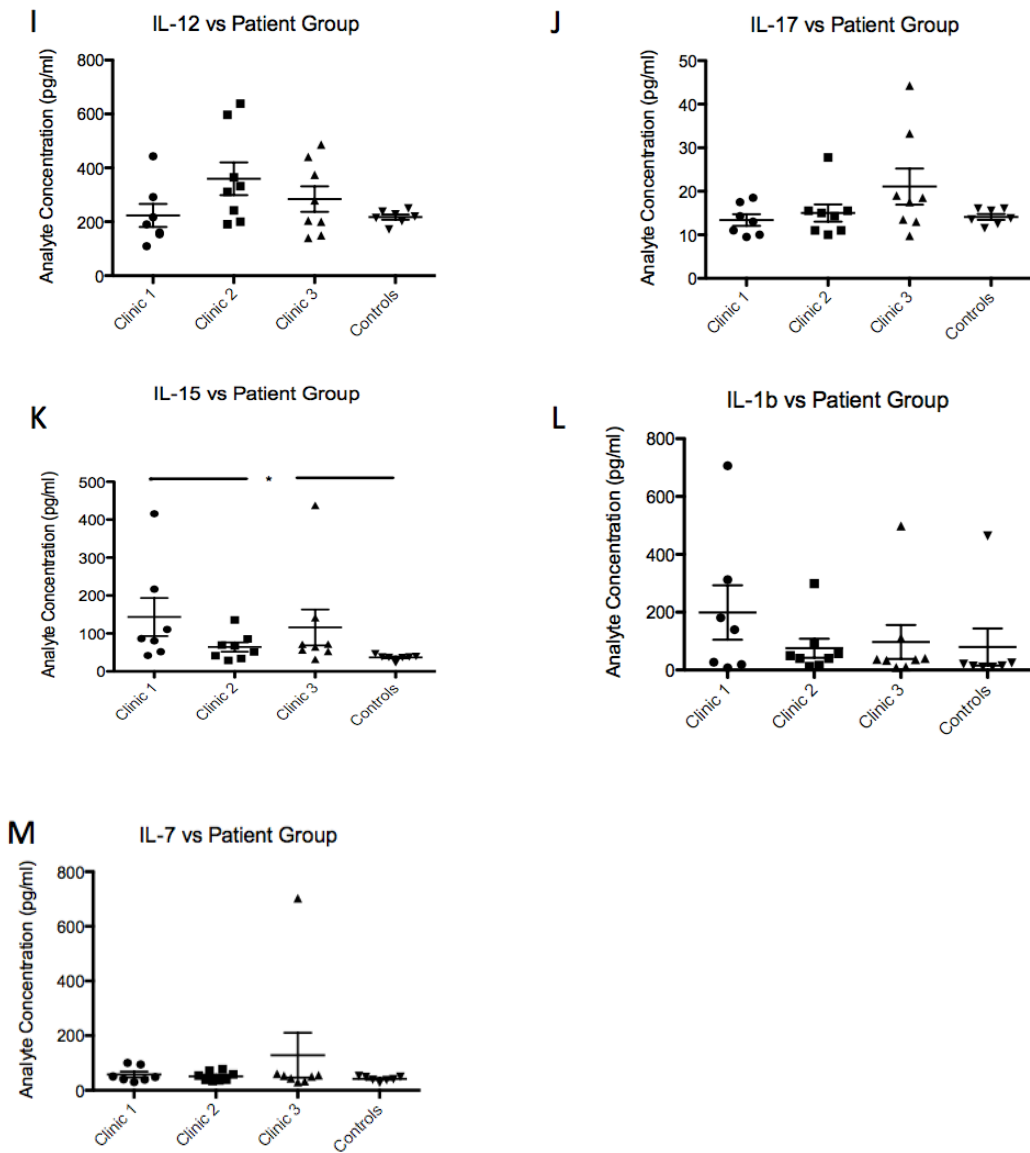


Figure 5.6- The graphs above show the levels of interleukins present in the plasma of patient samples. It can be seen that there is significantly higher expression of IL-15 in patients from clinic 1 when compared to control samples (K). It can also be seen that IL-6 is upregulated in all RA patient groups when compared to control samples (C). Data analysed by one-way ANOVA (* $p < 0.05$, ** $p < 0.01$). Clinic 1 (Escalating RA), Clinic 2 (Established RA), Clinic 3 (Early RA).

Figure 5.6 represents the levels of each of the interleukins. In (A) the level of IL-1 receptor antagonist (IL-1RA) is shown and it can be seen that between all clinical groups and the healthy control cohort there is no difference in the level of this analyte. (B) depicts the levels of the IL-2 receptor (IL-2R). This graph shows that in patients from clinic 3 there is a slight increase in the mean levels of IL-2R when compared to all other groups. This increase does not, however, reach statistical significance. (C) shows IL-6 levels. It can be seen that in all patient groups there is significantly higher levels of IL-6 when compared to the

healthy control samples. Patients from clinic 3 (newly diagnosed RA) have the most significantly raised IL-6 levels compared to healthy controls. The graph in part (D) of figure 5.6 shows the levels of IL-2 from the multiplex assay. All groups showed similar levels of this cytokine. In part (E) you can see the levels of the T_H2 cytokine IL-10. Some of the patients from clinic 3 seem to have raised IL-10 when compared to the other groups but this does not reach statistical significance. Related cytokines IL-13 and IL-4 can be seen in (F) and (G), respectively. All groups have similar levels of these cytokines. In part (H) the levels of IL-5 can be seen and apart from minor individuals with markedly increased levels of IL-5 the general trend is that there is no difference between any of the groups here. For IL-12, depicted in part (I) there is no major difference between the mean levels of the cytokine across the groups. What may be worth noting is that the range of IL-12 levels in the patient groups are more varied than the control cohort of which all samples are around 200 pg/ml. Part (J) shows the levels of IL-17 which shows similar means of the expression of the cytokine in all groups except the samples from clinic 3. The increased mean in this population is due to a more varied range in cytokine a concentration with the other groups having a smaller spread in levels. This increase is not significant. (K) shows the concentrations of IL-15 in the samples. Samples from group 1 have significantly increased concentrations of this cytokine in their plasma than patients from all other groups. Samples from clinic 3 patients are slightly increased IL-15 concentrations too but this is not a significant increase. The concentrations of IL-1 β are shown in figure 5.6 (L). Patients from group 1 have quite varied levels of this inflammatory cytokine and an increased mean level of expression when compared to all of the other groups. This increase is not significant. Finally, part (M) of this figure shows the levels of IL-7. Apart from a single patient sample from clinic 3, the concentrations of this cytokine in the plasma seem to be at a very similar level across all samples. As well as some of the interleukins, this multiplex assay also had other inflammatory cytokines.

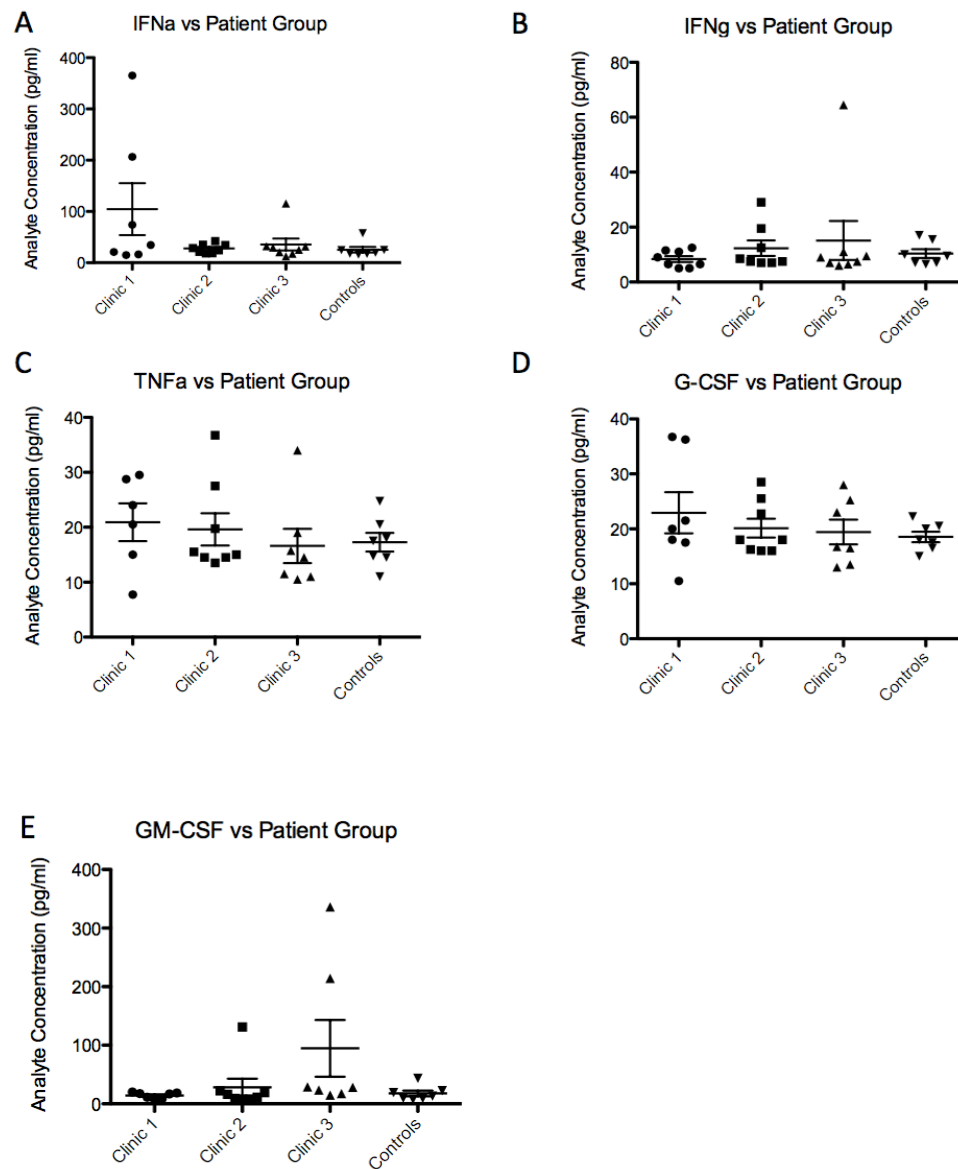


Figure 5.7- The figures above show the plasma levels of other cytokines and colony-stimulating factors. There are no significant differences in levels here between clinics or between patient and control samples. Data analysed by one-way ANOVA. Clinic 1 (Escalating RA), Clinic 2 (Established RA), Clinic 3 (Early RA).

Figure 5.7 depicts some key inflammatory cytokines involved in RA. (A) shows the levels of interferon (IFN)- α , a type I interferon. Clinic 1 patients have quite variable levels of IFN- α and the result of this is an increase in the mean concentration of this cytokine in the plasma of these patients. The other groups have similar levels and there is no significant difference between any of the groups. In figure 5.7 (B) the concentrations of IFN- γ are shown. All groups have very similar mean concentrations of this inflammatory cytokine. (C) depicts levels of TNF. The graph here shows an increase in the mean levels of TNF in the

patients of group 1 and 2 when compared to clinic 3 and healthy control samples. This is not a significant increase. In the penultimate graph of this figure (D) the concentrations of granulocyte-colony stimulating factor (G-CSF) are shown. The mean levels of all of the groups here are similar and there is no significant increase or decrease in G-CSF concentrations. Finally, (E) shows the concentrations of granulocyte-macrophage-colony stimulating factor (GM-CSF) and, mainly due to two patients who show anomalously high levels of the cytokine, in clinic 3 there is an increased mean compared to the other groups.

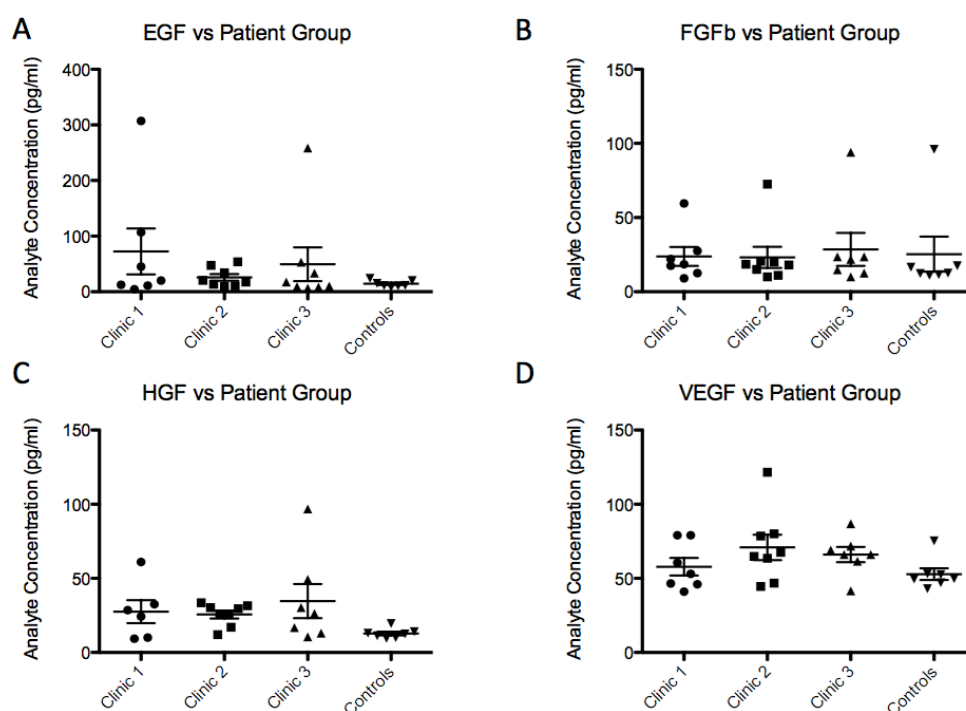


Figure 5.8- This set of graphs depict the plasma levels of various growth factors in the plasma of RA patients and control samples. Data analysed by one-way ANOVA. There are no significant findings from this analysis. Clinic 1 (Escalating RA), Clinic 2 (Established RA), Clinic 3 (Early RA).

The growth factors present on the multiplex are shown in figure 5.8. (A) shows epidermal growth factor (EGF) concentrations and you can see that the levels of this in each of the groups are broadly similar. Basic fibroblast growth factor (FGFb) is shown in (B) and apart from one patient in each of the groups the concentration of this is expressed at a consistent level across all groups. Hepatocyte growth factor (HGF) is shown in (C). There are increased concentrations of HGF in all patient groups but this increase is slight and does

not reach statistical significance. In (D) the levels of vascular endothelial growth factor (VEGF) there is a very slight increase in clinic 1 patients when compared to healthy control samples. Clinics 2 and 3 have a higher level of VEGF again but this is a small increase and is not significant.

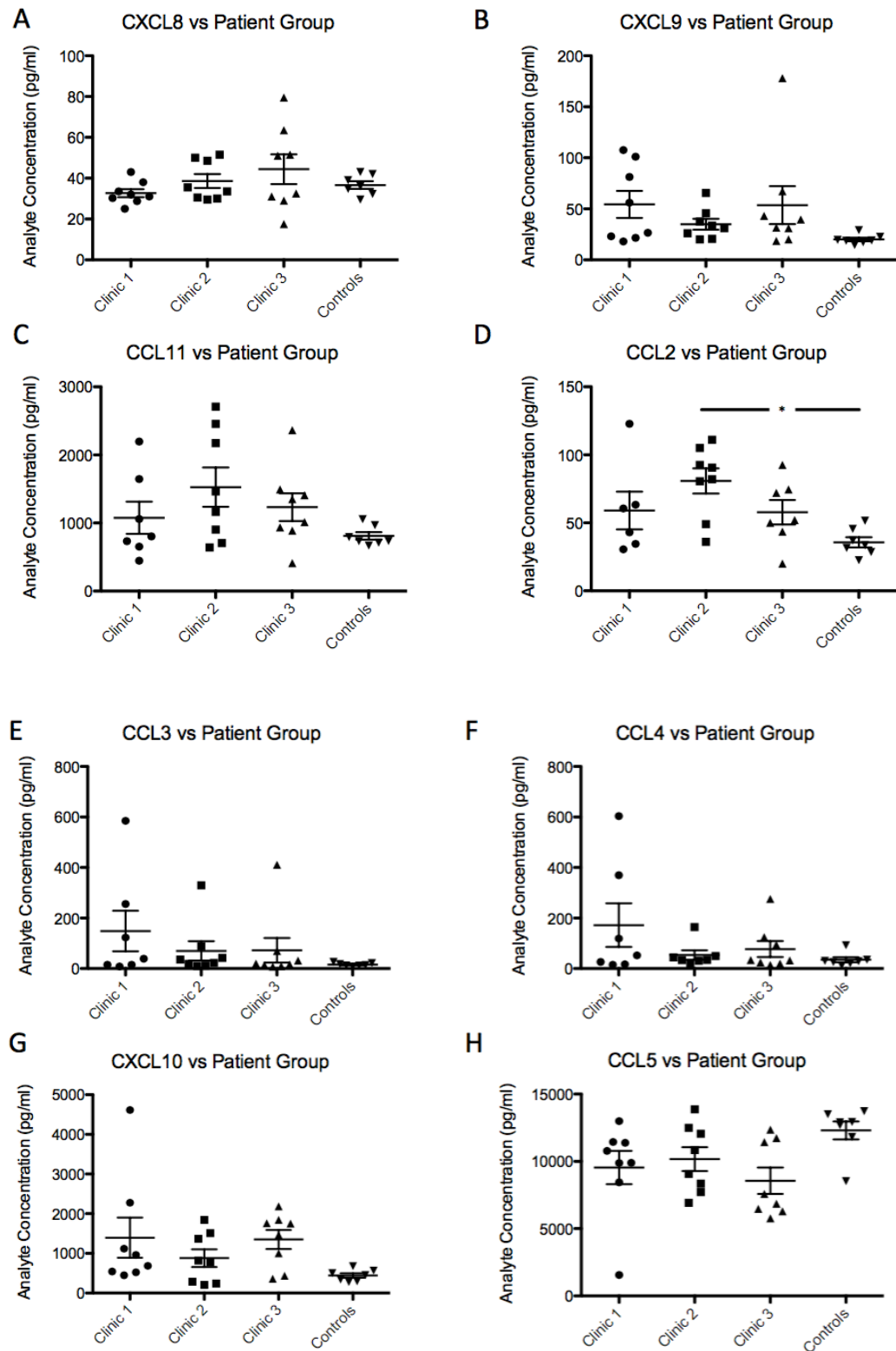


Figure 5.9- The data above show the levels of chemokines in the plasma of RA patients and the control cohort. In (D) one can see that patients of clinic 2 have significantly more CCL2 in their plasma when compared to controls. Data analysed by one-way ANOVA (* $p < 0.05$). Clinic 1 (Escalating RA), Clinic 2 (Established RA), Clinic 3 (Early RA).

The final grouping of analytes from the multiplex assay is the chemokine family. In (A) you can see the concentration of CXCL8, the key neutrophil chemoattractant, in the experimental samples. The levels of this chemokine do not differ significantly between the samples however, due to a large spread of concentrations in clinic 3, there is an increase in the mean level of CXCL8 in this group. CXCL9, the CXCR3 ligand, is shown in (B). Whilst the control group show little variation in the concentrations of CXCL9 the patients from clinic 3, and more so clinic 1, show highly variable concentrations of this chemokine. There is no significant change in the mean concentrations of this chemokine however. The patient samples show very little variation in the concentrations of CCL11 in part (C) however patient samples in all groups are highly variable. The mean concentration of CCL11 is raised in patient samples when compared to the healthy control samples, especially in clinic 2 patients however this is not a significant difference. The graph in part (D) shows the values for the concentration of CCL2 in the plasma of the cohort. The data in this graph suggests that patients from clinic 2 have significantly higher levels of CCL2 in their blood when compared to all other groups. The mean concentration of CCL2 in clinic 1 and 3 patients are at a similar level and are both raised when compared to healthy controls although this is not significant. In (E) and (F) the plasma concentrations of CCL3 and CCL4 are shown, respectively. In these cases, which are similar, there is a slight increase in the level of these chemokines in clinic 1 patients but in general there is not much difference between groups. Figure 5.9 (G) shows the sample levels of CXCL10, all of the patient sample groups have a higher mean concentration of this chemokine when compared to healthy controls. The levels of this chemokine are markedly more variable amongst patient samples than control samples. Finally, (H) shows the levels of CCL5 and in this case the concentrations seem very high. In fact the healthy controls appear to have higher levels of CCL5 in their plasma than the patient samples which seems like there could have been an issue with this particular analyte as this seems highly counterintuitive.

From the data shown above there are very few significant correlations between the levels of Acker2 transcription and the analytes in this plate. A couple of interesting results did appear after analysis however. Firstly, there is increased IL-6 in RA patients of every clinical grouping when compared to healthy control samples. Also, the levels of CCL2 in patients from clinic 2 (well controlled disease) is significantly up when compared to all other groups. This finding becomes even more interesting when compared with the graph of Acker2 transcription vs. clinical grouping. The same patient group have significantly more Acker2 transcription and CCL2 is an ACKR2 ligand.

5.2.3 Effects of Disease-Modifying Anti-Rheumatic Drugs (DMARDs) on ACKR2 Expression

During the treatment of RA DMARDs are very widely used in an attempt to control the pathology of the disease. DMARDs are defined as a group of non-related drugs that slow down the progression of RA. After finding that patients with well-controlled RA (clinic 2) had a significant correlation between Acker2 transcription and DAS score we wanted to look into this further. Patients in this group were all on a regimen of DMARDs so we wanted to see if any of the main drugs of this grouping had an effect on ACKR2 expression.

To do this we harvested peripheral blood mononuclear cells (PBMCs) from a buffy coat and treated those cells with the most common DMARDs, namely methotrexate (MTX), dexamethasone (DXM) and acitretin (ACI). Cells were either incubated along side the drug for 6 or 24 hours before lysing the cells and analysing for transcriptional changes. The figure below shows the effect that each of these drugs had on the transcription of Acker2.

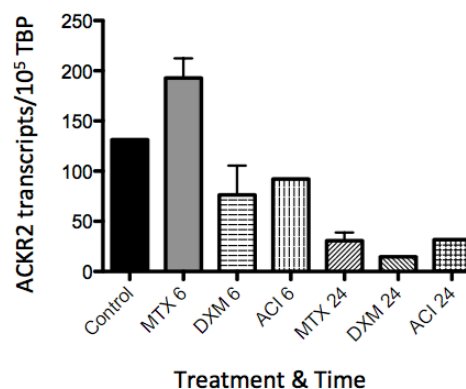


Figure 5.10- The effects of DMARDs on Ackr2 transcription in human PBMCs- The graph above shows the levels of Ackr2 transcription on PBLs when treated with DMARDs for the times stated. A 6 hour treatment with methotrexate (MTX) brought about an increase in transcription when compared to controls. All other treatments resulted in a reduction in Ackr2 transcription. This was a stand-alone experiment (n=1) and error bars represent technical replicates. MTX- Methotrexate (20nM) , DXM-Dexamethasone (1 μ M), ACI- Acitretin (100nM).

As can be observed in figure 5.10 every treatment condition of DMARDs caused a reduction in Ackr2 after 24 hours incubation when compared to a control sample. Interestingly, methotrexate, which is the most commonly prescribed DMARD, brought about an increase in the transcription of Ackr2 after a treatment time of 6 hours.

5.2.4 The Effects of Hypoxia on Ackr2 Transcription

It has been shown that one of the effects of cells being exposed to hypoxic conditions is that it can mimic inflammatory conditions. In the rheumatic joint there is a hypoxic environment as angiogenesis cannot keep up with the neoformation of the pannus (Strehl et al., 2014). It is suggested that this is a contributor to the generally inflammatory milieu in the rheumatic joint. Using the gene hypoxia inducible factor (HIF)-1 α to confirm that a hypoxic state had been induced, we then investigated the transcription of ACKR2 within this environment. As mentioned in more details in the materials and methods section of this thesis, cells were incubated in normoxic conditions as a control or hypoxic conditions for 24 hours. After this the cells were lysed and analysed for ACKR2 transcription. The results from this assay are shown below.

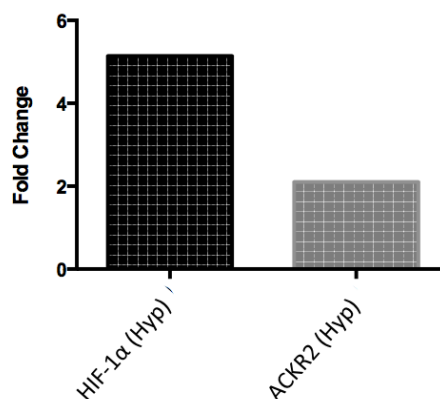


Figure 5.11- HDLEC cells in a hypoxic environment increase transcription of *Ackr2*- HDLEC cells were incubated in a hypoxic chamber for 24 hours then analysed by QPCR for changes in *Ackr2* compared to normoxic controls. *HIF-1α* is a control gene to ensure a state of hypoxia was achieved.

The results above suggest that in a hypoxic environment *ACKR2* transcription is increased by around two-fold compared with those in a normoxic environment. These findings are in corroborate previous findings of our group in similar assays, but also seem to rule out a role for *ACKR2* as a potential biomarker in differing stages of RA.

5.3 Discussion of Chapter 5

Taken together the findings from this work are inconclusive. The results presented above suggest that during well-controlled RA the levels of *Ackr2* transcription in the peripheral blood leukocytes of patients correlates with the DAS28 score. This finding does not seem to extend beyond this patient group however. Patients who have escalating RA or are newly diagnosed with the disease do not seem to display this. The reasons for this are many and further and more complex study would be require to tease out the details of this phenomenon. It has been previously shown that increased inflammation can result in increased expression of *ACKR2*.

What is clear is that when simulating some of the conditions of the rheumatoid joint the level of *Ackr2* transcription is increased. When cells were treated with a selection of the most common drugs that patients receive the transcription of the decoy receptor increased as it did in hypoxic conditions too. These findings

provide some basic evidence to suggest from this work that Akr2 may have a role to play in the regulation of inflammation in RA.

One of the main limiting factors of this study was the number of patient samples collected. Human samples are notoriously heterogeneous and so large sample sizes are required in order to create studies with the appropriate statistical power to draw the most appropriate conclusions. There was not enough time to collect enough patient samples from all three clinical groupings. The data presented shows a cohort of ten samples collected from each clinic along with ten healthy controls. Ideally we would have liked to increase the number of samples collected but time did not allow this.

From the work done previously in our group by Dr Helen Baldwin, as mentioned, the levels of Akr2 transcription in patients with RA was significantly increased when compared to patients with psoriatic arthritis, osteoarthritis and healthy controls. When all of the clinical samples were combined for this study we were unable to recapitulate this finding. The patients that were used for Dr Baldwin's study would all have fallen into group two, as defined by this work, and so when we looked specifically at this group of patients we were able to see a similar finding.

Another limiting factor to this work is the age of patient samples. Although the diagnosis of this condition usually happens around middle-age in patients, due to the chronic nature of the condition patients live for many years with this pathological burden (Innala et al., 2014). As a result of this, many of the samples collected during this study were of quite elderly patients. The average age of the total patient cohort was 56 whilst the average age of the control group was 37. Of course this could be an issue but since the control group were recruited in the lab this is the result. These controls would be more appropriate if they were more rigorously age matched.

There are also other factors that could have been matched. Patients were not asked if they smoked or not and consideration was not taken on comorbidities patients presented with therefore there is no data on this. It has been noted that some diseases may have an effect on the progression of RA and taking into

account further conditions patients presented with may have added further validity to this study.

The patients were grouped according to the clinic that they were attending. This was the best way in which to categorise the samples however with hindsight this could possibly have been improved. Obviously some patients would arrive at a given clinic and be told that they should change what clinic they attend upon the next visit. If this was due to an increased pathology and say a patient was recommended changing from clinic 2 to clinic 3 then this could skew the results. If this were to be done again it would be worth reviewing the patient's pathological state after the interview with their clinician. This may lead to more exclusion but could bring about a more controlled patient cohort. This may have improved the study and possibly change the findings. Another condition that could have been better matched is gender. In appendix IV supplemental figure 5 the gender of the patients recruited can be seen. It would possibly be worth recruiting a more evenly distributed gender cohort in the future.

RA is a disease that can be affected by weather conditions (Cutolo et al., 2006). It is said that during cold and wet weather, which is not uncommon in Glasgow, patients tend to feel worse. This interesting occurrence means that it could have been useful to take notes on the conditions on given days when samples were taken to add more detail to the study. This statement is not a suggestion that the transcription of *Ackr2* may be regulated by weather but it may have an effect on the DAS28 score. During the clinician's interview of patients, just before the blood would be taken for this study, a DAS28 score for that patient is assessed. One of the factors that go into making up this score is to ask the patient how their condition has been over the previous 7 days. The point here is that if the week before the hospital visit and clinical interview had been inclement then the patient may feel worse than if it had been good. This would result in a patient having a higher DAS28, as mentioned, and may have played a role in altering the results presented here. To make this slightly easier, patients could have been 'season-matched' in order to investigate what, if any, effect this had on the results of the study.

Having investigated the effects of individual DMARDs on Akr2 transcription in cell a line, it would be of great interest to look at this in RA patients. Patient's notes could be investigated to see the current DMARD regimen that an individual was on and then by taking a blood sample and doing the QPCR assay used above this could be quantified. This would provide more physiologically relevant detail to the *in vitro* study described here.

Of course, to truly assess any role for ACKR2 in RA the very best patients to study would be those that have had very little or no clinical intervention. This is, of course, extremely difficult and ultimately unfeasible. This study has shown again that the most common drug-type used in the treatment of RA can have a downstream effect on the transcription of the Akr2 gene. It would be interesting to select patients on regimens of single DMARDs and investigate if this had any affect on Akr2 activity. This could have been done for this work but, unfortunately, was overlooked during the design of the study.

To re-assess the hypoxia study in more relevant physiological terms it would be worth carrying out another QPCR and looking at the transcription of the Hif-1 α . This is a marker of hypoxia in cells and so could be correlated with the activity of Akr2.

Chapter 6- Discussion

Chapter 6 Discussion

6.1 Introduction

The atypical chemokine receptor ACKR2 has been shown to have key non-redundant roles in mammalian biology (Graham and Locati, 2013). It has also been shown to be functionally distinct from classical chemokine receptors with the ability to scavenge inflammatory CC chemokines without desensitisation and efficiently limit their bioavailability (Blackburn et al., 2004, Weber et al., 2004) which, in turn, has been shown to limit inflammation *in vivo* (Jamieson et al., 2005). As research has progressed into ACKR2 it has become apparent that it has roles beyond the limitation of inflammation including altering lymphatic vessel densities (Lee et al., 2014) and tumourigenesis (Nibbs et al., 2007).

Studies have shown that this atypical chemokine receptor has key roles in multiple human pathologies related to this work. ACKR2 has been shown to alter the pathology shown in patients with the skin conditions psoriasis (Singh et al., 2012) and scleroderma (Codullo et al., 2011) (a symptom of the disease systemic sclerosis), in allograft rejection in the eye (Hajrasouliha et al., 2013) and in rheumatoid arthritis (Dr Helen Baldwin, appendix III). It was therefore our goal to attempt to build on this body of evidence whilst investigating possible novel facets of ACKR2 function in the skin, the eye and in rheumatoid arthritis.

6.2 General Overview

The overall aim of this work was to investigate the role played by ACKR2 in tissue-specific inflammatory responses. It has been shown that ACKR2 plays a key role in controlling the resolution of inflammation *in vivo* (Jamieson et al., 2005). Previous work on ACKR2 has illustrated the importance of this atypical chemokine receptor in the skin (Jamieson et al., 2005) (Singh et al., 2012) and in the rheumatoid joint (Dr Helen Baldwin, appendix III). This work set about to further our knowledge into the mechanisms of these findings as well as investigate the role of ACKR2 in inflammation of the eye.

The work presented here has compared the inflammatory response of ACKR2-null mice to that of wild-type mice in three anatomically and physiologically distinct tissues. The advantage of this is that we have been able to observe some parallels between the separate models and settings. This work has further demonstrated the key role that ACKR2 plays in the limitation of inflammation through its ability to scavenge inflammatory CC chemokines (Weber et al., 2004). The results suggest that, not only is inflammation increased in the absence of ACKR2, but that the infiltrating cellular profile is also significantly altered. This alteration has down-stream effects on local cells.

6.2.1 Overview of Chapter 3: The Role of ACKR2 in Cutaneous Inflammation

6.2.1.1 ACKR2 in Wound Healing

The work carried out here in two of the anatomical contexts, the skin and the eye, presented phenotypes that could be attributed to macrophages. In both the tissues mentioned there was an increase in collagen deposition during the resolution phases following inflammatory challenge.

The main finding from the wound healing models carried out in chapter 3 was that mice lacking ACKR2 had less-well organised collagen fibres upon scar maturation compared to wild-type littermates. It was unclear if this phenotype was due to increased collagen synthesis or a failure to organise the fibres efficiently. Interestingly, when we removed macrophages from the mice before initiating, and during the development of the wounding model we found a complete reversal of the phenotype. It was also of note that removing these cells caused a temporal delay in wound closure in mice also lacking ACKR2. This allowed us to postulate that ACKR2 plays a fundamental role in regulating macrophage recruitment, a key cell in all stages of the wound healing process. During this work we also found a psoriasis-like inflammation at the injection site of ACKR2 null mice that was not present in wild type mice.

In order to gain insight into possible mechanisms for this hyperfibrotic response, parallels may be drawn with the autoimmune condition systemic sclerosis (SSc).

One of the main symptoms of SSc is aberrant deposition of collagen which causes fibrosis. The ACKR2 ligands CCL2, CCL5 and CCL7 have all been implicated in the initiation and perpetuation of this overactivation of fibroblasts (Distler et al., 2006, Yanaba et al., 2006). Work by Codullo et al. suggested that the transcription of *Ackr2* in peripheral blood leukocytes is increased in leukocytes of patients with SSc (Codullo et al., 2011). The hypothesis from this work was that ACKR2 was aiding the regulation of the systemic levels of its ligands and in turn was protecting the patient (Codullo et al., 2011).

It is reasonable to draw parallels from SSc to the pro-fibrotic responses seen in the inflamed skin and eyes of ACKR2-null mice. Firstly it would be interesting to see if the expression of the same ACKR2 ligands were increased in the plasma of the mice during these models in knockout mice. This would allow one to postulate that a similar mechanism may be responsible for this hypercollagenous response. The proposed model here would be that both systemic and local expression of ACKR2 alters the number of infiltrating cells which indirectly reduces the activation of collagen producing cells such as fibroblasts.

To test this hypothesis a small molecule inhibitor of ACKR2 could be used to provide insights into this process. By carrying out a wounding model, for example, in a wild-type mouse one could control at what time-point after wounding to block the activity of ACKR2 and investigate the phenotypes brought about by intervention at set times. It would be of interest to take this further and investigate the effect of an absence of ACKR2 in other fibrosis contexts. A lung or liver fibrosis model, as examples, would allow us to determine whether this phenomenon was purely a skin-based function of ACKR2 or if it is more widespread.

One of the key sites of expression of ACKR2 is on lymphatic endothelial cells (McKimmie *et al.*, 2013). This expression has a key role in the efficiency of the immune response (Lee et al., 2011) but what is not clear is how much of a contribution this expression makes to the phenotypes found in this work. In order to address this it would be useful to repeat these models in chimeric mice. By taking wild-type mice and reconstituting them with ACKR2-null bone marrow

and vice versa it would allow us to determine the contributions of ACKR2 expression on stromal tissue and leukocytes, respectively.

6.2.1.2 ACKR2 in Other Models of Cutaneous Inflammation

We also demonstrated a protective effect of ACKR2 in a mast cell degranulation model in mouse skin. Throughout this model, mice lacking the receptor had exaggerated inflammatory pathology when compared to wild-type mice in a process which in many ways mimics the cutaneous reaction to thermal damage. This was most obvious in the fact that ACKR2-null mice had significantly thicker skin than wild-type animals after the initiation of this inflammation. In the model of allergic contact dermatitis we saw very little difference between the responses of mice with and without ACKR2.

The results shown in this section suggest a multi-faceted role for ACKR2 in cutaneous tissue. Not only have our results added to the body of evidence showing that the absence of ACKR2 results in a reduced capacity to resolve inflammation, but they have demonstrated a novel role for this molecule in the formation and maturation of collagen fibres in scar tissue. The proposed mechanism for this involves ACKR2 acting indirectly on collagen producing cells such as fibroblasts. The absence of ACKR2 would result in a higher local concentration of inflammatory chemokines. This would, in turn, bring about an increased influx of inflammatory cells such as macrophages. One of the key cytokines produced and released by alternatively activated macrophages is TGF- β 1 which has been shown to increase the levels of collagen synthesis by fibroblasts. In addition to this macrophages have a key role to play in the organisation of collagen fibres in the maturing scar.

6.2.2 Overview of Chapter 4: The Role of ACKR2 in Ocular Inflammation

In the second results chapter of this thesis we set about investigating the role that ACKR2 plays in the experimental autoimmune uveoretinitis model.

This was done alongside Prof. John Forrester from the University of Aberdeen who has published on the subject of chemokines and chemokine receptors in

uveitis previously (Crane et al., 2001, 2006). The results gained from this series of experiments indicated that ACKR2 has a protective role in the model and that mice lacking the receptor have significantly increased pathology.

Having shown this, we wanted to take a more translational approach and assess the likelihood of this finding being feasible in the human eye. To do this we investigated the human retinal pigmented epithelial cell line, ARPE-19 (Dunn et al., 1996), for expression of ACKR2. Our findings show that these cells express the ACKR2 and that it is functional.

The results from in this chapter suggested that ACKR2 has a protective role to play in the prevention of pathological inflammation of the eye. They also suggest that this protection may be relevant to human tissue too. The next step in this study would be to gain human tissue and stain it for ACKR2 to see if the expression pattern of the atypical chemokine receptor is comparable between mouse and human tissue. The proposed mechanism here is that ACKR2 is reducing the local concentration of inflammatory chemokines resulting in a reduction of the numbers of recruited inflammatory cells which could go on to cause pathology in the local tissue.

6.2.3 Overview of Chapter 5: The Role of ACKR2 in Rheumatoid Arthritis

The study carried out in rheumatoid arthritis, the only work not carried-out on mice for this thesis, showed potential and added to work done previously in our lab (appendix III). The aim of this section was to assess the suitability of ACKR2 as a biomarker of disease state and/or progression in patients with this inflammatory autoimmune disease. Although the results from this work suggested that ACKR2 was not appropriate for this task, they did add further validity to Dr Helen Baldwin's previous finding that *Ackr2* transcription is increased in peripheral blood leukocytes of patients with well-controlled disease. This did not seem to be the case for patients with newly diagnosed disease (diagnosis less than 12 months before participation in the study) or patients who were being screened for escalation of therapy. It would be of

interest to further investigate the well-controlled cohort of patients in an attempt to disseminate the possible mechanisms behind this.

After investigating ACKR2 as a potential biomarker we looked into functional roles that the receptor may play in RA through *in vitro* studies. Again drawing on findings from previous studies carried out in our lab, we investigated the transcriptional response of Ackr2 when cells were exposed to hypoxic conditions and disease-modifying anti-rheumatic drugs (the most common therapeutic compounds used in clinics).

The results from these experiments suggested that ACKR2 is not a particularly relevant biomarker for disease progression/state in rheumatoid arthritis. They did, however, show that in the environment of the rheumatic joints, cells may increase the levels of Ackr2 transcription. This may lead to greater control of the inflammatory state of the local cells.

6.3 Additional Studies and Limitations

A chimeric mouse study would give additional insights into the EAU model carried out in chapter 4.2.1 and the allergic contact dermatitis model presented in section 3.5.6. Both of these models have extensive involvement of the adaptive immune system and so the efficient transit of cells to the local draining lymph node is of great importance. Using this system one could observe the phenotypic individual contributions of ACKR2 expression on lymphatic endothelial cells and leukocytes more distinctly. Using this system would also provide insight into contributions made by tissue-resident cells that express the receptor, such as keratinocytes of the skin (Singh et al., 2012), in the limitation of inflammatory responses in specific tissues.

Throughout the work done on murine models, one of the biggest limiting factors, as already mentioned in each chapter, was the lack of a reliable anti-ACKR2 antibody for use on mouse tissue. Although we have developed a reliable monoclonal anti-ACKR2 antibody for human tissues in our lab, along with the availability of commercial equivalents, there is a distinct lack of antibodies that recognise the murine receptor. Having this would have allowed us to gain real

insight into both the temporal and spatial expression of ACKR2-expressing cells during the various models employed for this work. It would also have given a key insight into the kinetics of accumulation of inflammatory cells in the presence or absence of ACKR2. This would have given added detail to the results that we gained from carrying out experiments using ACKR2-null mice.

Another great tool for investigating the mechanistic details of this work further would be an ACKR2-reporter mouse, although this is currently being made at the time of writing. Using this we could visualise, in real-time, where the ACKR2-positive cells were being recruited and how they were interacting with other cells in the local area. Other advantages of this system over an antibody staining method include the ability to harvest these cells in order to thoroughly investigate their phenotype and an avoidance of antibody staining artefacts. The 'biological toolbox' that has been available for this study has given great insight into the functions of ACKR2 but with some minor additions a great amount of added detail could be gained.

6.4 Future Directions

The results shown here have potential to provide the basis for the development of an ACKR2-based therapeutic intervention, certainly in inflammation of the skin and the eye. As explained in the introduction (section 1.4.4.2), the cell-surface expression of ACKR2 under normal circumstances is very low (Blackburn et al., 2004). If a molecule could be developed to increase the levels of expression of this molecule on the cell surface, i.e. increase its bioavailability, and then this could potentially reduce inflammatory pathology further. This could also have a positive effect on scar formation after excisional wounding of the skin.

In order to test if this would be beneficial to patients it would be useful to carry out ACKR2 overexpression studies. This could be achieved using one of two approaches. Firstly genomic engineering methods could be used or the other would be to use a viral vector to increase the levels of the receptor.

Having done this it would be of interest to carry out many of the studies presented in this thesis, as well as others, to investigate the resultant phenotypes. It would be intriguing to see if enhancing the expression of ACKR2 would further reduce inflammatory pathology in these models and, perhaps, further reduce inflammatory damage in the eye or improve scar organisation in the skin. The work shown here suggests that a molecule that could increase ACKR2 expression could have wide-ranging positive effects for patients, certainly in inflammation of the skin and/or the eye.

6.5 Conclusions

The data reported in this study suggest that the absence of ACKR2 both increases inflammatory pathology in murine models of skin and eye inflammation and can alter the profile of the infiltrating cell population in these cases. The functional ablation of macrophages resulted in a phenotypic reversal in the pathology of the skin leading to the hypothesis that a lack of ACKR2 alters the macrophage response during inflammation. This finding is in keeping with our current knowledge on the mechanisms of ACKR2 function and helps build on this. We show, for the first time, that ACKR2 may have a role in the maturation of scars.

Our findings further back-up and expand the repertoire of functions of ACKR2. These results demonstrate the importance of developing molecules that can regulate the expression of ACKR2 both positively and negatively.

Appendix 1: Publications arising from this work

The following publications are included in this appendix:

- BALDWIN, H.M., **PALLAS, K.**, KING, V., JAMIESON, T., MCKIMMIE, C.S., NIBBS, R.J.B., CARBALLIDO, J.M., JARITZ, M., ROT, A., and GRAHAM, G.J., 2013. Microarray analyses demonstrate the involvement of type I interferons in psoriasiform pathology development in D6-deficient mice. *Journal of Biological Chemistry*. 288 (51), pp. 36473–36483.
- LEE, K.M., MCKIMMIE, C.S., GILCHRIST, D.S., **PALLAS, K.J.**, NIBBS, R.J., GARSIDE, P., MCDONALD, V., JENKINS, C., RANSOHOFF, R., LIU, L., MILLING, S., CEROVIC, V., and GRAHAM, G.J., 2011. D6 facilitates cellular migration and fluid flow to lymph nodes by suppressing lymphatic congestion. *Blood*. 118 (23), pp. 6220–6229.
- ROT, A., MCKIMMIE, C., BURT, C.L., **PALLAS, K.J.**, JAMIESON, T., PRUENSTER, M., HORUK, R., NIBBS, R.J.B., and GRAHAM, G.J., 2013. Cell-autonomous regulation of neutrophil migration by the D6 chemokine decoy receptor. *The Journal of Immunology*. 190 (12), pp. 6450–6456.
- SINGH, M.D., KING, V., BALDWIN, H., BURDEN, D., THORRAT, A., HOLMES, S., MCINNES, I.B., NICOLL, R., SHAMS, K., **PALLAS, K.**, JAMIESON, T., LEE, K.M., CARBALLIDO, J.M., ROT, A., and GRAHAM, G.J., 2012. Elevated expression of the chemokine-scavenging receptor D6 is associated with impaired lesion development in psoriasis. *The American Journal of Pathology*. 181 (4), pp. 1158–1164.

Appendix II: Ethical Approval Letter for RA Study

WoSRES

West of Scotland Research Ethics Service

West of Scotland REC 4
Ground Floor – The Tennent Institute
Western Infirmary
38 Church Street
Glasgow G11 6NT
www.nhs.gov.uk

Dr James Dale
Ward 7B, Department of Rheumatology
Gartnavel General Hospital
Great Western Road
Glasgow, G12 0YN

Date 15 June 2011
Your Ref
Our Ref
Direct line 0141 211 2482
Fax 0141 211 1847
E-mail rose.gallacher@ggc.scot.nhs.uk

Dear Dr Dale

Title of the Research Tissue Bank: Institute of Infection, Immunity and
Inflammation Research Tissue Bank
REC reference: 11/S0704/7
Designated Individual: Dr James Dale

Thank you for your letter of 27 May 2011, responding to the Committee's request for further information on the above research tissue bank and submitting revised documentation.

The further information was considered at the meeting of the Sub-Committee of the REC held on 21 June 2011. A list of the members who were present at the meeting is attached.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion of the above research tissue bank on the basis described in the application form and supporting documentation as revised.

The Committee has also confirmed that the favourable ethical opinion applies to all research projects conducted in the UK using tissue or data supplied by the tissue bank, provided that the release of tissue or data complies with the attached conditions. It will not be necessary for these researchers to make project-based applications for ethical approval. They will be deemed to have ethical approval from this committee. You should provide the researcher with a copy of this letter as confirmation of this. The Committee should be notified of all projects receiving tissue and data from this tissue bank by means of an annual report.

Duration of ethical opinion

The favourable opinion is given for a period of five years from the date of this letter and provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully. The opinion may be renewed for a further period of up to five years on receipt of a fresh application. It is suggested that the fresh application is made 3-6 months before the 5 years expires, to ensure continuous approval for the research tissue bank.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Covering Letter	-	05 January 2011
Other: Application for Access to Stored Tissue Samples	1.1	25 April 2011
Other: Dr J Dale's CV	-	
Other: Dr I McInnes's CV	-	
Other: Letter from Jane Hair	-	28 April 2011
Participant Consent Form: Authorisation to donation of surplus tissue for medial research and teaching	-	
Participant Consent Form: Consent to anaesthesia, operation, investigation or treatment, medical photography and research	-	
Participant Consent Form	2	11 July 2005
Participant Information Sheet: The use of surplus tissue for medical research and education	-	
Protocol for Management of the Tissue Bank	1.3	08 December 2010
REC application	-	05 January 2011
Response to Request for Further Information	-	27 May 2011

Research governance

A copy of this letter is being sent to the R&D office responsible for NHS Greater Glasgow & Clyde. You are advised to check their requirements for approval of the research tissue bank.

Under the Research Governance Framework (RGF), there is no requirement for NHS research permission for the establishment of research tissue banks in the NHS. Applications to NHS R&D offices through IRAS are not required as all NHS organisations are expected to have included management review in the process of establishing the research tissue bank.

Research permission is also not required by collaborators at tissue collection centres (TCCs) who provide tissue or data under the terms of a supply agreement between the organisation and the research tissue bank. TCCs are not research sites for the purposes of the RGF.

Research tissue bank managers are advised to provide R&D offices at all TCCs with a copy of the REC application for information, together with a copy of the favourable opinion letter when available. All TCCs should be listed in Part C of the REC application.

NHS researchers undertaking specific research projects using tissue or data supplied by a research tissue bank must apply for permission to R&D offices at all organisations where the research is conducted, whether or not the research tissue bank has ethical approval.

Site-specific assessment (SSA) is not a requirement for ethical review of research tissue banks. There is no need to inform Local Research Ethics Committees.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

Here you will find links to the following:

- a) Providing feedback. You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.
- b) Annual Reports. Please refer to the attached conditions of approval.
- c) Amendments. Please refer to the attached conditions of approval.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk

11/S0704/7	Please quote this number on all correspondence
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Yours sincerely

Dr Brian Neilly
Chair

Enclosures: Standard approval conditions [SL-AC3]

Copy to: R&D office for NHS Greater Glasgow & Clyde

West of Scotland REC 4**Attendance at Sub-Committee in correspondence - deadline 21 June 2011****Committee Members:**

<i>Name</i>	<i>Profession</i>	<i>Present</i>	<i>Notes</i>
Dr Brian Neilly	Consultant Physician	Yes	

Appendix III: Dr Helen Baldwin's RA Study

Elevated ACKR2 expression is a common feature of inflammatory arthropathies

Helen M Baldwin, Mark D Singh, Veronica Codullo, Vicky King, Hilary Wilson, Iain McInnes and Gerard J Graham

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Running title: Regulation and expression of ACKR2 in inflammatory arthritis

Keywords: Chemokine, ACKR2, Arthritis, Inflammation

Correspondence to: Professor Gerard J. Graham, Chemokine Research Group, B3/27, Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow Biomedical Research Centre, Glasgow, G12 8TA, United Kingdom. Email: gerard.graham@glasgow.ac.uk

Funding: This work was funded by Arthritis Research UK (grant number 18448). Work in GJG's laboratory is funded by an MRC Programme Grant and a Wellcome Trust Senior Investigator Award. MDS was supported by a grant from the Scottish Chief Scientist's Office.

Abstract

Chemokines are essential contributors to leukocyte accumulation at sites of inflammatory pathology. Interfering with chemokine or chemokine receptor function therefore represents a plausible therapeutic option. However, our currently limited understanding of chemokine orchestration of inflammatory responses means that such therapies have not yet been fully developed. We have a particular interest in the family of atypical chemokine receptors which fine-tune, or resolve, chemokine driven responses. In particular we are interested in ACKR2 which is a scavenging receptor for inflammatory CC-chemokines and which therefore helps to resolve in vivo inflammatory responses. Here we show elevated expression of ACKR2 on peripheral blood cells as well as on leukocytes and stromal cells in synovial tissue. Expression on peripheral blood leukocytes correlates with, and can be regulated by circulating cytokines with particularly strong associations being seen with IL-6 and HGF. In addition, expression within the synovium is coincident with aggregates of lymphocytes, potentially atopic follicles, and sites of high inflammatory chemokine expression. Similarly increased levels of ACKR2 have been reported in psoriasis and systemic sclerosis. Therefore we propose that elevated ACKR2 expression is a common feature of inflammatory pathologies.

Introduction

The *in vivo* migration of leukocytes is regulated by proteins belonging to the chemokine family[1]. This family is defined on the basis of the presence of a conserved cysteine motif in the mature protein sequence and is further divided into CC, CXC, XC and CX3C subfamilies according to the specific nature of the cysteine motif. Chemokines can be broadly classified as being either inflammatory or homeostatic depending on the contexts in which they function[2, 3]. Inflammatory chemokines are predominantly involved in recruiting inflammatory leukocytes to damaged or infected tissue sites, whereas homeostatic chemokines are involved in more precise tissue localisation of leukocytes. Chemokines interact with their target cells through receptors belonging to the seven transmembrane spanning family of G-protein coupled receptors[4]. There are 18 identified chemokine receptors and, again, these broadly distribute themselves into inflammatory and homeostatic subclasses. Given the essential roles played by chemokines and their receptors in regulating immune and inflammatory leukocyte migration, it is not surprising that they play essential roles in a broad range of autoimmune and inflammatory pathologies[5, 6]. Disappointingly, despite two decades of study, there are currently no antagonists of chemokine receptor function licenced for use in inflammatory diseases. This represents a significant failing in the field and is likely to be related to our currently poorly-developed understanding of the full complexities of the chemokine orchestration of inflammatory responses[6].

In addition to the classical signalling chemokine receptors there exists a separate subclass of receptors characterised by an inability to mount typical signalling responses to chemokine binding[7]. These receptors also possess an altered DRYLAIV motif in the second intracellular loop and have been named 'atypical chemokine receptors' on this basis. There are currently four members of the atypical chemokine receptor family[7-9]: ACKR1 (formerly known as DARC); ACKR2 (formerly known as D6 or ccbp2); ACKR3 (formerly known as RDC1 or CXCR7) and ACKR4 (formerly known as CCRL1 or CCXCKR). These molecules serve essential *in vivo* functions in fine-tuning, or resolving, chemokine-driven responses and represent important new contributors to the overall regulation of chemokine function.

We have a particular interest in ACKR2 which is a highly promiscuous receptor for inflammatory CC-chemokines and which is expressed by lymphatic

endothelial cells, syncytiotrophoblasts and some subsets of leukocytes[10, 11]. Following ligand binding, ACKR2 internalises ligands and targets them for intracellular degradation[12, 13]. It is therefore a 'scavenging-receptor' for inflammatory CC-chemokines and its broad promiscuity ensures that it can scavenge essentially all CC-chemokines involved in inflammatory responses. Work from us, and others, has demonstrated a clear role for ACKR2 in the resolution of inflammatory responses at all tissue sites at which it is expressed[14-20]. In addition we recently demonstrated a role for ACKR2 in lymphatic vessel development[21]. We have also previously shown marked up-regulation of ACKR2 expression in the epidermis and peripheral blood in psoriasis[22], and whole skin and peripheral blood in systemic sclerosis[23] suggesting an, as yet poorly defined, association with inflammatory disease. The purpose of the current study was to extend these analyses to incorporate inflammatory arthropathies, where ACKR2-binding inflammatory CC chemokines such as CCL1, 2, 3 and 5 play key roles in the propagation and maintenance of synovitis [24-28] [29, 30].

Materials and Methods

Isolation of Peripheral Blood Mononuclear Cells (PBMC) and plasma

Heparinised whole blood (10ml) was taken from healthy donors and inflammatory arthritis patients [(early rheumatoid (early RA), established rheumatoid arthritis (RA) and psoriatic arthritis (PsA)] with ethical permission from the West of Scotland Ethics Committee. Matched serum samples (10ml) were taken from each patient. Heparinised blood was diluted 1:1 with wash buffer (PBS supplemented with 1% FCS and 2mM EDTA) and layered onto Histopaque (Sigma #1077-1) according to the manufacturer's instructions. Blood was centrifuged at 400g for 30mins (with the brake removed) and the buffy layer was extracted. Cells were washed twice with wash buffer and counted using a haemocytometer. Cells were either re-suspended in RLT buffer (QIAGEN #74004), containing β -mercaptoethanol, and stored at -80°C for future RNA isolation or re-suspended at 1×10^6 /ml in complete media (RPMI+10% FCS+2mM L-Glutamine+Pencillin/Streptomycin) for future cell culture. Plasma samples were centrifuged at 8000 rpm for 10 minutes at 4°C and serum was stored in 500 μ l aliquots at -80°C for later analysis.

Quantitative real time RT-PCR

PBMC samples stored in RLT buffer at -80°C were thawed at room temperature and RNA was extracted using the RNeasy micro kit (QIAGEN #74004) according to the manufacturer's instructions. RNA concentrations and quality were measured using the Nanodrop-2000 (Thermo Scientific) and stored at -80°C for future cDNA synthesis. RNA was thawed and was reverse-transcribed to cDNA (500ng) using Affinity Script (Stratagene #600559) according to the manufacturer's instructions. cDNA was stored at -20°C until used for quantitative real-time RT-PCR (qPCR) using methodology, and primers, as described previously[31-33]. Samples were run for 40 cycles on the 7900HT Fast Real-Time PCR System (ABI) and data were analysed using ABI Prism SDS software. The absolute number of ACKR2 copies was normalised to TATA-binding protein (TATA-BP) copy number.

Quantification of cytokines and chemokines

Plasma cytokines and chemokines were quantified using the human cytokine 30-plex luminex kit (Invitrogen #LHC6003). The serum was analysed

undiluted. The detection level of the luminex was 30pg/ml; all readings lower than this were designated non-detectable.

Immunofluorescence staining of synovial tissue

Paraffin-embedded RA synovial tissue was sliced into 5µm sections and mounted onto Superfrost slides (VWR #631-0108). Sections were rehydrated to water through xylene and successive concentrations of alcohol. Antigen was retrieved by boiling slides in 0.05M citrate buffer (pH6) for 8 min before slides were blocked for 30 mins at room temperature (RT) in 20% horse serum (Vector Labs #S-2000) and Avidin-D block (Vector Labs #SP-2001) diluted in Tris-Buffered Saline containing 0.01% Tween-20 (TBST). Slides were then incubated overnight at 4°C with rabbit anti-human CCBP2 (ACKR2, 2.5µg/ml, Sigma #HPA013819) at 4°C in Dako REAL antibody diluent (Dako #S2022) supplemented with 2.5% horse serum/2.5% human serum (Sigma #H4522) and Biotin block (Vector Labs #SP-2001). The following morning, slides were washed in TBST and incubated for a further 30 mins at RT with biotinylated horse anti-rabbit IgG (1/200, Vector Labs #BA-1100) in Dako REAL antibody diluent supplemented with 2.5% horse serum/2.5% human serum. Slides were washed in TBST and incubated with Fluorescein Avidin-D (1/500, Vector Labs #A2001) for 40 mins at RT.

Slides were then washed twice in TBST, and re-blocked with 20% horse serum (Vector Labs #S-2000) and Avidin-D block (Vector Labs #SP-2001) diluted in TBST for 30 mins at RT. Slides were washed and stained with antibodies against lineage markers overnight at 4°C in order to detect ACKR2⁺ macrophages: mouse IgG1 anti-human CD68 (clone PG-M1, 1µg/ml; Dako #M0876), mast cells: mouse IgG anti-human mast cell tryptase (clone AA1, 0.43µg/ml; Dako #M7052), CD3⁺ T cells: mouse IgG₁ anti-human CD3 (clone LN10, 1ug/ml, Vector labs #VP-C429), CD20⁺ B cells: mouse IgG_{2a} anti-human CD20cy (clone L26, Dako #M0755) or mouse IgG₁ anti-human CCL3 (clone 93321, R&D Systems #MAB270). The following day, sections were incubated with biotinylated horse anti-mouse IgG H+L (Vector Labs #BA-2000) and subsequently stained with Avidin-D Texas Red (Vector Labs #A2006) for 40min. Slides were washed, mounted using Vectashield mounting medium with DAPI (Vector Labs #H1200) and analyzed on an epifluorescent imaging microscope (Carl Zeiss). Images were captured using Axiovision software Rel 4.8.2.

Immunofluorescence staining of cell cytopins

PBMC were isolated as described above and re-suspended in PBS containing 2mM EDTA at 0.5×10^6 /ml. Cells (0.1×10^6) were spun onto superfrost slides (VWR) at 800 rpm for 3 mins using a Shadon Cytospin Centrifuge (Thermo Scientific), fixed in 100% methanol for 10mins at RT and left to air dry. Slides were then blocked with 20% horse serum (Vector Labs #S-2000) diluted in TBST, before being stained overnight at 4°C with rabbit anti-human CCR2 (ACKR2, 2.5µg/ml, Sigma #HPA013819), mouse IgG₁ anti-human CD3 (Dako) and mouse IgG_{2a} anti-human CD20 (Dako). Antibodies were diluted in TBST supplemented with 2.5% horse serum (Vector Labs #S-2000) and 2.5% human serum (Sigma #H4522). The following day, slides were washed and incubated with goat anti-rabbit FITC (1/300, #4030-02), goat anti-mouse IgG₁ Cy5 (1/300 #1070-15), and goat anti-mouse IgG_{2a} TRITC (1/300, #1080-03, all from Southern Biotech) in TBST containing 2.5% horse serum/2.5% human serum for 30 mins at RT. Slides were washed, mounted using Vectashield mounting medium with DAPI (Vector Labs #H1200) and analyzed on an epifluorescent imaging microscope (Carl Zeiss). Images were captured using Axiovision software Rel 4.8.2.

Stimulation of PBMC to detect ACKR2 expression

PBMC were cultured at 1×10^6 /ml in 6-well plates in complete media (RPMI+10% FCS+ 2mM L-Glutamine + Penicillin/Streptomycin) at 37°C for 6, 24 or 48 hours with combinations of cytokines, or chemokines, all from Peprotech at 100ng/ml. Cells were harvested on ice, and processed for qPCR as described above.

Statistical analysis

For all statistical tests, non-parametric data were analysed using the Mann Whitney U test and parametric data using an unpaired t-test. To detect significant correlation between variables, Spearman's correlation-coefficient was used, where $r=1$ denotes a perfect positive correlation and $r=-1$ a perfect negative correlation. $p<0.05$ denotes significant differences.

Results

Patient Cohort

A summary of the patient details is shown in Supplementary Table 1. Patients classified as early RA (early inflammatory disease) were defined as having a disease course of less than 1 year and had been taking disease modifying anti-rheumatic drugs (DMARDs) for less than 6 months. All patients with RA and PsA had been treated with DMARDs for the majority of the duration of their disease (over 1 year). There were no significant differences in ESR or CRP between the patient groups (Supplementary Figures 1A and 1B).

ACKR2 expression is elevated in PBMC from inflammatory arthritis patients

To determine the expression levels of ACKR2 in PBMC from established psoriatic arthritis (PsA), rheumatoid arthritis (RA) or early rheumatoid arthritis (Early RA) in comparison with healthy controls we used qPCR as previously described. As shown in Figures 1A and B all three patient populations displayed significantly elevated PBMC ACKR2 expression relative to healthy controls. Specifically we found ACKR2 expression to be increased by a median of approximately 2-fold in established PsA ($p=0.005$), 5-fold in established RA ($P=0.004$) and 9-fold in early RA ($p=0.0002$). There was also a significant difference in ACKR2 expression between PsA and early RA patients ($p=0.04$). Some patients displayed extremely elevated levels of ACKR2 expression and, overall, there was an almost '5 Log' spread in ACKR2 levels when data from healthy controls, and all three arthropathy groups, were pooled (Figure 1C).

To attempt to identify the peripheral blood leukocyte subpopulations responsible for the elevated ACKR2 expression we performed immunostaining of PBMC cytopins from RA patients (Figure 1D). Quantitation of the numbers of ACKR2-positive cells per field of view revealed a highly significant increase in the % ACKR2+ve cells in RA patients compared to healthy controls (Figure 1E). Co-staining for ACKR2 and CD14, CD3 or CD20 indicated that ACKR2 expression was detectable in peripheral blood monocytes, T cells and B cells (Figure 1F).

Thus elevated PBMC ACKR2 expression is seen on a variety of leukocyte subtypes in a range of inflammatory arthropathies.

ACKR2 expression levels do not correlate with age or markers of disease severity

Whilst there were significant differences between the ages of the healthy controls and the patient groups, and also between patient groups (Supplementary Figure 1C), there was no correlation between age and ACKR2 expression in PBMC (Supplementary Figure 2A). This suggests that age was not a contributing factor to the data obtained. We next tested for correlations between ACKR2 expression and markers of disease severity (ESR and CRP). This analysis showed no significant correlation between ACKR2 and ESR or CRP (Supplementary Figures 2B and 2C). Thus PBMC ACKR2 expression levels do not correlate with age or disease severity in arthropathies.

ACKR2 expression correlates with peripheral blood cytokine and chemokine levels.

To attempt to define possible regulators of ACKR2 expression on PBMCs, we measured levels of a broad range of circulating cytokines and chemokines using Luminex and tested for correlations with PBMC ACKR2 expression levels. As shown in Figure 2, when data from all patient and control groups were combined, statistically significant correlations with ACKR2 expression levels were seen for IL-6, IL-1 β , IL-2, TNF, IL-7, IL-15, IL5 and HGF. Each correlation detected was positive in the sense that increased cytokine levels correlated with increased ACKR2 levels. By far the most persuasive correlations were with IL-6 and HGF concentrations. In addition, for chemokines, there was a positive correlation between CCL2 levels and ACKR2 expression (Figure 3A) and whilst no overall correlation was detected with CCL3 (Figure 3B), patients expressing ACKR2 levels above the median of the whole population display significantly higher CCL3 levels than those below the median (Figure 3C). Interestingly, when broken down to disease subtypes, the strongest correlation between IL-6 levels and ACKR2 expression was seen for Early RA and between IL-1 and IL-2 and ACKR2 for PsA

Thus ACKR2 expression on PBMCs correlates positively with circulating levels of select cytokines and chemokines.

Combinations of cytokines and chemokines can up-regulate ACKR2 expression on PBMCs

To determine whether the correlating cytokines described above were functionally involved in regulating ACKR2 expression we took PBMC from healthy

individuals, with low levels of ACKR2, and tested the ability of cytokines to increase expression levels. By far the strongest overall correlation between circulating cytokine levels and PBMC ACKR2 levels were seen for IL-6 and HGF. As shown in Figure 4A, IL-6 was unable to modulate ACKR2 expression levels on PBMC. In a similar manner HGF was also unable to modulate expression levels (data not shown). Thus these cytokines are unable to independently induce ACKR2 expression over a 48 hour time frame. Next, to test combinatorial cytokine regulation of ACKR2 expression, we combined the cytokines that correlated with ACKR2 to produce a ‘cytomix’ containing IL-6, IL-1 β , IL-2, TNF- α , IL-7, IL-15, IL-5 and HGF. In addition we generated a ‘chemomix’ containing the chemokines CCL2 and CCL3. Healthy PBMC were stimulated *in-vitro* with these agents following which we detected a significant and sustained increase in ACKR2 expression after stimulation with the cytomix but no increase in expression after stimulation with the chemomix (Figures 4B and C). Thus PBMC expression of ACKR2 correlates with select circulating cytokines and mixtures of these cytokines are capable of increasing ACKR2 expression suggesting a functional association in peripheral blood.

Expression of ACKR2 within Rheumatoid Arthritis Synovium

Next we used immunohistochemistry to examine expression of ACKR2 in RA synovium. Initial staining (Figure 5A) revealed ACKR2 positive signals associated with cells at the tissue/synovial fluid interface as well as on numerous cells internal to the synovial tissue structure. Co-staining for CD45 and ACKR2 (Figure 5B) demonstrated that the receptor is expressed by numerous leukocytes but also by stromal cells throughout the tissue. Interestingly, ACKR2+ve leukocytes were generally not randomly distributed within the tissue and were present predominantly within leukocyte aggregates. Notably, co-staining for ACKR2 and one of its ligands, CCL3, indicates that there is significant overlap in their expression patterns and that ACKR2 is therefore expressed at appropriate tissue positions for effective intra-synovial chemokine scavenging (Figure 5C). We have previously reported expression of ACKR2 by T cells, B cells, monocytic cells and mast cells[31]. In agreement with this, co-staining for markers specific to these leukocyte populations demonstrated ACKR2 expression on these cellular populations (Figure 6A-D). Particularly strong co-staining was seen with aggregates of T and B cells (Figure 6E) suggestive of ACKR2 expression

within ectopic follicles. Thus ACKR2 is expressed on both leukocyte, and non-leukocyte, populations within the synovium, coincident with expression of inflammatory CC-chemokines.

Discussion

ACKR2 is an important regulator of inflammation and we have previously reported marked up-regulation of expression in skin, and peripheral blood leukocytes, in psoriasis and systemic sclerosis[23]. The purpose of the present study was to extend these observations and to examine expression of ACKR2 in arthropathies. Here we present data demonstrating up-regulation of ACKR2 in PsA, RA and early RA. We conclude, on this basis, that elevated peripheral blood ACKR2 expression is a consistent feature of inflammatory pathologies. We further demonstrate an association between select circulating cytokines and ACKR2 expression and show that mixtures of these cytokines are capable of increasing ACKR2 expression levels on healthy PBMC. The strongest association between circulating cytokines and ACKR2 expression was seen for IL-6 and HGF although neither cytokine alone was capable of inducing ACKR2 expression. Interestingly, whilst a similar association with HGF was seen in psoriatic patients, these patients displayed no association between IL-6 and ACKR2 (data not shown). This contrasts with the ACKR2-inducing effects of IL-6 seen on lymphatic endothelial cells[32]. Together our observations suggest alternative molecular drivers for enhanced ACKR2 expression in different inflammatory pathologies and cell types. Surprisingly in the arthropathy groups studied (as well as in psoriatic patients) there is no apparent correlation between peripheral blood ACKR2 expression and disease severity. As circulating inflammatory CC-chemokines will predominantly have derived from the original inflamed site, one intriguing possibility is that ACKR2 limits the activity of circulating chemokines and therefore potentially ameliorates the development of chemokine associated comorbidities (e.g. atherosclerosis) in patients. This possibility, however, remains to be tested.

In addition we demonstrate expression of ACKR2 on both leukocytes, and resident stromal populations, in patient synovial biopsies. The demonstration of expression on stromal cells, especially those at the synovium/synovial fluid interface, extends observations made in psoriatic skin which demonstrated

inducible expression on keratinocytes[22]. We hypothesise that stromal cell expression serves to reduce inflammatory chemokine bio-availability, or to limit its domain of influence, and this is supported by the coincident expression of ACKR2 and CCL3 in synovial tissues. Strikingly, many of the leukocytes that are positive for ACKR2 expression are B and T cells present in large leukocyte aggregates within the synovial tissues. We propose that these correspond to ectopic follicles although the roles for ACKR2 within these structures is not immediately apparent. It may be that ACKR2 is important to ensure the relative absence of inflammatory leukocytes within these ectopic lymphoid follicles[34, 35].

In summary, therefore, we provide evidence demonstrating up-regulation of ACKR2 in peripheral blood cells and in cells within the synovium. This, coupled with previous observations from psoriasis, and systemic sclerosis, patients suggests that elevated ACKR2 levels are a consistent feature of human inflammatory pathologies. Further work is required to determine the functional implications of elevated expression in these pathological contexts.

Figure Legends

Figure 1. ACKR2 expression is elevated in PBMCs from inflammatory arthropathy patients.

A) ACKR2 expression was measured by qPCR in PBMC from PsA, RA and Early RA patients using absolute quantification and normalised to TATA-BP expression levels.

B) Fold change in expression was calculated by dividing the ACKR2 expression/ 1×10^6 TATA-BP by the median ACKR2 expression/ 1×10^6 TATA-BP of healthy controls.

C) A graph of compiled data showing the overall spread of ACKR2 expression across healthy controls and the three patient groups.

D) Cytospins of PBMC from healthy controls and RA patients were stained with rabbit anti-human ACKR2 (2.5 $\mu\text{g/ml}$) which was detected using biotinylated horse anti rabbit IgG/Streptavidin FITC.

E) The numbers of ACKR2⁺ cells per view were counted over ten fields of view and the mean ACKR2 expression is shown from 7 healthy donors and 10 RA donors.

F) Co-staining for ACKR2 (green), and CD14, CD3 or CD20 (each in red) on cytopins of PBMC from RA patients. The overlay is shown in the 3rd panel of the images. Slides were mounted with DAPI and expression was measured using a Zeiss epifluorescent microscope (Axiovision rel 4.8.2).

Figure 2. Correlation of ACKR2 expression in PBMC with plasma cytokines.

Peripheral blood mononuclear cells (PBMC) and paired serum samples were taken from healthy controls, psoriatic arthritis patients (PsA), rheumatoid arthritis patients (RA) or early rheumatoid arthritis patients (early RA). ACKR2 expression was measured using qPCR with absolute quantification and normalised to 10^6 copies of TATA binding protein (TATA-BP). Plasma was analysed by 30-plex luminex analysis. ACKR2 expression is shown against significantly correlating cytokines: IL-6, IL-1 β , IL-2, TNF- α , IL-7, IL-15, IL5 and HGF. Correlation analysis was performed using Spearman's Correlation Coefficient.

Figure 3. Correlation of ACKR2 expression in PBMC with ACKR2-binding chemokines. Peripheral blood mononuclear cells (PBMC) and paired plasma

samples were taken from healthy controls, psoriatic arthritis patients (PsA), rheumatoid arthritis patients (RA) or early rheumatoid arthritis patients (early RA). ACKR2 expression was measured using qPCR with absolute quantification and normalised to 10^6 copies of TATA binding protein (TATA-BP). Plasma was analysed by 30-plex luminex analysis. ACKR2 expression is shown correlated with against CCL2 (A) and CCL3 (B). CCL3 levels between ACKR2-high and ACKR2-low patients (C). Correlation analysis was performed using Spearman's Correlation Coefficient.

Figure 4. Regulation of ACKR2 expression by cytokines and chemokines.

- A) Healthy PBMCs were treated with PBS or with IL-6 in the presence, or absence, of soluble IL-6 receptor for 6, 24 and 48 hours. RNA was isolated and ACKR2 expression assessed using qPCR. Data at each time point are expressed relative to ACKR2 expression in fresh untreated PBMCs.
- B) Cytokines correlating with ACKR2 expression were pooled into a 'cytomix' containing IL-6, IL-1 β , IL-2, TNF- α , IL-7, IL-15 and IL-5 and HGF (all at 100ng/ml) and the mix used to stimulate PBMCs for 6, 24 and 48 hours. Expression of ACKR2 was measured and expressed as described above.
- C) A 'chemomix' containing CCL2 and CCL3 (both at 10 μ g/ml) was similarly used to stimulate PBMC, and ACKR2 expression measured and expressed as described above.

Figure 5. ACKR2 is expressed within the rheumatoid arthritis synovium in leukocytes and stromal cells.

- A) Paraffin embedded rheumatoid arthritis synovial tissue sections (5 μ m) were stained for ACKR2 using rabbit anti-human ACKR2 (2.5 μ g/ml) and expression was visualised using DAB. Slides were counterstained with haematoxylin, visualised under a Zeiss light microscope and analysed using Axiovision Rel 4.8.2 software.
- B) Co-staining for ACKR2 (green) and CD45 (red) and showing the overlay of the 2 stains (yellow) on paraffin-embedded synovial tissue sections. Slides were counterstained with DAPI (blue) to reveal nuclei. Images were taken using a Zeiss epifluorescent microscope and Axiovision Rel 4.8.2 software.
- C) Paraffin embedded rheumatoid arthritis synovial tissue sections were stained for ACKR2 (green) and CCL3 (red). The third image shows the overlay of the two

colours. Slides were counterstained with DAPI and visualised on a Zeiss epifluorescent microscope with AxioVision rel 4.8.2 software.

Figure 6. ACKR2 is expressed within rheumatoid arthritis synovium on defined leukocyte subpopulations.

Paraffin embedded rheumatoid arthritis synovial tissue sections (5 μ m) were stained for ACKR2 (green) together with CD3 (A), CD20 (B), CD68(C), or MCT (D) to identify leukocytes within the synovium expressing ACKR2. Yellow represents the overlay of the 2 colours and indicates co-incident expression. Slides were counterstained with DAPI and visualised on a Zeiss epifluorescent microscope with AxioVision rel 4.8.2 software. Percentages of ACKR2⁺ Lineage⁺ cells were quantified over 10 fields of view and the results are shown in (E).

Supplementary Figure 1. ESR, CRP and age of the individuals used in this study.

Patients were recruited from Stobhill hospital and the Victoria Infirmary, Glasgow. ESR (A), CRP (B) and age (C) were plotted for each patient group.

Supplementary Figure 2. Correlation between ACKR2 expression in PBMC and age, ESR and CRP.

Peripheral blood mononuclear cells (PBMC) were taken from healthy controls, psoriatic arthritis patients (PsA), rheumatoid arthritis patients (RA) or early rheumatoid arthritis patients (early RA). ACKR2 expression was measured using qPCR with absolute quantification and normalised to 10⁶ copies of TATA binding protein (TATA-BP). ACKR2 expression is shown against age (A), ESR (B) and CRP (C). Correlation analysis was performed using Spearman's Correlation Coefficient.

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Figure 1

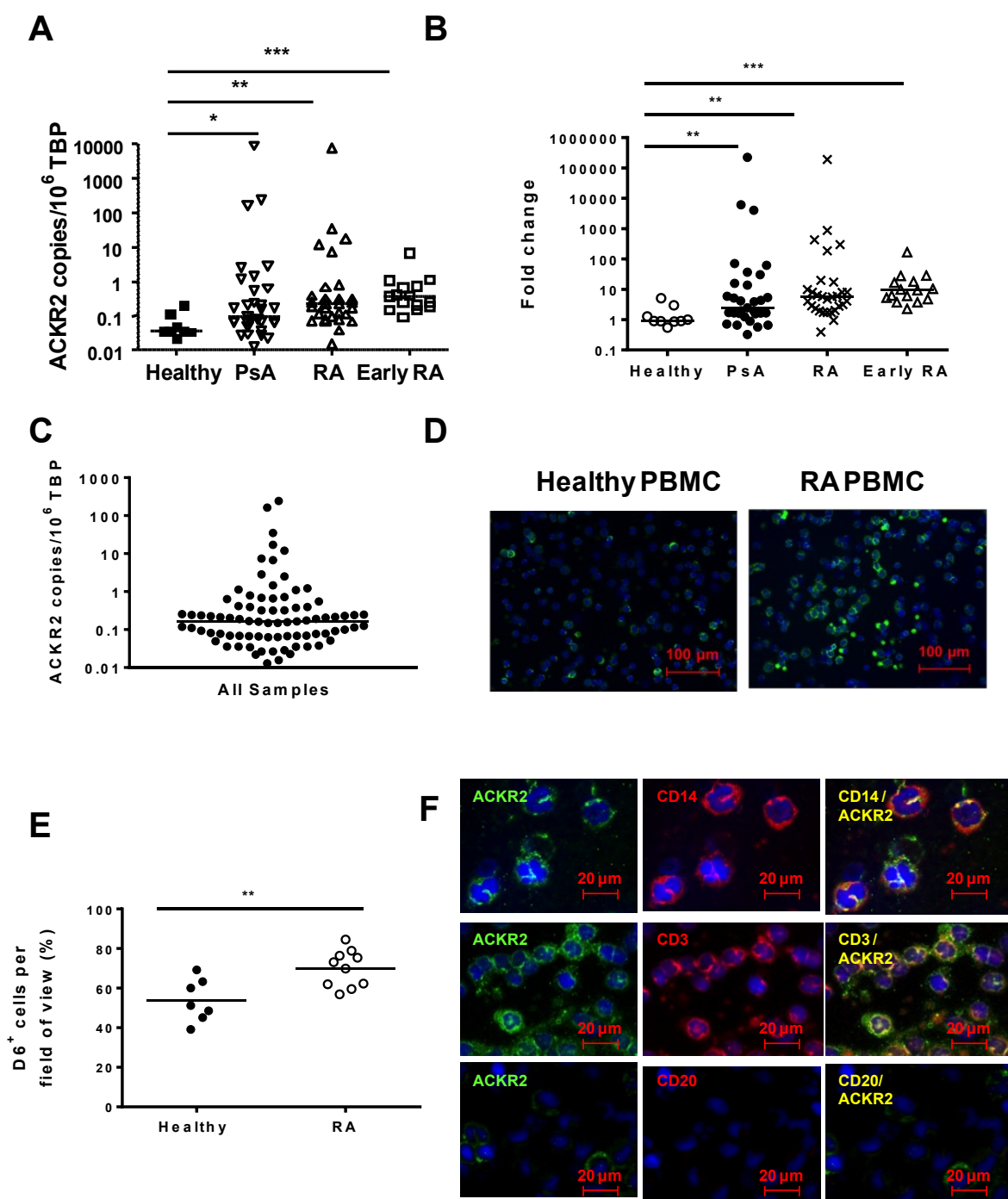


Figure 2

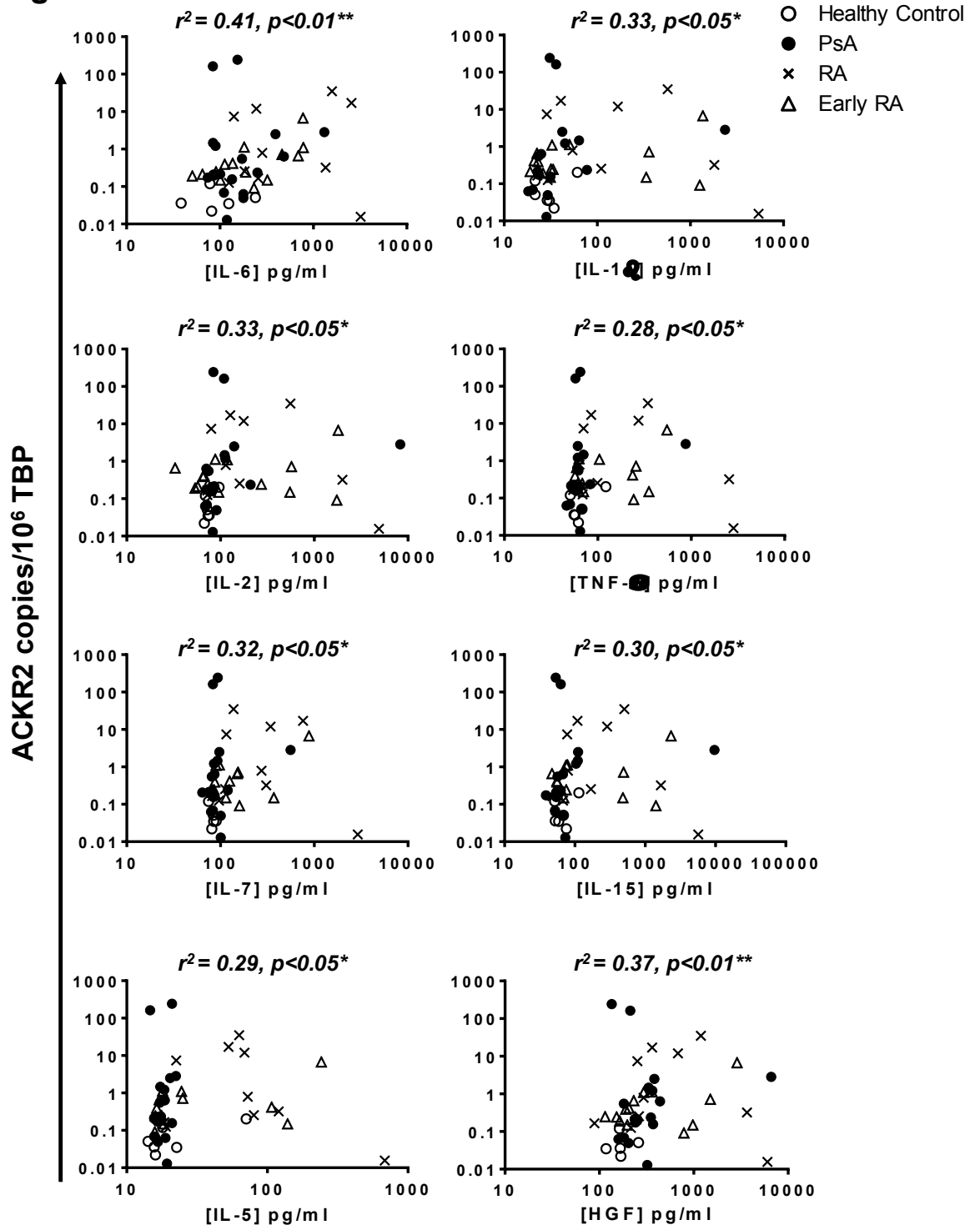


Figure 3

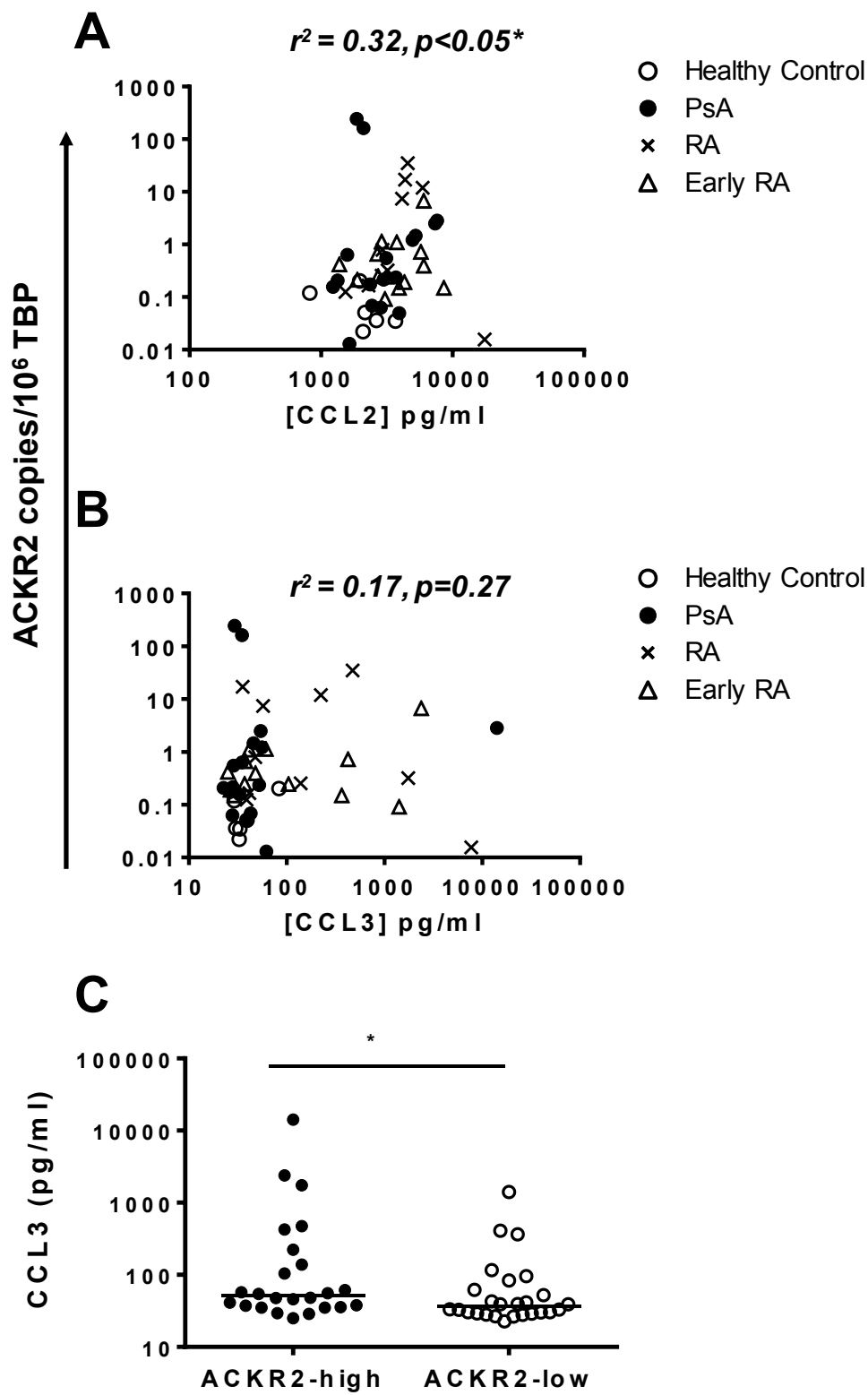
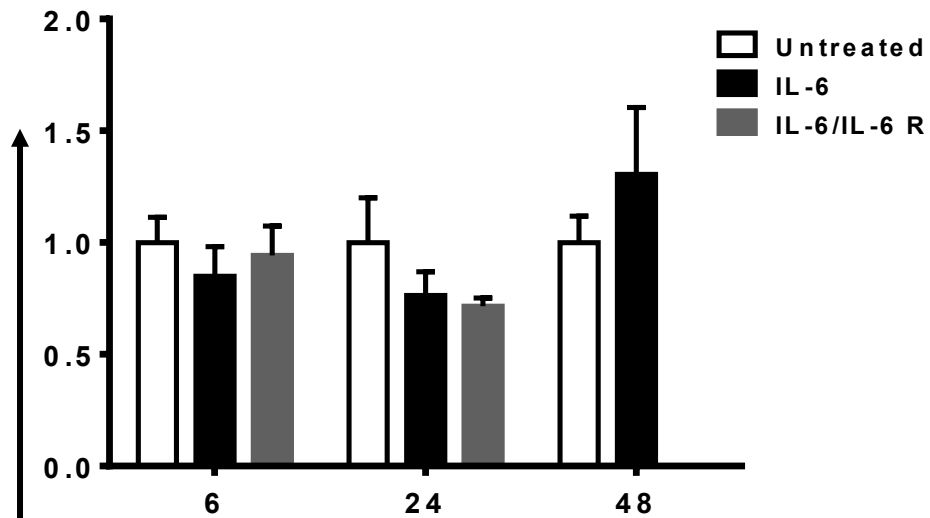
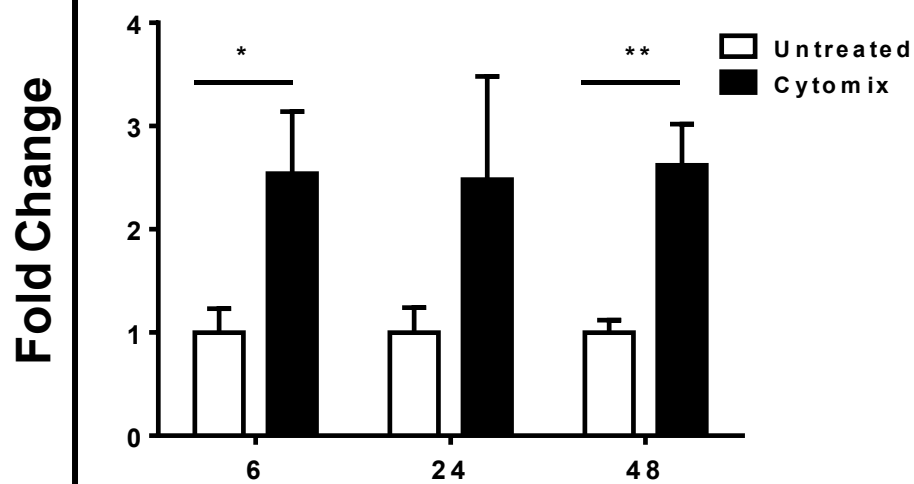


Figure 4

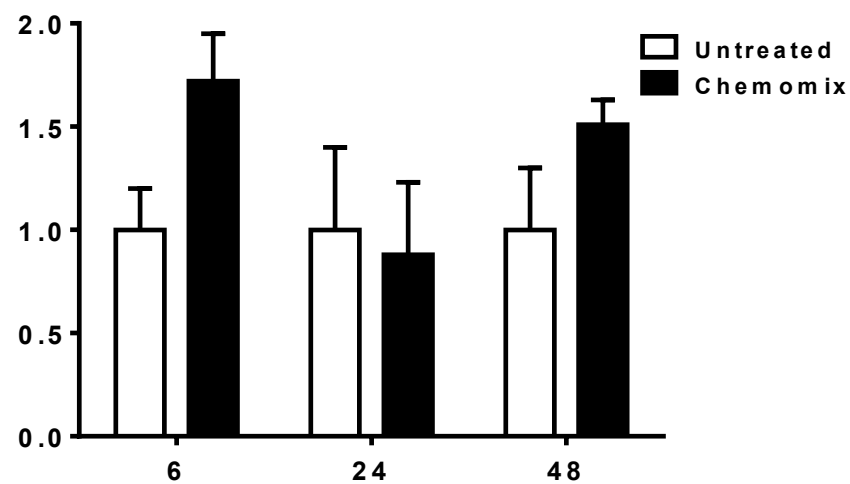
A



B



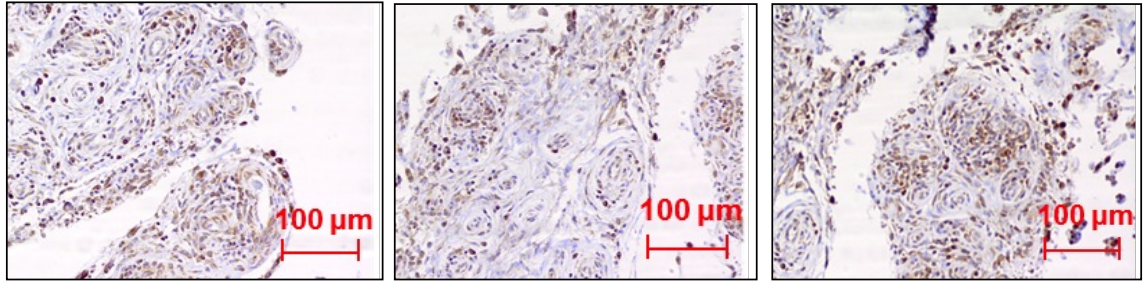
C



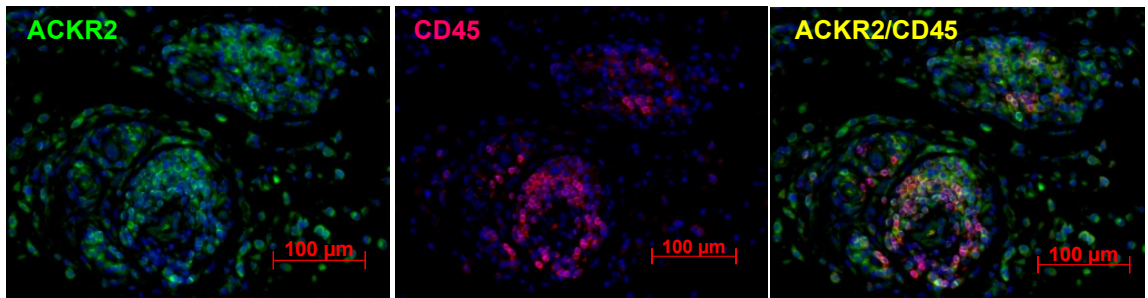
Time (hours)

Figure 5

A



B



C

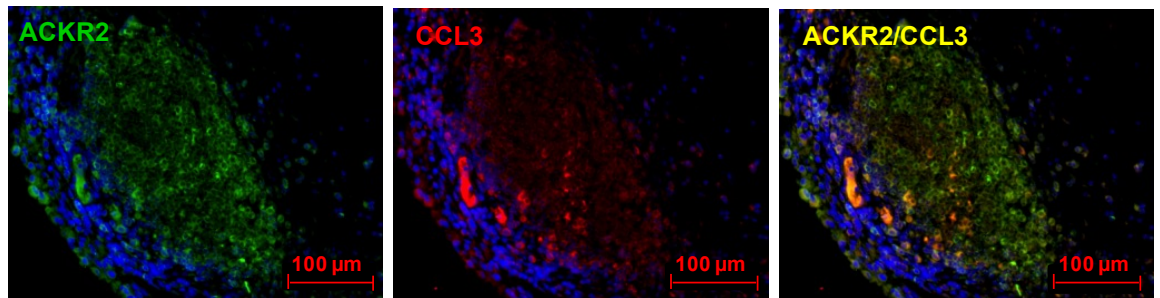
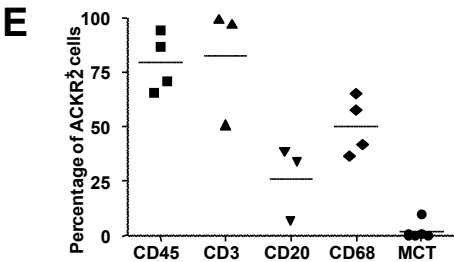
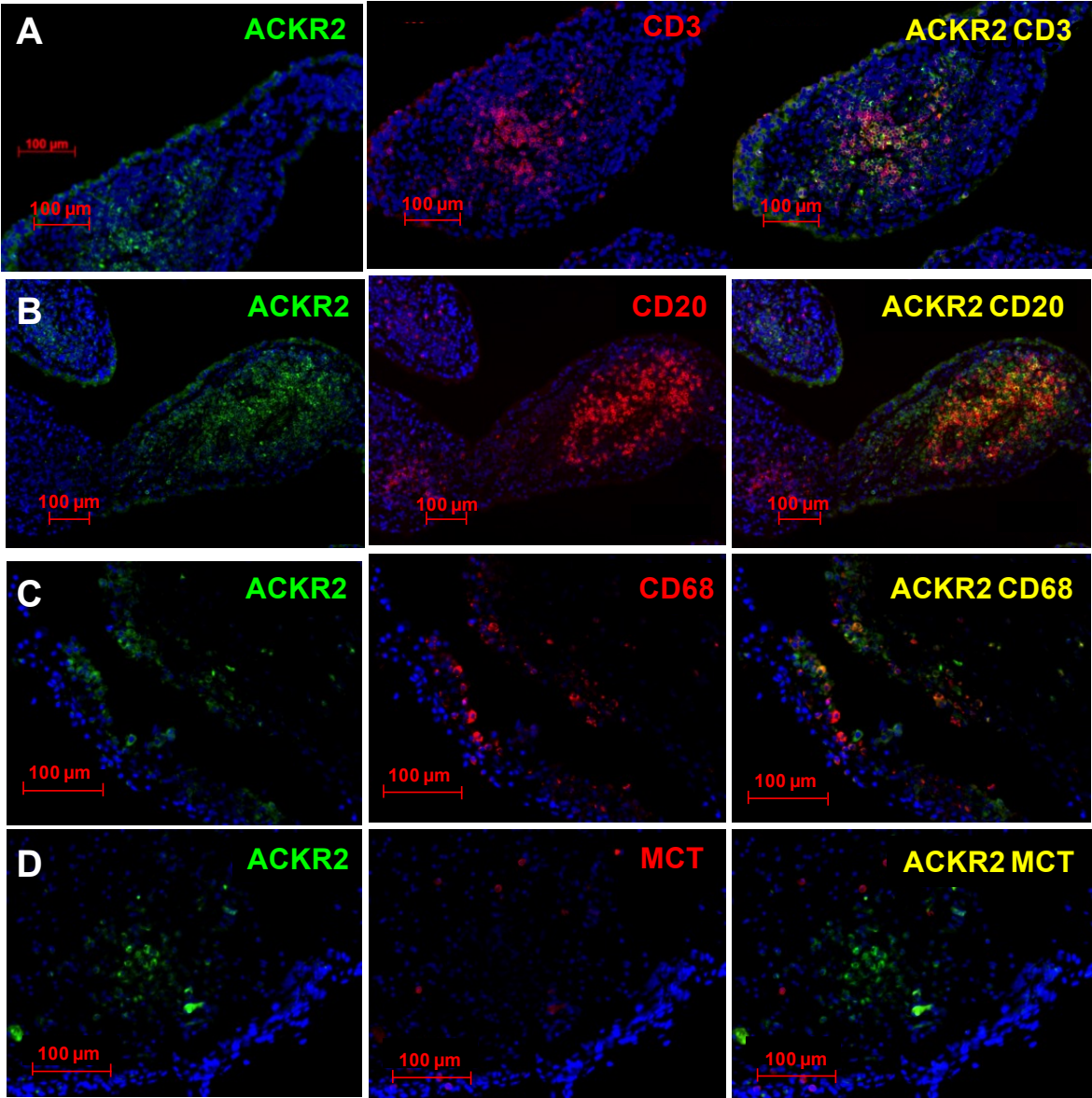


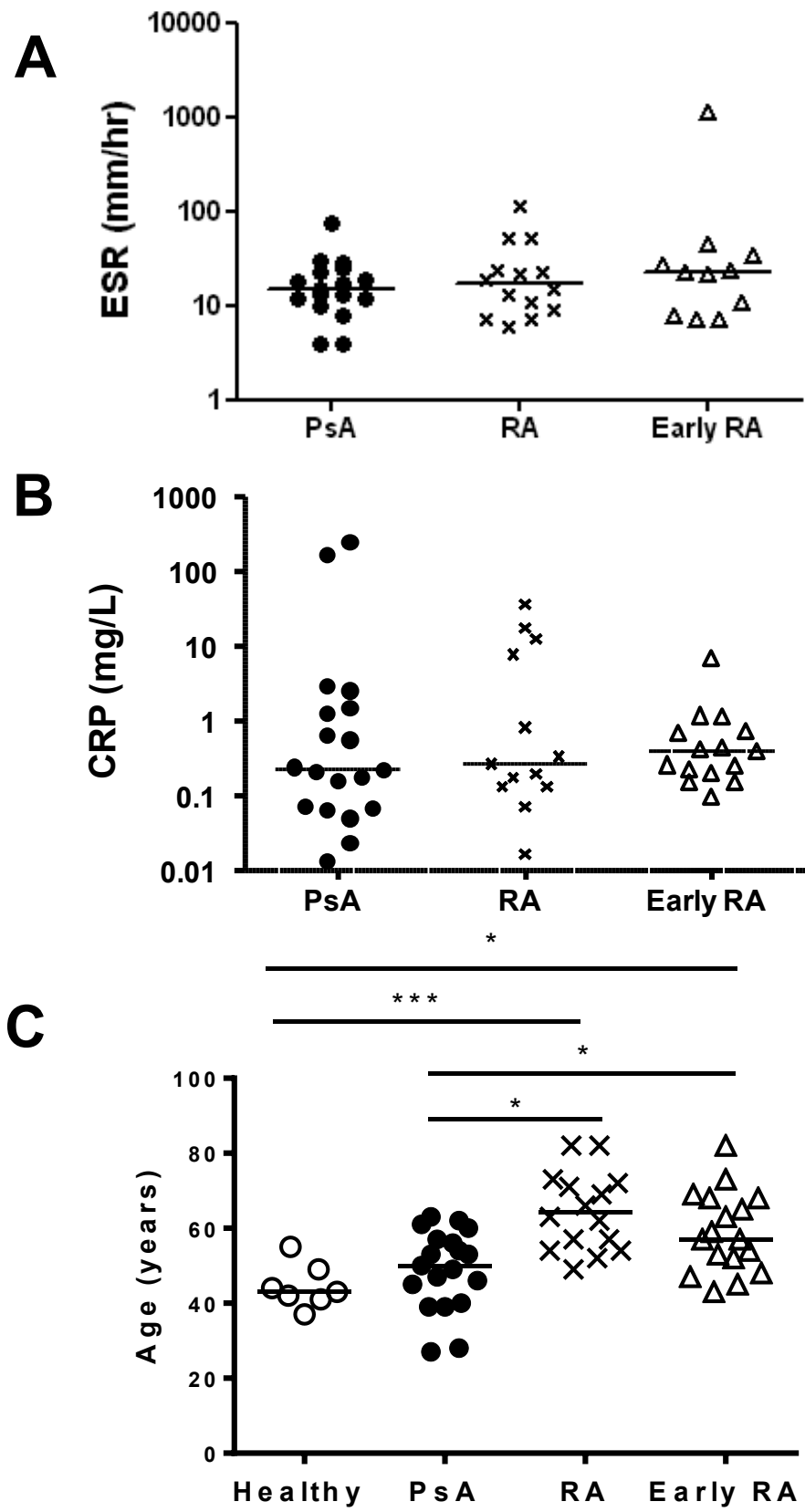
Figure 6



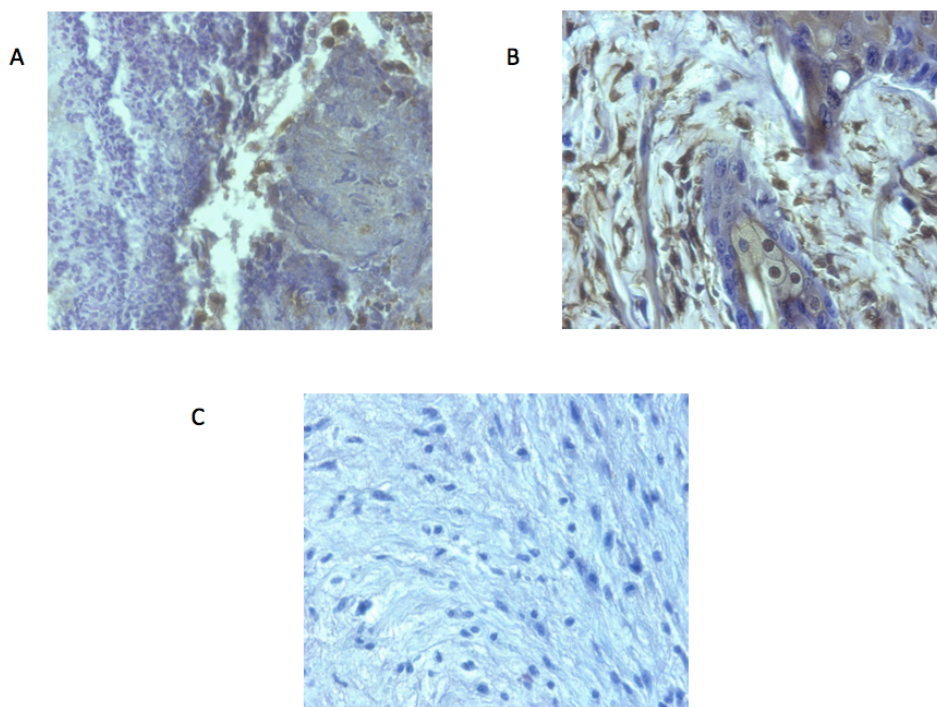
Supplementary Table 1

Parameter	Healthy controls	Psoriatic Arthritis	Rheumatoid Arthritis	Early Inflammatory arthritis
Age (mean±SD)	44.4 ± 5.9	48.9±10.6	60.8±10.5	57.7±10.1
Sex (F:M)	7:4	17:2	10:5	14:1
Disease duration (years±SD)	N/A	11.4±6.5	19.1±6.9	0.7±0.3
Treatment None/ DMARDs/ Etanercept/ Adalimumab/ Rituximab	N/A	6/8/1/3/1	7/5/0/2/1	0/12/0/3/0
ESR	N/A	19.1±16.1	25.5±51.4	19.2±12.8
CRP	N/A	16.6±18.2	26.5±28.8	7±9.1
RhF +/-/unknown	N/A	1/17/1	9/6	4/9/2
ACPA+/-/unknown	N/A	0/18/1	4/11	2/11/2
Total No	11	19	15	15

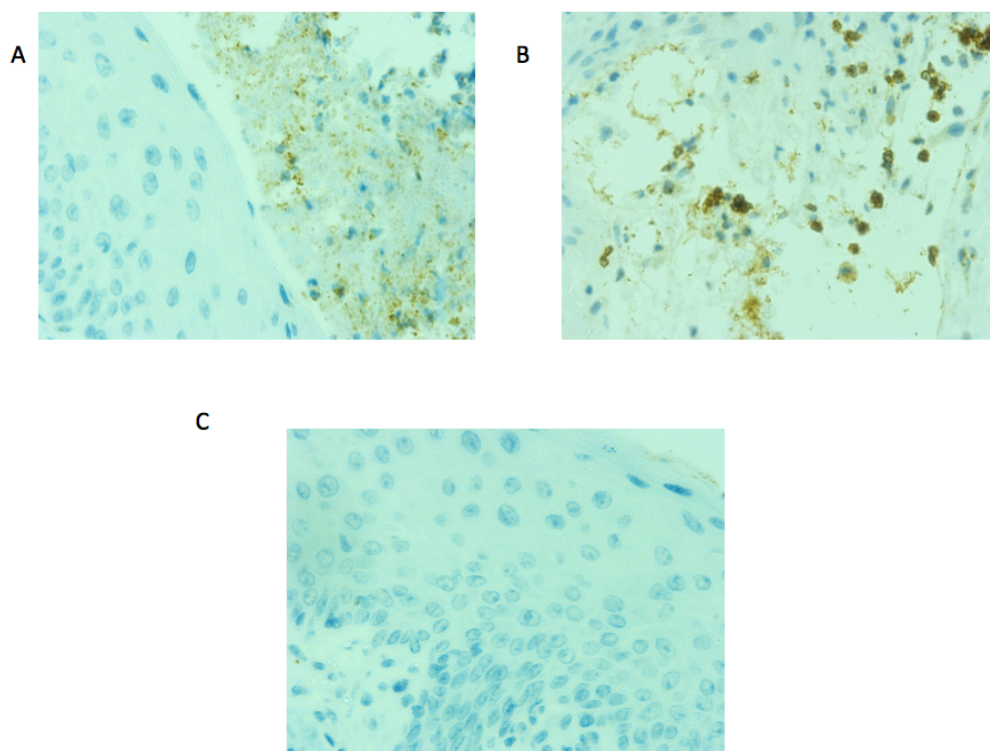
Supplementary Figure 1



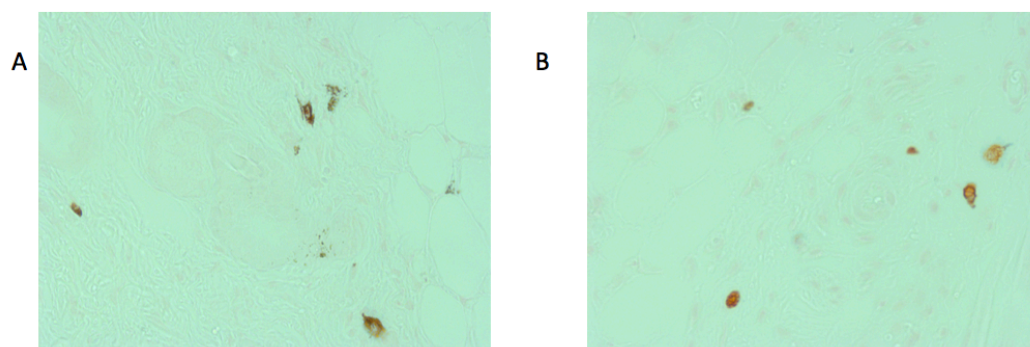
Appendix IV: Immunohistochemistry/Histology Supplemental Figures



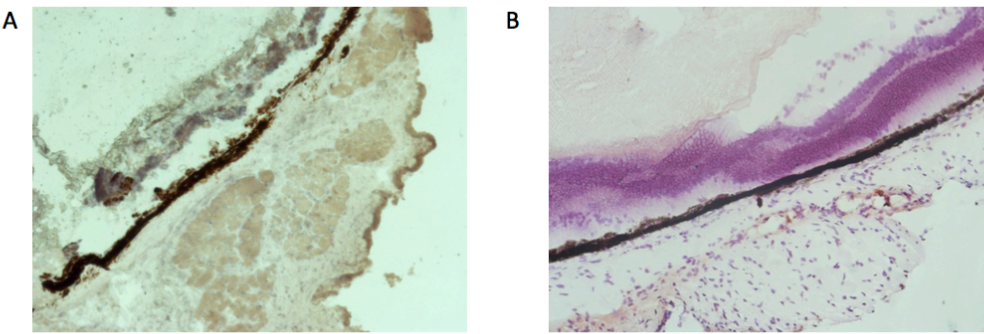
Supplemental Figure 1- The images above show the MAC-2 staining corresponding to figure 3.4B. The images show example viewfields for (A) wild-type mice (B) ACKR2 null mice and (C) is an isotype control.



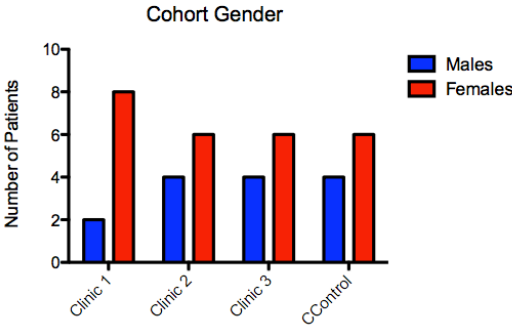
Supplemental Figure 2- The images above represent examples to show the myeloperoxidase (MPO) staining corresponding to figure 3.4A. The images show example viewfields for (A) wild-type mice (B) ACKR2 null mice and (C) is an isotype control.



Supplemental Figure 3- The images above show representative astra blue staining corresponding to figure 3.4C. The images show example viewfields for (A) wild-type mice and (B) ACKR2 null mice.



Supplemental Figure 4- The images above show isotype control samples of immunocytochemistry for the EAU model (section 4.2.1.2). (A) depicts isotype control for the anti-CD45 stain in figure 4.5 and (B) is the isotype control sample corresponding to the anti-MAC-2 staining in figure 4.6.



Supplemental Figure 5- The bar chart above shows the ratio of male vs. female samples that were collected for the human study into rheumatoid arthritis in chapter 5.

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