MODULATION OF CHEMOKINE FUNCTION DURING INFLAMMATION

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Abstract

Oxidative stress is a key feature of inflammatory diseases. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated by many cell types during inflammation. ROS are known to induce chemokines, however it is increasingly apparent that RNS also impact on inflammation. This study was designed to investigate the effects of tissue stress on both chemokine production and function.

How stress alters chemokine production in epithelium was established by qPCR. A distinct tissue, stress and chemokine specific response was elicited; of those studied, CXCL8 showed the greatest induction. The chemokines produced by epithelial cells were functional but post-translational modification occurred and so these chemokines may not have their predicted function. The effects of stress *in vivo* were also assessed. Immunohistochemistry showed association between RNS activity and ischaemic time in a model of kidney ischaemia-reperfusion injury. These observations were extended to human inflammatory liver disease, with increased RNS activity at sites of inflammation, a situation in which chemokines such as CCL2 are also present.

RNS also modulates inflammation by post-translational modification. CCL2 was nitrated by RNS creating a chemokine, nCCL2, with decreased chemotactic activity in a diffusion gradient. Similar results were seen for nCCL5 and nCXCL8. Recruitment of HEK-CCR2b cells was decreased following CCL2 nitration and radio-ligand binding experiments confirmed there was some loss of receptor binding. However, the biological significance of this was uncertain. Glycosaminoglycan interactions were prevented by CCL2 nitration as was proportion of transendothelial migration. The ability of nitration to decrease the chemotactic potential of CCL2 was confirmed by *in vivo* assays.

These data show the complexities of the chemokine system. Increased chemokine production by oxidative stress and concurrent modification of those chemokines by RNS represents a powerful paradigm for the regulation of inflammation.

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LIST OF ABBREVIATIONS

ALD	Alcoholic liver disease
APC	Allophycocyanin
APS	Ammonium persulfate
ATCC	American Type Culture Collection
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CDK	Cyclin dependant kinase
cDNA	Complementary DNA
CGD	Chronic granulomatous disease
DAB	3, 3’ diaminobenzidine tetrahydochloride
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco’s Modified Eagle’s medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-to-meshenchymal transition
ESI-MS	Electrospray ionisation mass spectrometry
FBS	Foetal bovine serum
FFPE	Formalin fixed paraffin embedded
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPCR	G protein coupled receptor
GRK	G protein coupled receptor kinase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HIF-1α	Hypoxia inducible factor-1 alpha
HIV	Human immunodeficiency virus
HPF	High power field
HRP	Horse radish peroxidase

HSC	Hepatic stellate cells
H_2O_2	Hydrogen peroxide
ICAM	Intercellular adhesion molecule
IEX	Ion-exchange chromatography
IFNγ	Interferon gamma
IHC	Immunohistochemistry
IL	Interleukin
IRI	Ischaemia-reperfusion injury
JAM	Junctional adhesion molecule
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MMP	Matrix metalloproteinase
Mono S	Methyl sulfonate
MS	Mass spectrometry
MWCO	Molecular-weight cut off
3-NT	3-nitrotyrosine
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-κB
NOS	Nitric oxide synthase
NPA	No primary antibody control
OPD	*o*-phenylenediamine
p21	$p21^{WAF1/Cip1}$
PAD	Peptidylarginine Deiminase
PBC	Primary biliary cirrhosis
PBMC	Peripheral blood monocytes
PBS	Phosphate buffered saline
PE	Phycoerythrin
PECAM	Platelet-endothelial cell adhesion molecule
pI	Isoelectric point
PI3K	Phosphotidylinositol-3-OH kinase
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate

PPAD	Peptidylarginine deiminase from *Porphyromonas gingivalis*
PSC	Primary sclerosing cholangitis
PSGL	P-selectin glycoprotein ligand
PyBOP	Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
qPCR	Real-time quantitative polymerase chain reaction
RP-HPLC	Reversed phase – high performance liquid chromatography
RPMI	Roswell Park Memorial Institute medium 1640
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RU	Resonance units
SAB	Standard Assay Buffer
SASP	Senescence associated secretory phenotype
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
SPPS	Solid-phase peptide synthesis
SPR	Surface plasmon resonance
*Spy*CEP	*Streptococcus pyogenes* cell envelope protease
SSC	Side scatter
TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine
TGFβ	Transforming growth factor beta
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNFα	Tumour necrosis factor alpha
VCAM	Vascular cell adhesion molecule

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1 General Introduction

1.1 Background to this study

Inflammation, often resulting from tissue stress, is a significant clinical problem. Oxidative stress has a major role in inflammation, especially following organ transplantation, and can also lead to premature tissue ageing or senescence, contributing to loss of organ function. Chemokines are involved in the initiation and maintenance of both these pathologies and so this study looks at the relationship between oxidative stress and chemokine regulation. This is in terms of production of chemokines and their post-translational modifications.

1.2 Tissue Inflammation

Inflammation is a complex response to both infection and tissue injury and is a considerable burden to public health; inflammatory diseases were estimated to cause 44.5% of global mortality in 2008 (World Health Organisation). The cardinal signs of inflammation (rubor, calor, tumor and dolor) were first described in Roman times (Celsus, c. A.D. 25). These symptoms are due to a number of responses aimed to limit infection and damage. Redness and pain are caused by vasodilation increasing blood flow to the site of injury. Heat and further pain are due to the actions of pyrogens causing local tissue destruction and nerve irritation. Swelling results from oedema due to leukocyte infiltration and an increase in extravascular fluid. Later a fifth important consequence of inflammation was described, the loss of function of inflamed areas. This is particularly detrimental if crucial organs become inflamed.

Inflammation is a double edged sword. Its purpose is to limit and eliminate the agent causing inflammation and to remove damaged tissue, however an inappropriate and unregulated immune response can be damaging. Acute inflammation, directed by the innate immune system, is short lived and is generally beneficial in the resolution of damage. It focuses, for example, antimicrobial factors at the site of infection, helping remove the cause of inflammation. Some injury can occur, for instance from oxidative stress, but generally is not as severe as that

caused by prolonged inflammation. This response needs to be actively terminated as failure to resolve results in chronic inflammation.

Uncontrolled chronic inflammation can promote damage by the sustained recruitment of leukocytes, particularly monocytes and lymphocytes, causing persistent inflammation. Oxidative stress, produced by both infiltrating leukocytes and some surrounding cells, as discussed in section 1.4.1, can lead to tissue destruction and the resulting functional impairment. Both apoptotic and necrotic cell death occurs, the latter itself a strong inducer of inflammation by cell content release, and neutrophil recruitment to phagocytose debris. Tissue repair and regeneration following inflammation must also occur and if unsuccessful, fibrous scarring can result. Tight regulation of inflammation is crucial.

Inflammation occurs in a range of diseases, including cancers, rheumatoid arthritis and tuberculosis. Relevant to this study, inflammation is a problem at all stages of organ transplantation. Ischaemia-reperfusion injury (IRI) occurs when the blood supply returns to tissue after a period of ischaemia (Eltzschig and Eckle, 2011). This can occur for example following organ transplantation when organs are surgically isolated from the blood system until reperfusion in the recipient. IRI is also a major cause of organ dysfunction following surgery such as heart bypass (Hammond *et al.*, 2011). Intracellular oxidative stress under these conditions can be generated in the mitochondria and by oxidases in epithelial and endothelial cells. It causes both cellular and molecular damage as will be discussed in section 1.4.1. However the initial hypoxia followed by reperfusion is not the only source of oxidative stress in this situation. Initial stress causes the infiltration of immune cells capable of performing oxidative burst, a secondary source of oxidative stress. A study in hepatocytes demonstrated that this stress induces liver inflammation and may cause apoptosis and necrosis (Bhogal *et al.*, 2010). The Banff system (Demetris *et al.*, 1997) for grading liver allograft rejection highlights the role of such stress and inflammation in acute rejection. The three categories of the rejection activity index are portal inflammation, bile duct inflammation and damage, and venous endothelial inflammation. IRI not only has adverse effects in early transplantation but also has a major impact on chronic rejection (Gueler *et al.*, 2004).

Chemokines are prime drivers of inflammation and cell recruitment and will now be discussed in detail. These, like oxidative stress, can be produced by both the cells of the affected organ, including stressed epithelium, and by infiltrating leukocytes.

1.3 CHEMOKINES

Chemokines, *chemo*attractant cyto*kines*, are small (8-14kDa) proteins secreted by many cell types to direct leukocyte navigation. The chemokine system, present in a primitive form in *Drosophila* (Gerard and Rollins, 2001), evolved with immune cells to mediate their migration. Cells move along a concentration gradient towards high chemokine concentrations; chemotaxis. Gene duplication throughout evolution has created a large family consisting of at least 45 ligands and 20 receptors. Many chemokines interact with multiple receptors, which in turn can interact with more than one chemokine as shown in Table 1, the system is however non-redundant.

Chemokines are classified by both structure and function. Functionally they are classed as inflammatory or homeostatic (Zlotnik and Yoshie, 2000). Homeostatic chemokines have various roles including regulating angiogenesis, directing lymphocytes to the lymph nodes for immune surveillance, and cellular maturation. These chemokines are secreted without any specific stimulus and are generally selective in receptor binding (Rot and von Andrian, 2004). Inflammatory chemokines however are produced and released in response to infection or damage, and function to recruit leukocytes from the blood to the site of injury. These chemokines are more promiscuous, often binding multiple receptors (Rot and von Andrian, 2004). It is the regulation of these chemokines which is crucial for resolving inflammation. Although the categorisation of chemokines in this manner is useful, the distinction between homeostatic and inducible chemokines is increasingly blurred. For example CCL19 and CCL21 are all critical for the development and function of lymphoid tissues but are also induced during inflammation (Marsland *et al.*, 2005).

Structurally, the human chemokine family is divided into four classes (Zlotnik and Yoshie, 2000; 'Chemokine/chemokine receptor nomenclature,' 2002) defined by

the position and spacing of the N-terminal cysteine residues: C, CC, CXC and CX_3C, and receptors are named accordingly (see Figure 1-1). Within the CXC class there is further functional classification based on presence or absence of an ELR motif (glutamic acid-leucine-arginine) immediately adjacent to the CXC motif. ELR positive chemokines bind CXCR1 and/or CXCR2 and are potent angiogenic factors (Strieter *et al.*, 1995), and ELR negative chemokines bind CXCR3 and inhibit angiogenesis. CXCL12 is the exception, it binds CXCR4 and despite lacking the ELR motif can be pro-angiogenic (Verbeke *et al.*, 2012). Genetically the chemokine groups are also distinct (O'Donovan *et al.*, 1999; Mortier *et al.*, 2012). The genes for CXC chemokines are located on chromosome 4, with ELR positive and negative chemokines in separate subclusters. CC chemokines are in are two clusters on chromosome 17.

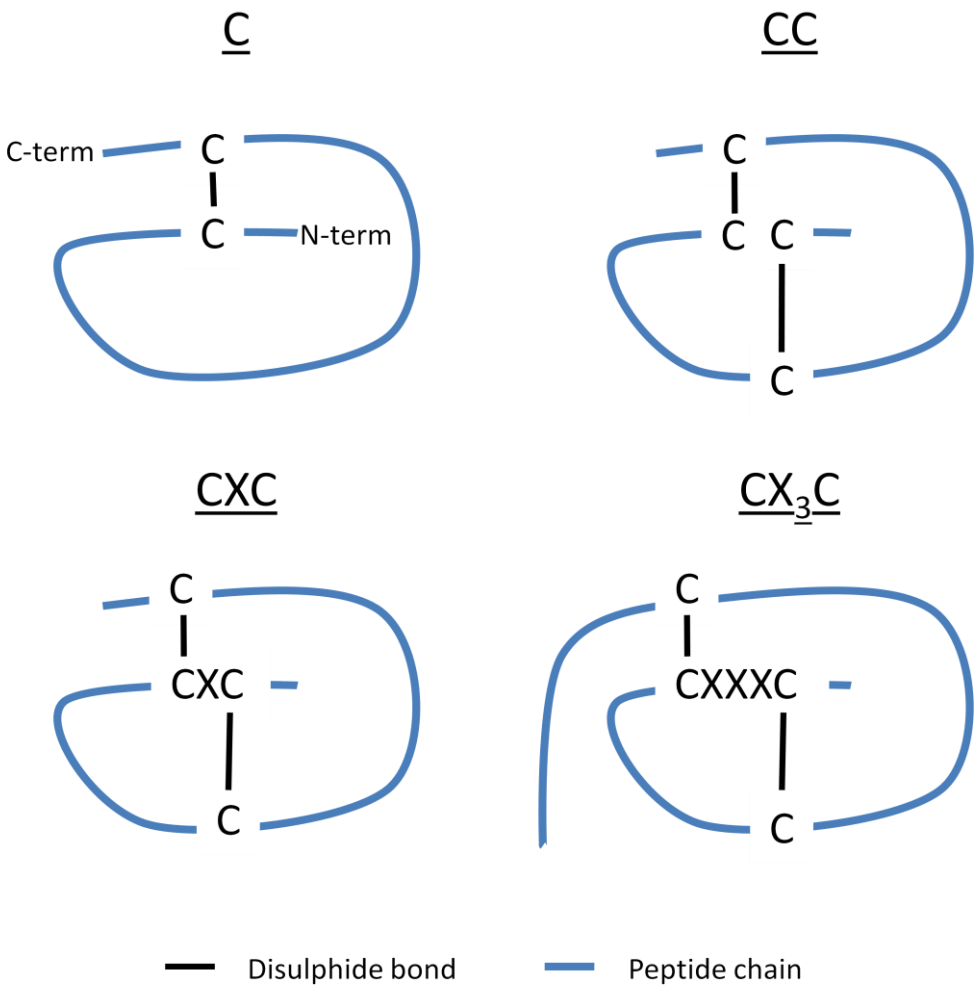


Figure 1-1 The four structural chemokine families
Schematic showing the peptide chains and connecting disulphide bonds in the four chemokine families – C, CC, CXC, CX_3C.

Name	Other names	Typical Receptor	Atypical Receptor
C family			
XCL1	Lymphotactin, SCM-1α	XCR1	
XCL2	SCM-1β	XCR1	
CC family			
CCL1	I-309	CCR8	
CCL2	MCP-1	CCR2	ACKR1, ACKR2
CCL3	MIP-1a	CCR1, CCR5	ACKR2
CCL4	MIP-1β	CCR1, CCR5	ACKR2
CCL5	RANTES	CCR1, CCR3, CCR5	ACKR1, ACKR2
CCL7	MCP-3	CCR1, CCR2, CCR3, CCR5	ACKR1, ACKR2
CCL8	MCP-2	CCR1, CCR2, CCR3, CCR5	ACKR2
CCL11	Eotaxin	CCR3	ACKR1, ACKR2
CCL13	MCP-4	CCR2, CCR3	ACKR1, ACKR2
CCL14	HCC-1	CCR1, CCR5	ACKR1, ACKR2
CCL15	HCC-2	CCR1, CCR3	
CCL16	HCC-4 LEC	CCR1, CCR2, CCR5	
CCL17	TARC	CCR4	ACKR1, ACKR2
CCL18	PARC, DC-CK1		
CCL19	ELC, Exodus-3, MIP-3β	CCR7	ACKR4
CCL20	LARC, Exodus-1, MIP-3α	CCR6	
CCL21	SLC, 6Ckine, exodus-6	CCR7	ACKR4
CCL22	MDC	CCR4	ACKR2
CCL23	MPIF-1, Ckβ8	CCR1	
CCL24	Eotaxin-2, MPIF-2, Ckβ6	CCR3	
CCL25	TECK	CCR9	ACKR4
CCL26	Eotaxin-3, MIP-4α	CCR3	

CCL27	CTACK, ILC	CCR10	
CCL28	MEC	CCR3, CCR10	
CXC family			
CXCL1	Gro-α	CXCR2	ACKR1
CXCL2	Gro-β, MIP-2α	CXCR2	ACKR1
CXCL3	Gro-γ, MIP-2β	CXCR2	ACKR1
CXCL4	PF-4	CXCR3b	
CXCL5	ENA-78	CXCR2	ACKR1
CXCL6	GCP-2	CXCR1, CXCR2	ACKR1
CXCL7	NAP-2	CXCR2	
CXCL8	IL-8, NAP-1	CXCR1, CXCR2	ACKR1
CXCL9	MIG	CXCR3	
CXCL10	IP-10	CXCR3	
CXCL11	I-TAC	CXCR3	ACKR1, ACKR3
CXCL12	SDF-1	CXCR4	ACKR3
CXCL13	BCA-1	CXCR5	
CXCL14	BRAK, bolekine		
CXCL16	SRPSOX	CXCR6	
CXCL17	DMC, VCC-1		
CX_3C family			
CX3CL1	Fractalkine, Neurotactin	CX3CR1	

Table 1 Human chemokines and receptors
The members of the four chemokine families and the receptors they bind. Atypical receptors have recently changed nomenclature, former names are given in brackets - ACKR1 (Duffy antigen/chemokine receptor; DARC), ACKR2 (D6), ACKR3 (CXCR7), ACKR4 (CCRL1, CCR11). This lsit is non-exhaustive and is adapted from the Subcommittee on Chemokine Nomenclature, 2003 and Bachelerie *et al.*, 2014.

CCL2, formerly known as monocyte chemotactic protein-1 (MCP-1), is the main chemokine studied in this thesis and will be used to demonstrate multiple aspects of chemokine biology including structure and function throughout the introduction. It is a widely expressed prototypical inflammatory chemokine produced by multiple cell types and is able to induce migration of many cells, predominantly monocytes.

CCL2 is an 8.7kDa protein encoded by the three exon *CCL2* gene on chromosome 17 (Shyy *et al.*, 1990) and like many chemokines has an N-terminal signal sequence which is cleaved forming the 76 amino acid mature protein (see Figure 1-6), first sequenced in 1988 (Robinson *et al.*, 1989). CCL2 has a discrete receptor system, binding only CCR2, although it can also bind the atypical receptors ACKR1 and ACKR2. Of interest for this study, CCL2 has roles in inflammation and transplantation, discussed in more detail in section 1.3.3. For example it is associated with acute kidney transplant rejection with increasing levels corresponding to increased severity of rejection (Robertson *et al.*, 2000).

CCL2 has multiple roles in immunity. It can influence innate immunity by the recruitment of monocytes, and also adaptive immunity by the control of T helper cell polarisation, skewing responses towards Th2 (Gu *et al.*, 2000). This Th2 polarisation is thought to be exploited by HIV to enhance viral replication by recruiting target cells, increasing disease progression (Ansari *et al.*, 2011). CCL2 is predominantly thought of as a monocyte recruiter, however not all monocytes are $CCR2^{+}$. $CCR2^{+}$ monocytes are classed as the inflammatory subset with $CCR2^{-}$ monocytes found to be recruited to non-inflamed tissues (Geissmann *et al.*, 2003). Expression of the chemokine receptor CX3CR1 is inversely correlated with CCR2 expression in such cells. Another example of the diverse effects of CCL2 is cancer metastasis, for example metastatic breast cancer. Inflammatory monocytes, recruited to the tumour by CCL2, promote the extravasation of tumour cells using monocyte-derived vascular endothelial growth factor (Qian *et al.*, 2011).

Other chemokines examined in this study include CCL5, CXCL8 and CXCL10. CCL5 is a potent chemoattractant for cells including monocytes, T cells and eosinophils and is associated with multiple pathologies including allograft rejection

(Robertson *et al.*, 1998) and also suppresses HIV replication (Coffey *et al.*, 1997). CXCL8 is produced by many cells types including both epithelial and endothelial cells as well as leukocytes. Although predominantly thought of as a neutrophil attractant, CXCL8 can recruit other cells including macrophages, and like many chemokines has a role in cancer (Verbeke *et al.*, 2012). CXCL10 is the final chemokine considered in this study. It is an interferon inducible chemokine (Luster *et al.*, 1985) involved in the recruitment of numerous cell types and the inhibition of angiogenesis (Angiolillo *et al.*, 1995; Dufour *et al.*, 2002), and is a predicator of response to therapy in hepatitis C patients (Falconer *et al.*, 2010).

1.3.1 *REGULATION*

Chemokine regulation is highly complex (Mortier *et al.*, 2012) and, due to their role in leukocyte recruitment, this tight regulation is crucial. This ensures, for example, that inflammation does not become uncontrolled and pathogenic. The regulation is both temporal and spatial and occurs at multiple levels as shown in Figure 1-2 and described in detail in later sections of this introduction.

The production of chemokines is a major level of regulation (section 1.3.2) but once produced, the functions of chemokines can be modified by post-translational modification (section 1.6), the main focus of this study. Many stresses mentioned in sections 1.2 and 1.4 are able to affect this, and other areas of chemokine biology, creating tighter regulation during times of inflammation when chemokine function is crucial.

Signalling is another level of regulation. Chemokines bind and signal through G-protein coupled receptors (see section 1.3.6.2) but atypical receptors (Bachelerie *et al.*, 2014) can also bind chemokines, generally with anti-inflammatory effects (O'Boyle *et al.*, 2011). They are scavenger receptors, down-regulating the bioavailability of chemokines in specific microenvironments. Biased agonism exists within the chemokine system as do synergy and antagonism (Gouwy *et al.*, 2012). The multimerisation of both chemokines and receptors also plays an important role (section 1.3.5.2).

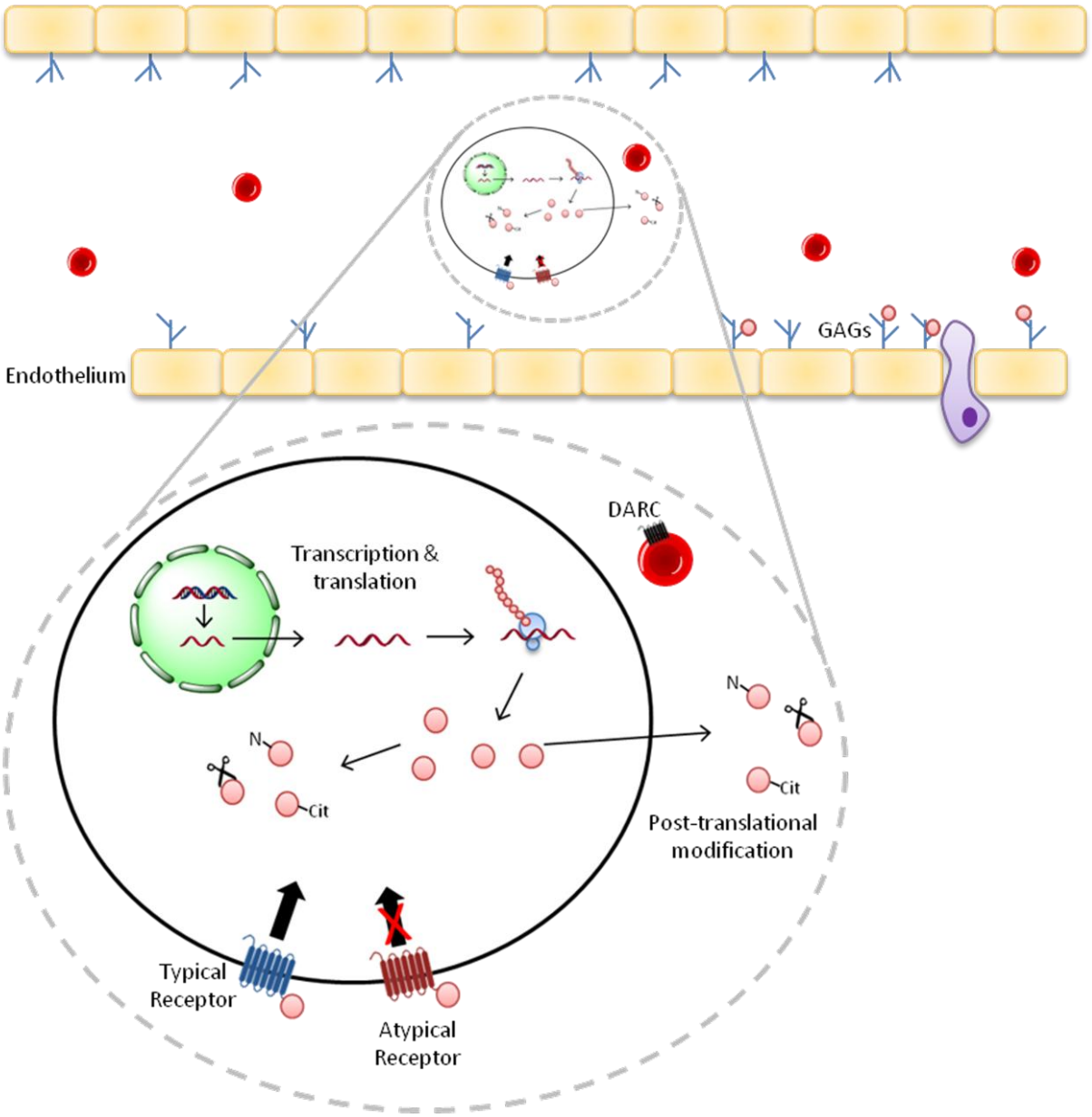


Figure 1-2 Mechanisms of chemokine regulation
Chemokine function is regulated at many levels. Protein production is regulated at both the transcriptional and translational level, with some microRNAs regulating mRNA levels. Chemokine post-translational modification occurs both intra- and extra-cellularly for example nitration, citrullination and protein cleavage, all of which can alter chemokine function. Chemokines bind and signal through 'typical' G-protein coupled receptors, this triggers downstream signalling and causes cell migration. Atypical receptors, including ACKR1 (DARC), however bind chemokine, reducing bioavaliability, but do not signal in the normal manner. Chemokines need to bind glycosaminoglycans (GAGs), for example heparan sulphate, for *in vivo* function. GAGs are presented on the endothelium and bind chemokine, enabling cell migration. N- chemokine nitration, Cit- chemokine citrullination, scissors – enzymatic cleavage. From Barker *et al.*, 2014.

In order to form the chemokine gradients needed for *in vivo* function (Weber *et al.*, 2013), chemokines bind to glycosaminoglycans (GAG) such as heparan sulphate. This chemokine immobilisation increases the concentration at the site of production, thus aiding the recruitment and infiltration of cells *in vivo* (section 1.3.4). GAG expression is increased during inflammation (Ali *et al.*, 2005a), and binding to GAGs affects other levels of regulation, further complicating the process. Chemokine cooperativity is also dependant on GAG binding (Verkaar *et al.*, 2014).

1.3.2 *CHEMOKINE EXPRESSION*

Chemokine function is strictly regulated at many levels, the first being expression. As mentioned, inflammatory chemokines are inducible and are produced in response to infection, tissue injury or stress, with regulation at both the transcriptional and translational level. The availability of chemokines is controlled by both temporal and spatial expression. Some chemokines can be preformed and are stored in endothelial cells in secretory granules including Weibel-Palade bodies, for a rapid response to insult (Øynebråten *et al.*, 2005) whereas in other situations *de novo* synthesis is required.

Many stimuli trigger the production of chemokines. For example through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), which recognise highly conserved pathogen-associated molecular patterns (PAMPs), including lipopolysaccharide (LPS), and danger-associated molecular patterns (DAMPs), including RNA and heat shock proteins as reviewed by Piccinini and Midwood, 2010 and Mortier *et al.*, 2012. PRRs are therefore able to induce chemokine production in response to both infection and sterile inflammation. The activation of these receptors often induces the transcription of chemokines via NFκB pathways, as is described in detail for CCL2 in section 1.3.2.1. Synergy between proinflammatory factors also occurs in the induction of chemokines. For CCL8 production the effects of IL-1β and IFNγ are synergistic but are additive for the production of CCL2 (Struyf *et al.*, 1998b). However the effects of this synergy are not always simple. Exposing fibroblasts and endothelial cells to proinflammatory cytokines and TLR ligands dramatically increases the production of CCL8. These same conditions also increase proteolytic cleavage of the chemokine (a post-translational modification described in detail in section 1.6), creating an antagonist

(Struyf *et al.*, 1998b). One chemokine can also influence the expression of others. A study in CCL2$^{-/-}$ mice showed decreased levels of several other chemokines during acute inflammation. Importantly this finding is not purely due to a failure to recruit inflammatory cells able to produce further chemokines to the site of inflammation, but is also seen in isolated macrophages from knock-out mice (Ferreira *et al.*, 2005).

Post-transcriptional control of chemokines also occurs. Many gene transcripts are innately unstable due to the presence of AU-rich elements (AREs) in their 3′-untranslated regions (3′-UTR) which are targeted for decay by RNA-binding proteins (Hitti and Khabar, 2012). These include chemokines and cytokines which require only transient expression (Bakheet *et al.*, 2006). Both the upregulation and downregulation of chemokines is crucial to preventing excessive chemokine production and hence uncontrolled inflammation. Increased stabilsation of CXCL8 mRNA occurs following IL-1 treatment (Winzen *et al.*, 1999) and in THP-1 cells on differentiation to macrophages (Mahmoud *et al.*, 2014). MicroRNA involvement in chemokine regulation is being increasingly studied (Tano *et al.*, 2011; van Solingen *et al.*, 2011; Kim *et al.*, 2012; Arabanian *et al.*, 2014). Pathogens also exploit microRNA modulation of chemokine production as a mechanism to evade the immune system, for example human cytomegalovirus can increase CCL5 mRNA degradation (Kim *et al.*, 2012).

Alternative splicing of both chemokines and receptors occurs, further adding to the complexity of the system. Six splice variants of CXCL12 have been identified (Yu *et al.*, 2006). CXCL12γ has an extended C-terminus and increased GAG binding affinity (Laguri *et al.*, 2007; Rueda *et al.*, 2008). Maintaining chemokines at the site of production is crucial for function and is achieved by GAG binding as discussed further in section 1.3.4.

1.3.2.1 Induction of CCL2

The induction of CCL2 is complex and not yet fully understood, with most regulation occurring at the level of transcription initiation. Mechanisms of induction are cell type and stimulator specific. Expression is regulated by distal and proximal promoters, separated by 2.2kb DNA; κB and SP-1 sites in these

regions are crucial for activation (Ping *et al.*, 1996; Ping *et al.*, 1999). TPA (PMA) responsive elements (TRE) are also present. Phosphorylation and subsequent ubiquitin-mediated degradation of IκB (Alkalay *et al.*, 1995), allowing NFκB to translocate to the nucleus and bind to the distal region (Boekhoudt *et al.*, 2003) is therefore critical for CCL2 transcription. This can be triggered by protein kinase C (PKC) activation (Ghosh and Baltimore, 1990) or tumour necrosis factor (TNF) induced phosphorylation (Miyamoto *et al.*, 1994), which, like LPS activation, can be PKC independent (Shyy *et al.*, 1993). LPS triggers CCL2 via NFκB (Shyy *et al.*, 1990) and unlike chemokines including CXCL10, CCL2 induction in response to LPS is mediated in a MyD88-dependant manner (Bandow *et al.*, 2012) in macrophages. NFκB binding leads to recruitment of CREB-binding protein and p300, leading to histone acetylation and chromatin remodelling, opening the structure allowing recruitment of transcription machinery and eventual gene expression. As with several TNF induced genes the arginine methyltransferase CARM1 is needed although its exact role is not understood (Teferedegne *et al.*, 2006). A p53 binding site has been identified and functional p53 is required for CCL2 expression (Hacke *et al.*, 2010), this may be connected to findings that TNF can activate p53 via NFκB and provides another possible link between oxidative stress, senescence and CCL2 production. Highlighting the need for tight regulation of this protein is the finding that a SNP in the distal promoter is associated with increased CCL2 production and inflammation. This A/G polymorphism creates an extra binding site for the TALE family of transcription factors (Page *et al.*, 2011).

1.3.3 *CHEMOKINES IN TRANSPLANTATION*

Problems at all stages of transplantation involve the chemokine system. As liver and kidney epithelial cells have chemokine-producing ability, these sites are particularly susceptible to increased lymphocyte recruitment, causing the bile-duct and peritubular inflammation seen in rejection (Hancock *et al.*, 2000). Tissue stress as early as brain death of the donor can lead to endothelial dysfunction and chemokine release, and IRI also causes the production of proinflammatory factors. Upon reperfusion, proinflammatory cytokines are produced, such as TNFα and IL-1β. These stimulate the release of preformed chemokines from vascular endothelium, including CCL2 and CXCL8, recruiting macrophages and neutrophils

respectively to the graft (El-Sawy *et al.*, 2002); increased CXCL8 expression in kidney correlates with ischaemia time (Lo *et al.*, 2011). CCR1 knockout mice have decreased recruitment of both neutrophils and macrophages in a model of kidney IRI. The temporal pattern of recruitment however is unaltered with rapid neutrophil recruitment peaking at 24hr, and more gradual monocyte recruitment throughout the seven days of the model used in this study (Furuichi *et al.*, 2008). The IFNγ-inducible chemokines CXCL9, CXCL10 and CXCL11 are rapidly increased following reperfusion (Lo *et al.*, 2011), and are abundant in rejecting allografts. Controversy however surrounds the role of their receptor, CXCR3, in transplantation (Halloran and Fairchild, 2008).

As well as ligands, chemokine receptors are also increased. CXCR1 and CXCR2 expression on hepatocytes increases in hepatic IRI but a murine study shows that their effects differ. CXCR2 is associated with delayed recovery, whereas CXCR1 seems to aid repair (Clarke *et al.*, 2011). Later stages of transplant rejection including arteriosclerosis (Gerard and Rollins, 2001) and fibrosis are increased by chemokines and growing research suggests that chemokine levels could be used as biomarkers, such as CXCL10 for monitoring graft inflammation and predicting fibrosis (Berres *et al.*, 2011; Romagnani and Crescioli, 2012).

CCL2 production is upregulated by epithelial cells in liver fibrosis (Kruglov *et al.*, 2006), this CCL2 may then 'activate' portal fibroblasts; an example of paracrine signalling. Studies report that blocking CCL2 decreases hepatic macrophage and monocyte recruitment and ameliorates steatosis (Baeck *et al.*, 2012). Chemokines are also involved in senescence (discussed further in section 1.5), with particular focus placed on the CXCR2 ligands CXCL1 and CXCL8 (Acosta *et al.*, 2008b), further increasing inflammation, senescence, and in the case of transplantation, organ loss.

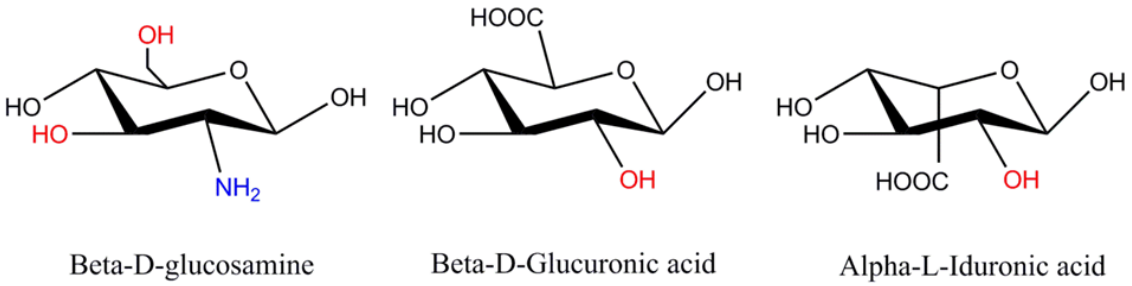
As with all transplant immunology, the picture is further complicated as chemokines can be donor or host derived, and determining which has most significant effects is important both clinically and in understanding the system. A study by Melzi *et al.* suggests that for islet transplantation a strategy to selectively decrease recipient, but not donor CCL2 will have the most benefit (Melzi *et al.*, 2010).

1.3.4 *GLYCOSAMINOGLYCAN BINDING*

For migration *in vivo* cells need vectoral cues. These come in the form of chemokine gradients, immobilised by binding to GAGs. GAGs, including heparin and heparan sulphate, are complex acidic polysaccharides both within the extracellular matrix and at the cell surface conjugated to proteins (proteoglycans). The main families of proteoglycans are syndecans, glypicans and perlecans. Syndecans are transmembrane molecules and there are four members of the mammalian family. Glypicans however are attached to the cell surface by a glycosylphosphatidylinositol anchor. The heparan sulphate chains attached to glypicans are closer to the cell surface than those attached to syndecans. The attachment of perlecans is different again. Cell adherence is by binding to integrins, not cells directly (Celie *et al.*, 2009).

These surface GAGs are important biological mediators for multiple fundamental cell processes including proliferation, cell death, haemostasis, adhesion and migration (Simon Davis and Parish, 2013). Chemokines bind GAGs by electrostatic interaction (Proudfoot *et al.*, 2003) and binding adds both another level of specificity to interactions and also immobilises chemokines, increasing concentration at the site of production (Whitelock and Iozzo, 2005). This forms a haptotactic chemokine gradient needed for *in vivo* chemokine function. Chemokine gradients have been visualised *in vivo* in numerous papers by the Sixt lab (Schumann *et al.*, 2010; Weber *et al.*, 2013; Stoler-Barak *et al.*, 2014).

GAG structures are complex but all contain disaccharide repeat units. The disaccharides for heparin and heparan sulphate (collectively HSGAGs) are shown in Figure 1-3. The different combinations of these units, combined with a number of modifications such as sulphation and acetylation, creates remarkable structural heterogeneity (Simon Davis and Parish, 2013). Variations are found in other GAGs for example chondroitin sulphate and dermatan sulphate. The strongly polyanionic nature of GAGs allows interaction with the basic amino acids found in the GAG binding sites of chemokines. These are not just non-specific electrostatic interactions however, structure and oligomerisation state alters the ability of GAGs to interact with proteins (section 1.3.5.2).



Beta-D-glucosamine Beta-D-Glucuronic acid Alpha-L-Iduronic acid

Figure 1-3 Glycosaminoglycan subunits for heparin and heparan sulphate
Each disaccharide repeating subunit contains a uronic acid (β-D-glucuronic acid or α-L-iduronic acid), with 2-O possible sulphation sites, and a β-D-glucosamine, with possible sulphation sites at the 3-O and 6-O positions, with additional sulphation or acetylation possible at the N position. All of these combinations result in 48 disaccharide building blocks for HSGAGs. The most common subunits in heparan sulphate are glucuronic acid and *N*-acetylglucosamine, however heparin has a higher level of sulphation with the predominant unit being iduronic acid(2*S*) and *N*-acetylglucosamine(6*S*). Red – possible sulphation sites, Blue – possible sulphation or acetylation site.

GAG distribution is relatively even across the surface of endothelium as shown in Figure 1-4 but chemokines bind to discrete regions due to multimerisation. Both heparin and heparan sulphate have been used in assays in this study to model chemokine-GAG interactions. Both GAGs are present *in vivo*; heparin is produced by mast cells and predominantly functions as an anti-coagulant, where as heparan sulphate can be produced by numerous cell types and is involved in chemokine immobilisation. Heparan sulphate is therefore the more physiologically relevant of the two for this work.

The importance of GAG binding is highlighted by studies showing that increasing the GAG binding ability of chemokines which lack signalling capabilities is anti-inflammatory (Piccinini *et al.*, 2010). Anti-inflammatory properties are also seen in the reverse mutation chemokines that signal normally but cannot bind GAGs (Ali *et al.*, 2005b; O'Boyle *et al.*, 2009). As well as forming *in vivo* chemokine gradients GAGs have other roles in chemokine biology. GAG binding can protect chemokines from proteolysis (Sadir *et al.*, 2004) (seezz section 1.6.1) and encourage chemokine oligomerisation, another fundamental part of chemokine biology, as discussed further in section 1.3.5.2. Endothelial expression of these GAGs increases during the stresses produced by transplantation, altering the potential to bind chemokines and so altering chemokine function (Ali *et al.*, 2005a). This is yet another level of chemokine regulation during stress.

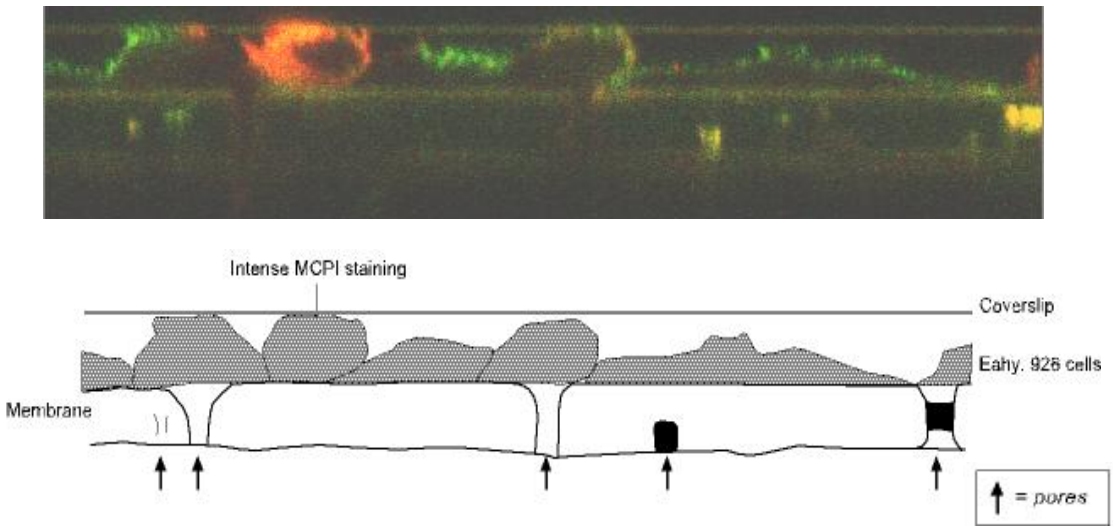


Figure 1-4 Chemokine-GAG interaction
Confocal microscopy image from Hardy *et al.*, 2004 showing immunofluorescence detection of CCL2 (red) binding to heparan sulphate (green) on EA.hy926 cells following incubation of the endothelium with 12.5nM CCL2 for 1hr at 37°C. X-Z sectional image shows characteristic distribution of CCL2 associated with single cells and concentrated to discrete regions of the apical cell surface.

1.3.5 *STRUCTURE*

Structurally the human chemokine family is divided into four classes, listed in Table 1, and defined by the position and spacing of the N-terminal cysteine residues. Despite relatively low sequence homology, chemokines have several highly conserved structural regions and all have basic domains for GAG binding. Chemokines contain the common tertiary fold, a Greek key motif (Richardson, 1977). In chemokines this contains three antiparallel β-sheets stabilised by multiple hydrogen bonds and hydrophobic interactions (Handel and Domaille, 1996). There is also a C-terminal α-helix, and the whole structure is stabilised by disulphide bonds between the conserved cysteine residues (Schwiebert, 2005) (see Figure 1-5). The N-terminus plays a crucial role in receptor binding and activation. It is generally disordered and the well ordered regions, although not being directly involved in binding, create a scaffold to ensure protein correct conformation (Clark-Lewis *et al.*, 1995).

1.3.5.1 Structure of CCL2

The structure of CCL2 has been determined (Handel and Domaille, 1996; Lubkowski *et al.*, 1997), greatly helping identification of the functional role of specific residues. What is known about these residues is shown in Figure 1-6 and

will now be discussed. Due to differences between techniques, the exact importance of each residue in a given aspect of biology, both *in vitro* and *in vivo*, is not definitive.

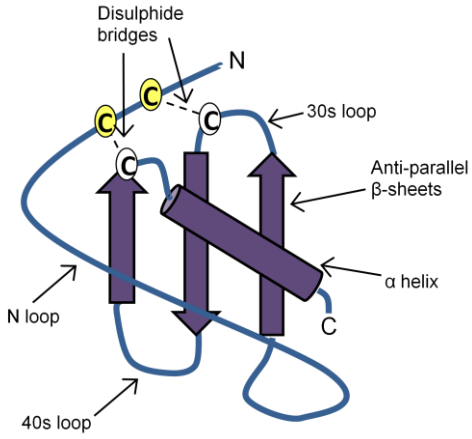


Figure 1-5 Chemokine structure
Cartoon of general chemokine structure. The spacing between the cysteine residues highlighted in yellow determines the chemokine family. The Greek key like motif containing three antiparallel β-sheets and an α-helix is shown.

In keeping with the two site model for receptor activation described in section 1.3.6.2, the N-terminus is crucial for signalling but contributes little to the binding affinity (Chakravarty *et al.*, 1998; Hemmerich *et al.*, 1999), deletion of residues 2-8 creates a potent antagonist (Jarnagin *et al.*, 1999). The exact N-terminal residues are not important suggesting receptor interaction may be with backbone atoms. Iso5Pro, a mutation predicted to disrupt helix formation also has little effect on binding, indicating that the secondary helical structure is also not crucial (Jarnagin *et al.*, 1999). The multiconformation hypothesis was suggested: the core domain of CCL2 is needed for G protein activation but the N-terminal is needed to create a conformation able to trigger calcium influx (Jarnagin *et al.*, 1999).

Most mutants alter CCL2 signalling to an extent which correlates with alteration in receptor binding but some, known as active triggering residues, have decreased signalling to a much greater extent than the decrease in affinity. Mutations including deletion of residues 2-8 and mutation of Tyr13 (Jarnagin *et al.*, 1999) demonstrate these two regions are crucial for signalling.

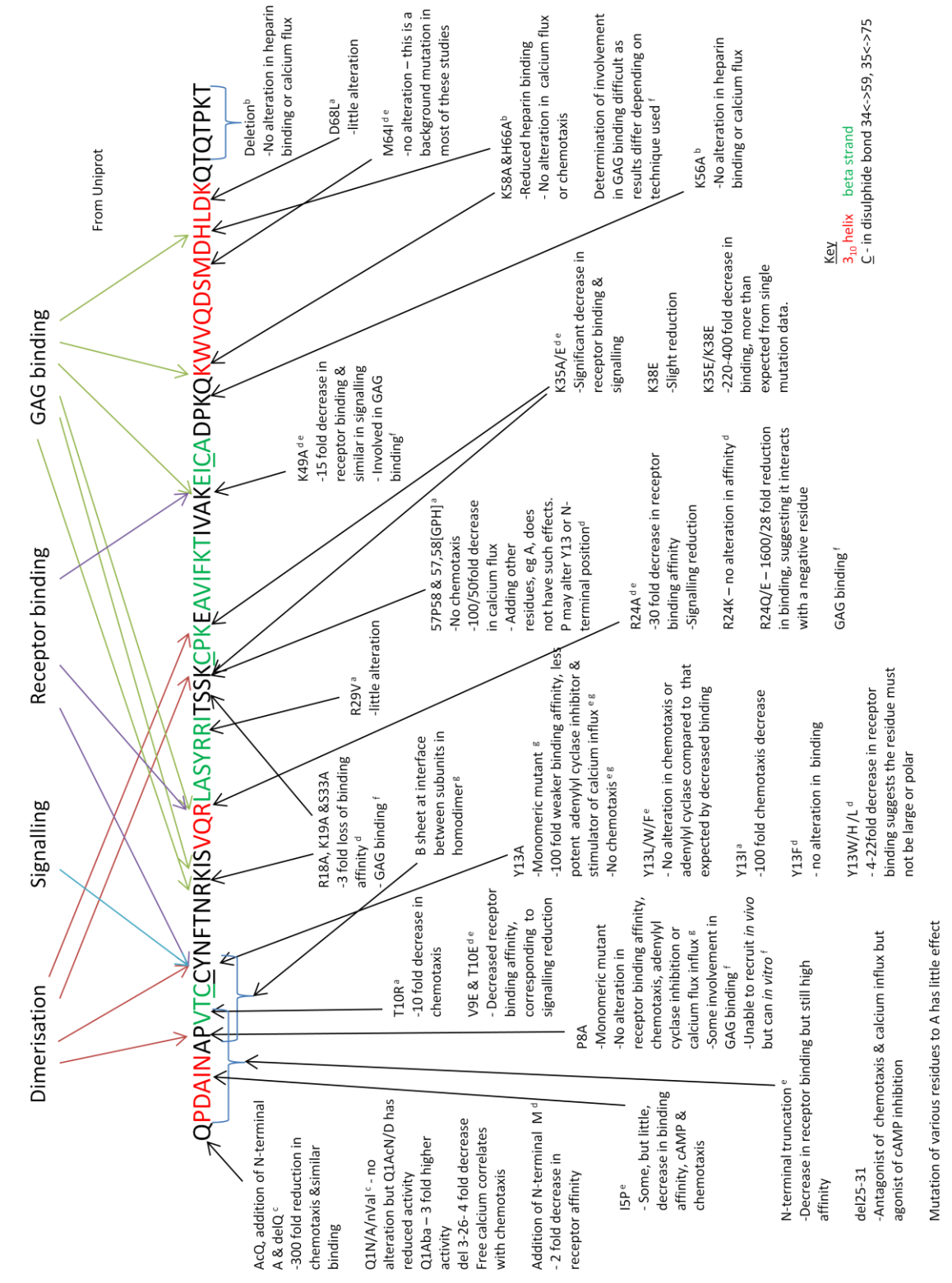


Figure 1-6 CCL2 amino acid sequence and function of specific residues
Shown is the amino acid sequence of mature human CCL2. References: [a]Beall *et al.*, 1996, [b]Chakravarty *et al.*, 1998, [c]Gong and Clark-Lewis, 1995, [d]Hemmerich *et al.*, 1999, [e] Jarnagin *et al.*, 1999, [f]Lau *et al.*, 2004, [g]Paavola *et al.*, 1998 and Uniprot.

The C-terminal region contains the positive residues Lys58 and His66, likely sites for binding of negative GAGs (Chakravarty *et al.*, 1998). Arginine and lysine are the residues normally associated with GAG binding but the participation of histidine is in agreement with the pH dependence of CCL2 GAG binding. A study by Lau *et al.*, 2004, shows some involvement of these residues depending on the technique used but finds that Arg18>Arg24>Lys19>Lys49 are the key residues in GAG binding, Arg18Ala/Lys19Ala mutants cannot recruit cells *in vivo* but are functional *in vitro* fitting with data previously discussed in section 1.3.4 regarding the importance of GAG binding *in vivo*.

Quaternary structure formation is important for function. The two monomers of the CCL2 homodimer are held together by hydrogen bonds which form between both Tyr13 residues via a water molecule. Val9 and Thr10 also interact via their hydroxyl groups (Paavola *et al.*, 1998). The mutation Pro8Ala creates an obligate CCL2 monomer, receptor binding is unaltered but it cannot recruit leukocytes *in vivo* (Paavola *et al.*, 1998; Lau *et al.*, 2004). Analysis of CCL2 structure shows that receptor and GAG binding cannot occur concurrently due to the proximity of the binding sites if present as a monomer (Lau *et al.*, 2004) and is confirmed by a recent study which created an obligate dimer (Tan *et al.*, 2012). Upon GAG binding, CCL2 forms a tetramer, allowing both GAG and receptor binding to occur. As a tetramer the residues important in GAG binding form a ring creating a positive surface for GAG binding (Lau *et al.*, 2004).

1.3.5.2 Multimerisation

Many chemokines can form oligomers, especially at the high chemokine concentrations reached at inflammatory sites on interaction with GAGs. Despite the similarity in tertiary structure there is great diversity in the quaternary interactions formed. For some chemokines, including CCL2, formation of higher order structures is necessary for function *in vivo* (Paavola *et al.*, 1998; Lau *et al.*, 2004). Not all chemokines however can form dimers. CCL7 for example lacks the conserved proline residue involved in CC dimerisation, Pro8 in CCL2 (Key-Sun *et al.*, 1996). *In vitro* chemically obligate monomers can still induce chemotaxis, *in vivo* but oligomerisation is needed (Proudfoot *et al.*, 2003; Campanella *et al.*, 2006). This requirement for oligomerisation is not fully understood but GAG binding is

involved. Wild type, not monomeric, CXCL10 is retained on endothelial surfaces, inducing transendothelial migration (Campanella *et al.*, 2006), presumably due to a difference in GAG binding. The natural obligate monomer CCL7 has multiple GAG binding sites so can function *in vivo* despite lack of dimerisation ability (Salanga *et al.*, 2014).

Crucial differences are seen between CC and CXC chemokines in their dimerisation biology. CC chemokine dimers are sterically blocked from binding their receptors due to important binding residues being situated on the dimer interface (Jin *et al.*, 2007; Tan *et al.*, 2012). CXC chemokines however are able to bind chemokine receptors while dimerised (Veldkamp *et al.*, 2008; Gangavarapu *et al.*, 2012).

As well as homodimers chemokines can form heterodimers, the CCL2-CCL8 heterodimer forms in preference in homodimers, and like homodimers can be influenced by GAGs (Crown *et al.*, 2006). CXCL4 and CXCL8 interact to form heterodimers, increasing the anti-proliferative effects, and ability to cause cell migration via CXCR2 (Nesmelova *et al.*, 2005).

1.3.6 *SIGNALLING*

For leukocyte recruitment during inflammation to occur the target cells need to come in contact with GAG bound chemokines. For this to occur cells must first adhere to the endothelium; this process is initially mediated by selectins.

1.3.6.1 Selectins

Shear forces in blood flow force cells to roll along the surface of blood vessels, allowing leukocytes to make contact with the endothelium. Weak associations are formed due to interaction with selectins (calcium dependent lectins), tethering lymphocytes to the inflamed endothelium, which in shear vascular flow results in leukocyte rolling. The three types of selectin are lymphocyte (L-selectin), constitutively expressed on leukocytes, platelet (P-selectin) and endothelial cell (E-selectin), the latter two being expressed on cytokine activated endothelial cells. P-selectin is pre-formed and is released from Weibel-Palade bodies in response to cytokines including IL-1β and TNFα; E-selectin is regulated by these at the level of transcription (Lowe, 2003). Initial adhesion is through P-selectin then L-selectin

reduces the leukocyte rolling allowing signalling through chemokine receptors (Parish, 2006). This then causes integrin activation and subsequent leukocyte migration as discussed in section 1.3.7.

Selectins are heavily glycosylated cell surface proteins which bind other cell-surface glycoproteins or glycolipids. For example the P-selectin counter receptor, P-selectin glycoprotein ligand-1 (PSGL-1) is expressed on neutrophils and other leukocytes and is characterised by abundance of a specific *O*-glycan. This is opposed to the broader range of polypeptides and glycolipids which E-selectin interacts with (Lowe, 2003). As well as aiding cell migration by immobilising chemokines, as discussed in section 1.3.4, heparan sulphate is a major L-selectin ligand especially during acute inflammation (Giuffre *et al.*, 1997; Wang *et al.*, 2005). During chronic inflammation however, the mucin 6-sulpho sialyl Lewis X is thought to play a more important role (Parish, 2006). The importance of selectins in the complex regulation of leukocyte rolling and potential subsequent inflammation is demonstrated by Ali *et al.*, 2008. α-L-Fucosidase is an enzyme able to cleave fucosylated residues from, for example, P-selectin ligands, and can therefore decrease cell rolling and subsequent cell migration. This enzyme can be upregulated by chemokines, a regulatory cycle.

Selectin – ligand interaction is biphasic. Increasing tensile force initially prolongs interactions but when the force becomes too great the interactions decrease again. This is a mechanism to regulate cell adhesion under varying levels of mechanical stress and is due to the nature of the interaction being both 'catch' and 'slip' bond like (Konstantopoulos *et al.*, 2003; Marshall *et al.*, 2003).

1.3.6.2 Chemokine Receptors

Chemokines signal by binding class A, rhodopsin-like, seven transmembrane domain G protein coupled receptors (GPCRs) (Gether, 2000). Upon chemokine binding there is coupling between the receptor and heterotrimeric G proteins. Receptor activation induces conformational changes and increased affinity of leukocyte integrins for their adhesion molecules, eliciting adhesion to the endothelium (Ward and Marelli-Berg, 2009).

Chemokine receptors couple via a $G\alpha_i$ subunit, inhibiting adenylyl cyclase and decreasing intracellular cAMP levels, they are pertussis toxin sensitive. $G\alpha_i$ is necessary but not sufficient for chemotaxis (Neptune and Bourne, 1997). The release of the Gβγ subunit alone can trigger chemotaxis (Neptune *et al.*, 1999) but it has to be from $G\alpha_i$, release of Gβγ from $G\alpha_s$ or $G\alpha_q$ trimers is not functional (Neptune and Bourne, 1997).

The two-site model is the current model for receptor activation (Monteclaro and Charo, 1996; Wells *et al.*, 1996; Crump *et al.*, 1997). A high affinity and specificity interaction between the chemokine and receptor, normally the N-terminal, allows chemokine docking and determines ligand specificity. The N-terminal of the chemokine then flips into the receptor binding groove, inducing a conformational change and allowing G protein binding. Whether the receptor needs to be present as a monomer or dimer for activation is yet to be fully determined (Thelen and Stein, 2008).

Following receptor activation, Gβγ (Thelen, 2001; Thelen and Stein, 2008) activates numerous downstream effectors including phospholipase C (PLC) and phosphotidylinositol-3-OH kinase (PI3K), leading to the activation of PKC and recruitment of signal transducers including protein kinase B (PKB) and PTEN. The later can also activate the mitogen activated protein kinase (MAPK) pathway via Shc and Ras. Most chemokines can interact with multiple receptors, which in turn can interact with more than one chemokine, often causing ligand specific effects. CCR2 for example has multiple ligands. CCL2, but not CCL8, induces ERK1 nuclear translocation and c-Myc expression (O'Boyle *et al.*, 2007) and CCL11, a CCR2 antagonist, also produces its effects via ERK but has different downstream pathways to the agonist CCL2 (Ogilvie *et al.*, 2004).

This downstream signalling results in an increase in cell movement and directionality but this is not due to receptor accumulation at the leading edge although there is an accumulation of membrane (Xiao *et al.*, 1997; Servant *et al.*, 1999). There is however an asymmetric distribution of intracellular proteins including PKB which are needed for signal transduction (Parent *et al.*, 1998; Meili *et al.*, 1999). Cholesterol is required for polarised redistribution of factors

including PI3K but is not needed for early events such as calcium flux (Rose *et al.*, 2008).

Chemokine receptors, like many other GPCRs can form homo and heterodimers and can also interact with, for example, opioid receptors (Suzuki *et al.*, 2002). These interactions have functional consequences. Receptor oligomerisation can in some pairs cause negative cooperativity but in others enhance signalling or activate new, specific signalling pathways (Stephens and Handel, 2013). The receptors CCR5 and CCR2 can form heterodimers but the exact functional consequence is unclear. One study suggests that the dimer can only bind a single chemokine, creating competition between ligands (El-Asmar *et al.*, 2005). A second study shows signalling through the dimer is pertussis toxin insensitive and triggers cell adhesion instead of chemotaxis (Mellado *et al.*, 2001).

Further complexity is added by the level of receptor expression. LPS signals via TLR4 to augment, but not directly induce, monocyte migration in response to CCL2 by modulating CCR2 surface expression in a G protein-coupled receptor 2 (GRK2) dependent manner (Liu *et al.*, 2013).

Chemokine receptors are internalised, and subsequently recycled or degraded. This mostly occurs following chemokine stimulation but is also constitutive. Receptor internalisation is predominantly β-arrestin-mediated following receptor phosphorylation and is reviewed by Borroni *et al.*, 2010. Many receptors reach the endosomal compartment by clathrin-coated pits and are then recycled to the surface but the rate is receptor dependant. CXCR3 is degraded and so for new surface CXCR3, *de novo* synthesis is required (Meiser *et al.*, 2008).

1.3.6.3 CCL2 Receptor

CCR2 is the only known receptor for CCL2. A study suggested CCR4 may be a receptor (Power *et al.*, 1995) but problems with the specificity of the transfectants used have since been established and it has been shown that CCL2 does not bind CCR4 (Imai *et al.*, 1998). Numerous, more recent studies also propose other receptors but none have been confirmed. Portal fibroblasts were reported to not express CCR2 and therefore effects on these cells must be via another receptor

(Kruglov *et al.*, 2006). Later studies however show that CCR2 expression on liver cells promotes fibrosis in mice and that $CCR2^{-/-}$ HSC are unresponsive to CCL2 *in vitro* (Seki *et al.*, 2009), suggesting that the findings of the earlier study are due to a lack of CCR2 detection rather signalling via another means. CCL2 heterodimerisation with other chemokines could explain the apparent existence of a second receptor. CCR2 itself can dimerise with CCR5 (Mellado *et al.*, 2001; El-Asmar *et al.*, 2005).

CCR2 has two isoforms, CCR2a and CCR2b, with alternatively spliced carboxyl tails (Charo *et al.*, 1994). The 47 amino acid carboxyl tail of CCR2b is in the same exon as the transmembrane domains, whereas the 61 amino acid tail of CCR2a is in a downstream exon. It is thought that the C-terminal is important in receptor trafficking as in monocytes CCR2a is found in low levels on the cell surface but is present in the cytoplasm (Wong *et al.*, 1997). The difference in C-terminal leads to a difference in G-protein coupling between the isoforms. CCR2a can bind $G\alpha_{14}$ and $G\alpha_{16}$ but CCR2b can bind neither (Kuang *et al.*, 1996), both receptors can couple to $G\alpha_i$ and so are pertussis toxin sensitive (Kuang *et al.*, 1996). Another functional difference between the two isoforms is the finding that Jurkat cells expressing CCR2a are chemotactic in response to CCL2 without calcium flux whereas CCR2b causes flux (Sanders *et al.*, 2000).

1.3.7 *CHEMOKINE FUNCTION*

Having discussed the regulation of chemokine production, their ability to bind GAGs and form gradients, and signalling through GPCRs, how do these proteins perform their main function: directing leukocyte migration into extravascular tissues? The key steps in this process are tethering, rolling, adhesion and diapedesis, each step being a prerequisite for the next (Schenkel *et al.*, 2004) and can be seen in Figure 1-7.

1.3.7.1 Polarisation

Polarisation occurs when leukocytes are stimulated, for example by chemokines. Two regions are created, the leading edge and the uropod, by cytoskeletal rearrangement (del Pozo *et al.*, 1995). To do this they must be able to detect differences in concentration across a very small distance indeed. A lot of work in

this field has been carried out using the amoeba *Dictyostelium discoideum* which use cAMP secretions from bacteria as a chemotactic signal (Devreotes and Janetopoulos, 2003). Accumulation of PIP_3 at the anterior is an early sign of polarisation (Devreotes and Janetopoulos, 2003). These physical changes occur by re-arrangement of the F-actin filaments throughout the cell (Coates *et al.*, 1992). The importance of this cytoskeletal rearrangement is highlighted by studies showing inhibition of actin polymerisation causes cells to lose both morphology and polarised sensitivity to chemoattractants (Devreotes and Janetopoulos, 2003). Polarisation leads to the re-distribution of many cell-surface molecules important for migration. These include chemokine and integrin receptors (del Pozo *et al.*, 1995).

1.3.7.2 Integrins

After tethering to, and rolling along the endothelium via selectins, as described in section 1.3.6.1, leukocytes use integrins for firm adhesion. Integrins are transmembrane heterodimers which not only attach the migrating cells to the vessel wall but activate intracellular signalling pathways. They consist of two noncovalently attached glycoproteins subunits, α and β. There are at least 18 α subunits and 8 β, combining to form 24 distinct integrins. Each of these has a specific function with β6 thought to have an important role in inflammation, and αL, αM, αE, β2, β7 knockout mice have defects in leukocyte function (Hynes, 2002).

Integrin α4β1 binds vascular cell adhesion molecule 1 (VCAM-1) and is important for leukocyte migration. Intracellular adhesion molecule-1 (ICAM-1) is the endothelial expressed molecule which interacts with the integrin αLβ7, or CD11a/CD18, expressed on numerous leukocytes. The expression of ICAM-1, like most, if not all, integrin ligands is regulated. In this case glycosylation is crucial for expression (Diamond *et al.*, 1991) and a failure to glycosylate leads to a decrease in cell migration and resulting recurrent infection (He *et al.*, 2014).

Integrins must become activated for firm adhesion to occur; this is by 'inside-out' signalling. Upon activation, integrins straighten, forming an open conformation with increased ligand affinity (Beglova *et al.*, 2002; Shimaoka *et al.*, 2002). The resting, or closed conformation only binds ligands with low affinity (Adair *et al.*,

2005). Integrin valency also increases upon activation by clustering. Positive feedback occurs with clustering increasing upon activation and binding (Welf *et al.*, 2012). The change of state to high-affinity, and lateral mobility of integrins, overall increases the avidity of the molecules and is triggered by chemokines (Constantin *et al.*, 2000). This activation is both rapid and transient.

1.3.7.1 Migration into Tissues

Following adhesion, leukocytes have to cross the endothelium. The majority of cells cross through endothelial cell junctions (para-cellular migration) but can also pass through the endothelial cells themselves (trans-cellular migration) (Hashimoto *et al.*, 2012). The latter has more recently been discovered and the exact signalling pathways involved have still to be fully identified (Muller, 2014). For para-cellular migration, cells need an additional step, termed locomotion, to efficiently move to cell junctions (Schenkel *et al.*, 2004). Interactions between platelet-endothelial cell adhesion molecule-1 (PECAM-1; CD31) on leukocytes and endothelium and similar interactions of CD99 are crucial for diapedesis. PECAM-1 blockade arrests cells mainly on the apical surface but CD99 inhibition arrests cells partially though the junction (Schenkel *et al.*, 2002). Junctional adhesion molecule (JAM) is also important in regulating para-cellular migration (Martin-Padura *et al.*, 1998).

Cells then cross the basal lamina and enter the tissue. Once in the affected tissue cells continue to migrate along a chemotactic gradient towards the site of infection or damage. Collagen networks increase during inflammation and this remodelling provides physical guidance to direct cells to the site of action. MMPs and other proteases are crucial for extravasation but the exact role of such enzymes in extravascular motility remains unclear (Lerchenberger *et al.*, 2013).

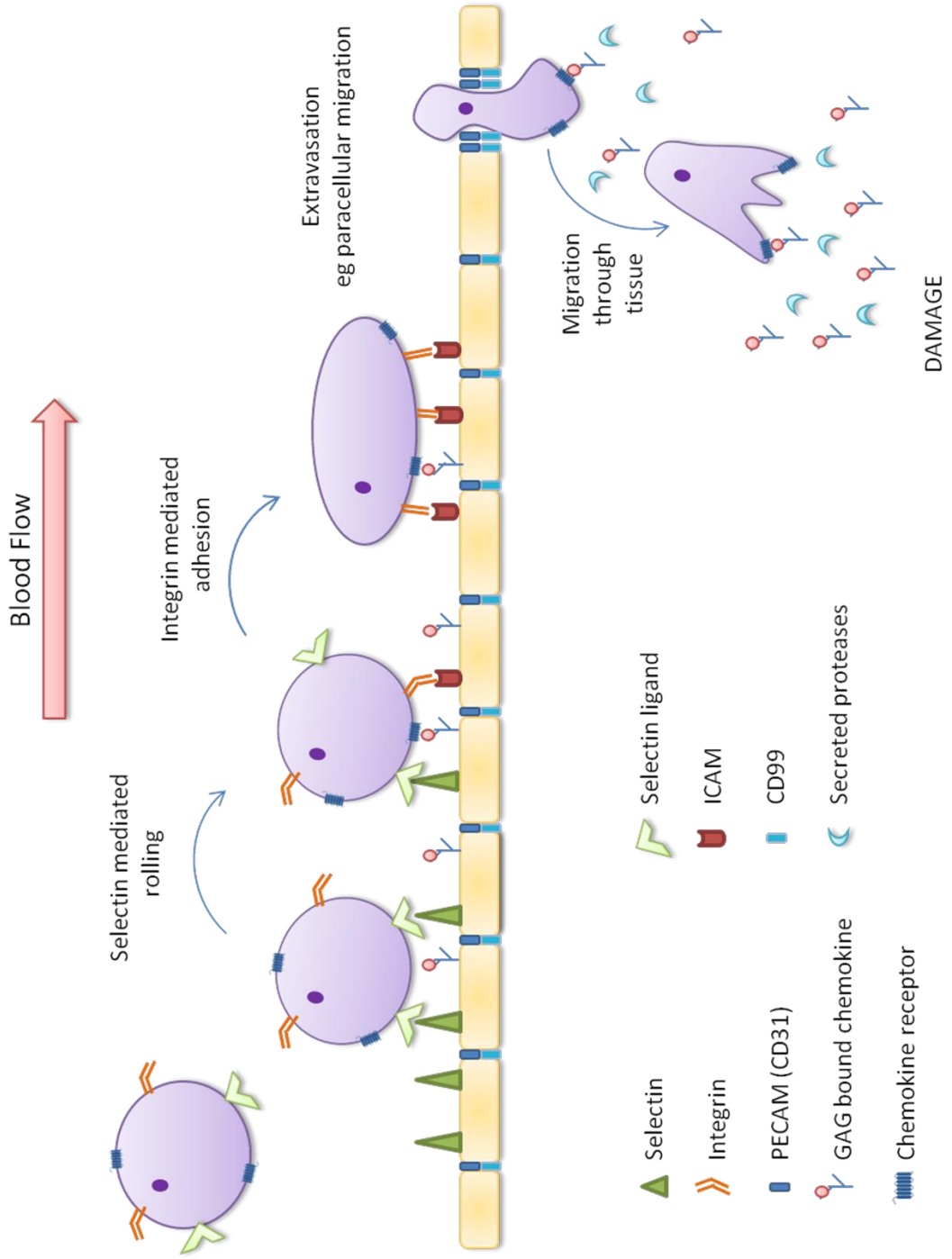


Figure 1-7 Leukocyte transendothelial migration
Leukocytes are recruited from the circulation to sites of inflammation by a series of distinct processes: tethering, rolling, adhesion and diapedesis, followed by chemotaxis through the tissue to the affected area. The initial interactions between leukocytes and the endothelium are transient low-affinity adhesions between selectins and their ligands, brought into contact by the shear forces in blood flow, and result in the rolling of leukocytes along the endothelial surface. Leukocytes rolling along the endothelium encounter GAG bound chemokines which bind and signal through appropriate receptors leading to the activation of integrins. Activated integrins then mediate high-affinity adhesive interactions between the leukocytes and the endothelium, by binding endothelial adhesion molecules such as ICAM, resulting in firm adhesion and arrest of rolling leukocytes. Following arrest, leukocytes extravasate through the endothelium into the tissue, either by a para-cellular route as depicted or through the endothelial cells directly (trans-cellular). Once in the tissue leukocytes migrate to sites of inflammation by following a chemokine concentration gradient and secreting proteases to aid movement through the tissue.

1.4 TISSUE STRESS

There are many causes of stress and damage to tissues which link to inflammation and chemokine biology. As previously mentioned oxidative stress has an important role in transplantation and so is used in this study. To model stress and inflammation in this study phorbol 12-myristate 13-acetate (PMA) and LPS were also used. These can activate numerous pathways shared with oxidative stress (Numata *et al.*, 1998; Gopalakrishna and Jaken, 2000).

1.4.1 *OXIDATIVE STRESS*

Oxidative stress is the accumulation of reactive oxygen species (ROS), and the related reactive nitrogen species (RNS). It arises due to an imbalance in ROS producers, including oxidases, and scavengers (or antioxidants) such as superoxide dismutase (SOD), altering the normal redox state of cells. This stress can be pathological as it has the potential for cellular dysfunction and tissue damage. ROS are oxygen based free radicals, or molecules involved in their formation as shown in Figure 1-8. Although closely related species, all have different effects, functions and half-lives. For example superoxide is often the first radical produced. It is short lived, damaging only macromolecules in the immediate vicinity. In contrast, hydrogen peroxide has a longer half-life and can act as a signalling molecule as well as effecting molecules directly.

Oxidative stress can be cell intrinsic as well as produced during inflammation and IRI as discussed in section 1.2. A major source of reactive species is electron leakage from the mitochondria during aerobic respiration (Bokov *et al.*, 2004; Ďuračková, 2010; Papadopoulos *et al.*, 2013). Other sources include peroxisomes, the site of lipid metabolism, xanthine oxidase (Ďuračková, 2010) and NADPH oxidase in the plasma membrane of activated macrophages (Dröge, 2002). Vascular adhesion protein-1, an amine oxidase present at increased levels on inflamed endothelium, for example in the liver, produces hydrogen peroxide and is linked to leukocyte recruitment during inflammation (Weston and Adams, 2011). Infiltration of immune cells able to perform oxidative burst, for example macrophages and neutrophils, is a further source of stress during inflammation by the release of multiple reactive species. This is an innate immune effector

mechanism to aid the killing of pathogens but can contribute to inflammation and stress with excess release of reactive species. Sufficient antioxidants are crucial to prevent damage by oxidative stress. *Sod1*$^{-/-}$ mice are deficient in the antioxidant CuZnSOD and although apparent normality in early life, have reduced lifespan and develop hepatocellular carcinoma (Elchuri *et al.*, 2005).

DNA, proteins and lipids are all affected by ROS. DNA damage due to oxidative stress includes both single–base damage and strand breaks (Rai, 2010; Woodbine *et al.*, 2011). The most common DNA modification is the oxidation of deoxyguanosine to 8-oxoG (Nakabeppu *et al.*, 2007). Detrimental double stranded breaks also occur if radicals cluster near the DNA, effectively causing multiple single strand breaks. This can introduce genetic mutations, cause downstream signalling effects and activate DNA damage repair mechanisms, potentially resulting in apoptosis or senescence (Rai, 2010). The damage is further worsened if proteins involved in DNA repair pathways are those damaged.

To proteins, oxidative stress can cause both conformational and covalent change, by alteration of protein folding and amino acids respectively (Cole *et al.*, 2010), as will be discussed in more detail in section 1.6.2, and this can alter function. Oxidation can also alter the oligomerisation states of proteins. Peroxynitrite, along with other reactive species can cause oxidative cross-linking leading to the formation of higher order structures (MacGregor *et al.*, 2011; Wang and Barger, 2012). Such damaged proteins need to be repaired. If repair is unsuccessful there are two mechanisms of degradation: the ubiquitin-proteosome system and the lysosomal, or autophagic, system. Protein aggregates, which can for example accumulate during cell ageing, resist degradation, blocking the proteosome and chaperones, further interfering with protein homeostasis (Martinez-Vicente *et al.*, 2005).

Peroxidation is the main form of lipid damage; the removal of hydrogen from unsaturated fatty acids creating radicals that react with oxygen (Lambert and Elias, 2010). This causes cell death both by peroxidation of membrane lipoproteins, disrupting membrane integrity, and by generation of reactive aldehydes including 4-hydroxynonenal (4-HNE), which interact with Fas pathways, including JNK,

leading to apoptosis (Ďuračková, 2010). This lipid modification is crucial in the pathogenesis of multiple diseases including atherosclerosis (Dröge, 2002).

Chronic oxidative stress causes considerable damage to cell structure and function, with further amplification by recruitment of inflammatory cells. Alteration to transcription factors such as NFκB, HIF-1α and p53 further develop the stress response and can lead to apoptosis of the parenchyma (Reuter *et al.*, 2010). Oxidative stress also reversibly increases mitochondrial permeability, allowing signalling molecules to transition to the nucleus and vice versa (Cole *et al.*, 2010). The regulation of levels of reactive species is crucial as at high levels, in pathological states, they are damaging, but some are necessary in steady states as signalling molecules.

Multiple, often age related, diseases including many cancers are associated with oxidative stress. Tissue injury, for example in the liver, generates oxidative stress (Urtasun *et al.*, 2009) and hepatocytes produce low levels of ROS, as can Kupffer cells. Oxidative stress is not only a consequence of injury but contributes to fibrosis and pathogenesis in multiple liver diseases (Nitti *et al.*, 2008). Fibrosis can be mediated by ROS via increased levels of TGFβ (Urtasun *et al.*, 2008), however oxidative stress is not always pro-fibrogenic. The antioxidant CuZnSOD increases active matrix metalloproteinase (MMP) levels, via TGFβ1, leading to the breakdown of scar tissue, decreasing fibrosis (Vozenin-Brotons *et al.*, 2001; McCord and Edeas, 2005) and in neuroinflammatory disorders ROS can activate MMPs (Haorah *et al.*, 2007). Some RNS may also reverse fibrosis as they upregulate MMPs (Urtasun *et al.*, 2008).

1.4.1.1 Peroxynitrite and associated radicals

Peroxynitrite is formed by the rapid reaction of nitric oxide and superoxide at a rate approaching the diffusion limit (Huie and Padmaja, 1993). This rate is 3 to 8 times faster than superoxide is degraded by SOD, or other biological sinks (Dedon and Tannenbaum, 2004) and therefore can evade decomposition by the antioxidant enzyme. Figure 1-8 shows peroxynitrite and related species including nitric oxide which is formed from the oxidation of the amino acid arginine by NOS, with the stable end product of L-citrulline (Marletta, 1993). *In vivo* production of

peroxynitrite has been estimated at 50-100µmoles per minute and due to its short half-life of only 10ms at physiological pH, peroxynitrite is generally spatially associated with sites of superoxide production such as the plasma membrane (Szabo *et al.*, 2007); it can however affect cells up to 20µm away (Szabo *et al.*, 2007). Peroxynitrite exists in *cis* and *trans* forms with the former being more stable and the latter having greater reactivity, this isomerisation creates complex kinetics (Beckman *et al.*, 1992).

The exact biological effects of peroxynitrite are hard to determine due to its short half life. For this reason the reactions and effects attributed to peroxynitrite in this study are likely to actually be caused by breakdown products and associated

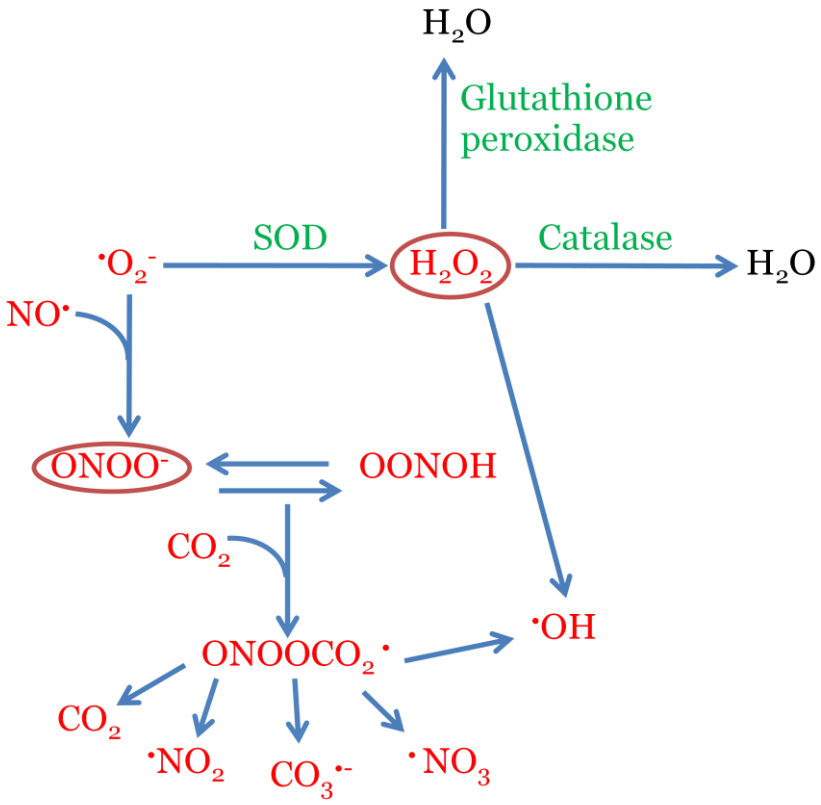


Figure 1-8 Reactive species in oxidative stress
Reactive oxygen species (ROS) and reactive nitrogen species (RNS) can both be formed from superoxide, itself a ROS and a byproduct of aerobic respiration. Antioxidant enzymes reduce the oxidative stress. Superoxide dismutase turns the highly reactive superoxide into the slightly less damaging hydrogen peroxide. ROS are turned to water to further remove damaging species. This is for example by the NADH dependant glutathione peroxidase or catalase. A non-exhaustive list of involved species include: $^{\bullet}O_2^-$ -superoxide, H_2O_2 – hydrogen peroxide, $^{\bullet}OH$ - hydroxyl radical, $ONOO^-$ - peroxynitrite, $NO^{\bullet}$ - nitric oxide, ONOOH - peroxynitrous acid, $ONOOCO_2$ - nitrocarbonate ion, $^{\bullet}NO_2$ – nitric dioxide, $^{\bullet}NO_3$ - nitrate, $^{\bullet}CO_3$ – carbonate radical. Antioxidant enzymes (green): superoxide dismutase (SOD).

radicals, for ease however they will be credited to peroxynitrite or RNS (Halliwell, 1997; Eiserich *et al.*, 1998; Pfeiffer *et al.*, 2000; Pfeiffer *et al.*, 2001; Thomas *et al.*, 2002). The protonated form of peroxynitrite is peroxynitrous acid, the proportion of which is determined by pH (pK_a=6.8). A crucial reaction for the *in vivo* effects of peroxynitrite is with carbon dioxide, forming the nitrocarbonate ion which can then undergo homolysis, creating the numerous reactive species shown in Figure 1-8 (Beckman *et al.*, 1990; Pryor *et al.*, 1997; Murphy *et al.*, 1998).

Macrophages produce nitric oxide and superoxide by inducible NOS (iNOS) and NADPH oxidase which react to form peroxynitrite. This aids macrophage antimicrobial function (Xia and Zweier, 1997; Bokov *et al.*, 2004) and is triggered by L-Arginine depletion occurring in inflammation. This is an innate immune effector mechanism to aid the killing of pathogens but contributes to inflammation and stress with excess release of reactive species. During chronic inflammation nitric oxide synthase 2 (NOS2) is upregulated in almost all liver cells (Urtasun *et al.*, 2008), further increasing the potential for peroxynitrite formation.

a) Effects on biomolecules

Peroxynitrite, or derivatives, can selectively oxidise or nitrate many molecules with wide ranging effects. In DNA, peroxynitrite reacts mainly with guanine to form 8-nitrodG, by nitrosation, or 8-oxodG (Dedon and Tannenbaum, 2004) as well as attacking the DNA backbone causing strand breaks (Dedon and Tannenbaum, 2004) and deoxyribose oxidation.

In proteins, peroxynitrite can spontaneously nitrate aromatic amino acids including tyrosine and tryptophan but SOD and Fe can also act as catalysts (Beckman *et al.*, 1992; Alvarez *et al.*, 1996), with the optimal pH reported to be pH7.5 (Beckman *et al.*, 1992). For tyrosine the sole nitration site is the third carbon however tryptophan has numerous reactive sites and can undergo oxidation, nitration and nitrosation (Nuriel *et al.*, 2011). Oxidation of the thiol group of methionine and cysteine to sulfoxide also occurs (Vogt, 1995). The short half-life of peroxynitrite prevents its detection *in vivo,* but it is generally accepted that the biomarker 3-nitrotyrosine (3-NT) is indicative of peroxynitrite presence. Antibodies have been raised against 3-nitrotyrosine and are used to identify

nitrated proteins and the presence of peroxynitrite or related species (Szabo *et al.*, 2007). I have previously shown that addition of peroxynitrite alone drastically increases levels of 3-NT in cells using such antibodies (Barker, 2011).

3-NT is not recognised during normal protein synthesis but can be incorporated both post-translationally and co-translationally by specific enzymes (Souza *et al.*, 2008). Nitration is not a silent modification and can have profound effects on protein function. Alteration of protein function occurs and if this leads to attenuation of antioxidants, a positive feedback loop is created further increasing peroxynitrite levels. Tyrosine nitration inactivates MnSOD by blocking the active site, and this has been observed in human renal allografts undergoing chronic rejection (Macmillan-Crow *et al.*, 1996). Peroxynitrite inactivation of CuZnSOD, however, is by oxidation of a histidine residue in the active site (Souza *et al.*, 2008). Lipid peroxidation by peroxynitrite not only alters lipids themselves but can also aid tyrosine nitration and oxidation (Szabo *et al.*, 2007). Nitrated lipids can also exert anti-inflammatory effects via PPAR-γ (Szabo *et al.*, 2007). Many of these modifications have been demonstrated *in vitro* but their relevance *in vivo* is unknown.

b) Cellular effects

The effects of peroxynitrite can be both damaging and beneficial, synergising with or antagonising the effects of ROS. This is dependent on the peroxynitrite concentration and surrounding microenvironment, but also the activation state of the cells involved and the antioxidant levels, specifically the peroxynitrite scavenger glutathione (Urtasun *et al.*, 2008). Peroxynitrite produces cell type dependant activation or inhibition of multiple signalling molecules including NFκB, phosphoinositide 3-kinase (PI3K), PKC, MAPK and histone deacetylase 2 (Szabo *et al.*, 2007). Peroxynitrite can be pro-apoptotic, triggering the release of mitochondrial apoptosis factors (Szabo *et al.*, 2007), but at low concentrations it initiates survival signals (Urtasun *et al.*, 2008), and is also involved in senescence induction. Nitric oxide and peroxynitrite are pro-apoptotic in neutrophils by triggering the release of cytochrome c from mitochondria (Meguro *et al.*, 2003), activating caspases 2 and 3, but macrophages are resistant to NO induced apoptosis (Shaw *et al.*, 2011).

As well as altering protein function, tyrosine nitration can also effect tyrosine phosphorylation, altering intracellular signalling. Generally, high peroxynitrite concentrations inhibit phosphorylation, possibly by direct competition between nitration and phosphorylation, but low concentrations lead to an increase in phosphorylation either by activation of tyrosine kinases or irreversible inhibition of phosphatases (Pacher *et al.*, 2007). There has also been a suggestion that nitration can mimic phosphorylation (Souza *et al.*, 2008) and can inhibit phosphatases, therefore increasing phosphorylation levels (Takakura *et al.*, 1999).

A study by Urtasun *et al*., 2009, suggests that peroxynitrite is protective in early stages of injury, although this is likely to be cell type and situation dependant. Treatment of hepatic stellate cells (HSC) with peroxynitrite leads to decreased cell activation and reduces the pro-fibrogenic effects of TGFβ. Peroxynitrite also increases MMP1 and MMP13 function by nitration and so decreasing fibrosis. However if HSC are activated prior to peroxynitrite treatment these anti-fibrogenic effects are not seen due to increased levels of glutathione, TGFβ and ROS. Peroxynitrite can also inhibit tissue inhibitor of metalloproteinase-1 function and therefore further increase the effects of MMPs (Frears *et al.*, 1996).

c) Involvement in disease

Peroxynitrite is toxic to pancreatic β-cells and there is increased 3-NT in the autoimmune attack of type 1 diabetes (Szabo *et al.*, 2007). As mentioned in section 1.4.1.1a, peroxynitrite in renal transplantation can further increase oxidative stress by inactivating MnSOD (Macmillan-Crow *et al.*, 1996). Peroxynitrite toxicity is increased by depletion of its scavenger glutathione, and this is associated with neurodegenerative disorders such as Parkinson's disease (Pacher *et al.*, 2007). 3-NT can also be seen in numerous cancers including colon, liver and prostate (Kasic *et al.*, 2011; Molon *et al.*, 2011)

Numerous vascular and cardiac conditions have been linked to peroxynitrite. The nitration of proteins including creatine kinase and voltage-gated potassium channels are important targets as well as nitration of structural proteins including desmin and myosin which can have damaging effects on cardiac contractility (Pacher *et al.*, 2007). At low levels however, peroxynitrite offers protection against

myocardial ischaemia-reperfusion injury by triggering cell survival signals (Urtasun *et al.*, 2008), in keeping with ideas that low level oxidative stress is protective.

1.4.2 *PMA*

PMA, a tumour promoting phorbol ester, is a potent, although not physiological, activator of PKC. Using a PKC activator is a relevant model to this study as PKC is one of several redox sensitive signalling molecules and so is involved in oxidative stress pathways. PKC is a superfamily of serine-threonine kinases which are involved in the stress response, cell growth and cell death. The isoform and location within the cell contributes to creating a cell or tissue specific response.

PMA stimulates PKC by binding its regulatory domain and mimicking the second messenger diacylglycerol (DAG) (Gopalakrishna and Jaken, 2000). Oxidative stress can have the same effect by oxidising cysteine (present as zinc-thiolates) in the zinc fingers which make up the PMA/DAG binding site. This alters conformation, relieving autoinhibition in the regulatory domain and causing PKC to be constantly active. High concentrations of oxidative stress however can inactivate the enzyme via alterations to cysteines in the catalytic domain (Gopalakrishna and Jaken, 2000), and chronic PMA exposure leads to a decrease in PKC due to proteosomal degradation.

PKC is also involved in the NFκB pathway which is important for redox-sensitive gene control. Superoxide increases NFκB DNA binding activity. In endothelial cells at least this is PKC dependent (Ogata *et al.*, 2000) and a study in T cells showed that PKC synergises with calcineurin to phosphorylate IκBα, activating the pathway (Steffan *et al.*, 1995). Hydrogen peroxide can activate tyrosine kinases which go on to regulate PKC-δ phosphorylation, further amplifying the effects of oxidative stress (Gopalakrishna and Jaken, 2000; Nitti *et al.*, 2008). Of importance to this study, as mentioned in section 1.3.2.1, CCL2 transcription can be triggered by PKC activation (Ghosh and Baltimore, 1990).

1.4.3 *LPS*

LPS, or endotoxin, is an essential component of the outer membrane of gram-negative bacteria. It is a ligand for Toll-like receptor 4 (TLR4), stimulating the production of proinflammatory cytokines by both MyD88 dependent and independent pathways, activating downstream signalling cascades including NFκB and MAPK. The chemokine CCL2 is induced in response to LPS in a MyD88-dependant manner in macrophages, however CXCL10 is independant (Shyy *et al.*, 1990; Bandow *et al.*, 2012).

LPS can therefore induce inflammation and can upregulate iNOS resulting in the production of radicals including peroxynitrite (Numata *et al.*, 1998). Previous studies have shown that LPS treatment of H69, the cholangiocyte cell line used in this study, induces cell proliferation by increasing production of the mitogen IL-6 (Park *et al.*, 1999).

1.5 SENESCENCE

Senescence is cell ageing, and premature, or accelerated senescence can be inflammation associated, contributing to the functional decline of tissues. Senescent cells are irreversibly arrested in the G1 phase of the cell cycle; the cells are not apoptosing but cannot divide further. Senescent cells generally have an enlarged and flattened morphology. Alterations in chromatin and transcription lead to the senescence associated secretory phenotype (SASP). Cellular senescence is implicated in the pathophysiology of multiple liver diseases, with increasing numbers of senescent cells seen in bile duct fibrosis (Sasaki *et al.*, 2010; Chiba *et al.*, 2011).

There are two categories of senescence, replicative and premature. Replicative senescence is associated with the Hayflick limit (Hayflick and Moorhead, 1961) and is determined by telomere length (Harley, 1991). Premature senescence however is linked to stress, including oxidative stress, and is independent of telomere length. Replicative senescence however, is not independent of oxidative stress as ROS damage can cause DNA breaks to accumulate in telomeres leading to accelerated shortening (von Zglinicki *et al.*, 2000). It is thought that senescence excludes cells from proliferation if there is a high chance of mutation; a tumour

suppressive mechanism (Laberge *et al.*). Due to other aspects of the biology of senescent cells however, this 'anti-tumour' effect is not clear cut.

Oxidative stress is involved in both establishing and maintaining senescence (Passos *et al.*, 2010). Chronic activation of $p21^{WAF1/Cip1}$ (p21; CDKN1A) causes mitochondrial dysfunction and ROS production. ROS then cause further DNA damage, reinforcing the phenotype. A minimum level of damage is needed to enter permanent senescence, although the damage itself does not have to be permanent (Passos *et al.*, 2010). Chronic activation of DNA damage repair pathways can lead to both senescence and apoptosis, depending on the degree of damage (Rai, 2010) and signalling through p21, MAPK and TGFβ are all important in linking senescence and ROS production. PKCδ is a downstream mediator of TGFβ-induced senescence (Katakura *et al.*, 2009).

1.5.1 *Senescence Associated Secretory Phenotype (SASP)*

The exact components of SASP vary between situations and cell types (Coppe *et al.*, 2008), but generally includes increased levels of proteases, matrix components, growth factors, for example TGFβ, and proinflammatory cytokines. Examples of such cytokines include the chemokines CXCL1 and CXCL8 which can both activate CXCR2, reinforcing senescence via activation of the p53 pathway (Acosta *et al.*, 2008b; Acosta and Gil, 2009). Downregulation of CXCR2 extends cellular lifespan (Acosta *et al.*, 2008a). SASP allows senescent cells to alter the surrounding microenvironment, spreading senescence by the bystander effect. This is generally via autocrine signalling and can be tumour suppressive, but paracrine signalling can be pro-tumourigenic (Coppe *et al.*, 2008). SASP also induces epithelial-to-mesenchymal transition (EMT), linking it to fibrosis (Laberge *et al.*; Coppe *et al.*, 2008). The nature of molecules in SASP means tissue repair may initially be stimulated but chronic secretion can be deleterious, disrupting tissue structure and decreasing the functional mass of the organ. This is in contrast to apoptosis which disrupts tissue integrity.

1.6 Chemokine Post-Translational Modifications

The function of chemokines, as with many proteins, can be profoundly altered by post-translational modification. These modifications can abrogate or enhance, and

in a few cases are mandatory for, chemokine function and thus act as a powerful regulatory mechanism to fine tune chemokine function. This has been previously reviewed by myself and others (Mortier *et al.*, 2008; Moelants *et al.*, 2013a; Barker *et al.*, 2014). Modification can be both enzymatic and chemical, altering both peptide length or modifying amino acids directly. These modifications can profoundly alter chemokine behaviour, changing which receptors they target, their presentation by GAGs and our ability to detect them with current antibodies. Chemokine receptors may also be modified, for example tyrosine sulphation of CXCR4 stabilises CXCR4:CXCL12 interaction (Rapp *et al.*, 2013; Ziarek *et al.*, 2013). Examples of different types of modification will now be given but these are not exhaustive.

1.6.1 *PROTEOLYSIS*

Multiple enzyme families can cleave chemokines, particularly at the disordered N-terminus although some examples of C-terminal cleavage have been found.

1.6.1.1 Matrix metalloproteinases (MMPs)

MMPs, zinc dependant endopeptidases, are involved in extracellular matrix degradation and remodelling. Multiple members of the family are upregulated following transplantation and during inflammation. MMP-2 and MMP-9, for example, are upregulated during ischaemia-reperfusion (Falk *et al.*, 2002; Kuyvenhoven *et al.*, 2004). Other MMPs are strongly associated with immune cells including the neutrophil associated MMP-8 and the macrophage specific MMP-12 (Dean *et al.*, 2008). Reactive nitrogen species can also upregulate MMPs (Urtasun *et al.*, 2009). As well as involvement in tissue remodelling these enzymes also have important roles in modifying bioactive molecules such as chemokines.

MMP-8 is released from inflammatory cells including infiltrating neutrophils. N-terminal cleavage of CXCL5 and CXCL8 by this enzyme increases chemotactic activity (Tester *et al.*, 2007). CXCL8 further increases MMP release from neutrophils and matrix component degradation by the enzyme further facilitates cell migration into the tissue, creating a feedback loop, amplifying cell recruitment and potentially leading to worsening damage if unresolved. MMP-1 and -9 also cleave CXCL8 (Van Den Steen *et al.*, 2000; Van Den Steen *et al.*, 2003b).

Chemokine inactivation also occurs by MMP cleavage. MMP-12 cleaves and inactivates ELR$^+$ CXC chemokines, including CXCL8 (Dean *et al.*, 2008), at the important E-LR motif. Later in inflammation, cleavage of CCL2, 7, 8 and 13 results in loss of activity and the formation of potent antagonists (Dean *et al.*, 2008) that may actively suppress macrophage recruitment, promoting resolution and preventing damage. There is specificity between ligand and substrate, indicating tight control of chemokine regulation. An example of this is CCL2 and CCL7 which are co-expressed and share the receptor CCR2. CCL2 can be cleaved by MMP-1 and MMP-3 but not by MMP-2 (McQuibban *et al.*, 2002). MMP-2 however can cleave and inactivate several chemokines including CCL7 (McQuibban *et al.*, 2001). These are all examples of N-terminal processing but MMP-8 and MMP-9 can also cleave C-terminal amino acids in CXCL9 and CXCL10, downregulating the function of both chemokines (Van den Steen *et al.*, 2003a).

1.6.1.2 CD26

The serine protease CD26 (dipeptidyl peptidase-4; DPP4) can also cleave many chemokines. CD26 is a membrane glycoprotein expressed by numerous cell types. It is highly expressed on T cells and is associated with several aspects of immune regulation including T cell proliferation. Inhibition of this enzyme has been investigated as a therapy for transplant rejection (Ohnuma *et al.*, 2008). CXCL11 cleavage by CD26 prevents chemotaxis but the chemokine is still able to desensitise the receptor CXCR3 and so can decrease the local activity of full-length CXCL11 (Ludwig *et al.*, 2002). CCL5 is also subject to post-translational processing by CD26, which cleaves the first two N-terminal amino acids (Lim *et al.*, 2005), preventing binding to CCR1 (Oravecz *et al.*, 1997) and CCR3 (Struyf *et al.*, 1998a), but CCR5 signalling is still intact, skewing the chemokine towards T cell recruitment. Interestingly, CD26 cleavage increases the anti-viral properties of CCL5 against HIV (Oravecz *et al.*, 1997). CCL4 also has altered receptor usage following CD26 cleavage, it still binds CCR5 but unlike the full length form also binds CCR1 and CCR2 (Guan *et al.*, 2002), altering the cell types it can recruit.

CD26 truncated CXCL10 has been isolated from patients with hepatitis C and is a negative prognostic factor in response to treatment (Albert *et al.*, 2011; Casrouge *et al.*, 2011; Ragab *et al.*, 2013; Riva *et al.*, 2014). This is presumably because of the

antagonistic properties of the truncated form of the chemokine, it has a 10-fold decrease in chemotactic potency (Proost *et al.*, 2001).

1.6.1.3 Other enzymes

Other enzymes can also alter chemokines. As well as CD26, CCL5 is subject to post-translational processing by cathepsin G which cleaves the first three N-terminal amino acids (Lim *et al.*, 2006). This variant has a 10 fold decrease in CCR5 binding and 100 fold lower chemotactic ability. This enzyme can also cleave platlet basic protein, forming the chemotactic CXCL7 (Walz and Baggiolini, 1990). C-terminal clipping of CCL1 by carboxypeptidase M results in increased signalling and anti-apoptotic activity but reduced CCR8 binding (Denis *et al.*, 2012).

The exact importance of this proteolytic activity *in vivo* however is uncertain as GAG binding can prevent chemokine cleavage. GAG binding protects CXCL8 and CXCL12 from proteolysis by elastase and CD26 respectively (Webb *et al.*, 1993; Sadir *et al.*, 2004) and cleavage of CCL11 by plasmin is also reduced (Ellyard *et al.*, 2007). This protection is not a universal finding however as CCL20 cleavage by cathepsin D still occurs to chemokine when GAG bound (Hasan *et al.*, 2006).

1.6.1.4 Infection

As well as enabling fine tuning of the chemokine system during inflammation, chemokine cleavage has been utilised by bacteria to evade the immune response. The pathogen *Streptococcus pyogenes* can express *Spy*CEP (*Streptococcus pyogenes* cell envelope protease) which cleaves ELR^+ chemokines, including CXCL8 (Edwards *et al.*, 2005). This cleavage inactivates the chemokine, unusually at the C-terminus, with functional effects most likely due to disruption of GAG binding and dimerisation rather than receptor binding. *Spy*CEP is a potent virulence factor, helping the bacteria evade immune response by decreasing neutrophil recruitment, and is crucial to aggressive bacterial dissemination (Kurupati *et al.*, 2010). This can lead to increasing disease, for example, lethal necrotising fasciitis (Edwards *et al.*, 2005).

Porphyromonas gingivalis, a bacterium implicated in periodontal disease, also produces enzymes capable of chemokine cleavage called gingipains (Mikolajczyk-

Pawlinska *et al.*, 1998; Moelants *et al.*, 2014). These enzymes can both cleave CXCL8 into more potent forms of the chemokine and degrade it, abrogating function, depending on whether the enzyme is soluble or membrane bound.

1.6.2 *RESIDUE MODIFICATION*

Modification of chemokines by direct alteration of amino acid residues will now be discussed, focusing on the two examples used in this study – nitration and citrullination. These are non-enzymatic and enzyme-catalysed modifications respectively. Other chemokine modifications can occur, including glycosylation, for example to CCL2 and CCL11 (Jiang *et al.*, 1990; Noso *et al.*, 1998) but these will not be described in detail.

1.6.2.1 Nitration

As discussed previously in sections 1.2 and 1.4.1, transplantation and associated inflammation involve oxidative stress. The RNS peroxynitrite can selectively oxidise and nitrate several residues, including the oxidation of histidine and the nitration of tyrosine and tryptophan. As shown in Figure 1-9, nitration of tyrosine results in the formation of 3-nitrotyrosine. This gives the modified residue a 45Da mass increase but the overall charge remains unaltered. 3-nitrotyrosine is a marker of nitro-oxidative stress and antibodies have been raised against the modified residue (Szabo *et al.*, 2007).

Chemokines are present at increased levels during periods of oxidative stress, such as transplantation (see section 1.3.3), and so could be targets of modification. Several chemokines have been found to be targets of peroxynitrite modification including CCL2 (Sato *et al.*, 1999a; Molon *et al.*, 2011) and CCL5 (Sato *et al.*, 1999b). Generally it is thought that modification of proinflammatory cytokines by peroxynitrite abrogates function, but this is dependent on numerous factors, including cell type. One study shows peroxynitrite-treated CCL2 loses its ability to recruit $CD8^+$ T cells but the recruitment of myeloid-derived suppressor cells is unaltered (Molon *et al.*, 2011). An earlier study however, shows CCL2 nitration impairs monocyte recruitment (Sato *et al.*, 1999a). Mechanisms of action have not been defined in these papers and this study aims to look at the effects in greater detail.

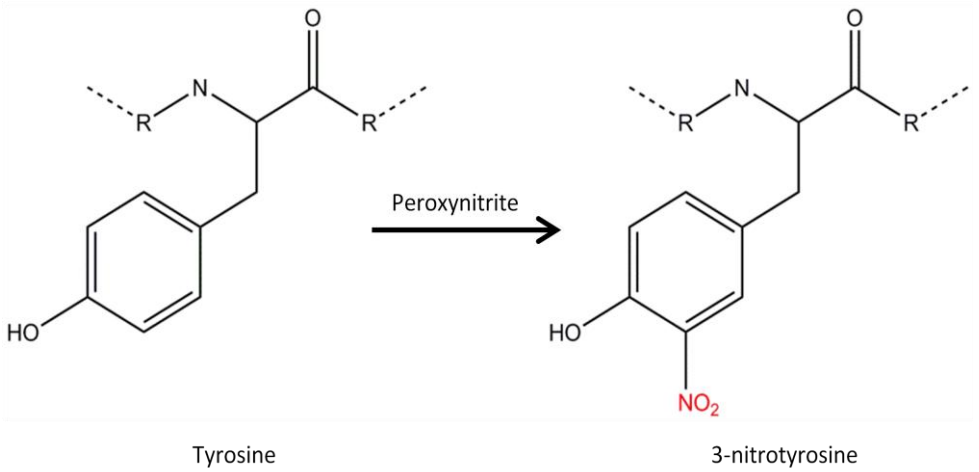


Figure 1-9 Tyrosine nitration
Nitration of tyrosine by peroxynitrite results in the formation of 3-nitrotyrosine. This reaction can occur to both peptidyl and free tyrosine residues.

Although not able to nitrate chemokines, the ROS hydrogen peroxide can modify chemokines by causing oxidative cross-linking of tyrosine residues in CCL5 (MacGregor *et al.*, 2011). This causes multimer formation of the chemokine but does not alter T cell chemotaxis or receptor usage. Interestingly, GAG binding prevents this modification occurring.

1.6.2.2 Citrullination

Enzymatic residue modification of chemokines includes citrullination. This is the formation of citrulline by the deimination of arginine, by peptidylarginine deiminase (PAD). This modification only occurs post-translationally as the enzyme substrate is peptidylarginine not free arginine.

As shown in Figure 1-10, citrullination gives a 1Da mass reduction per arginine residue modified but more profound functional consequences are likely to result from loss of the basic charge. This is especially important for chemokines which strongly rely on their cationic residues for GAG interactions, as explained in section 1.3.4. Arginine residues often play an important role in the structural integrity of proteins due to ionic interactions with negatively charged residues and cofactors, and by forming hydrogen bonds. Citrullination could logically therefore lead to changes in protein structure and hence function.

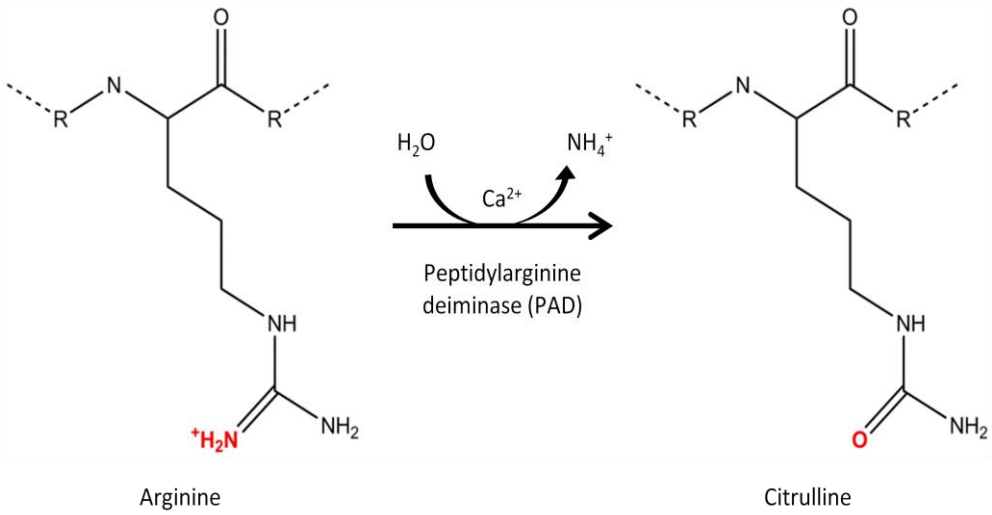


Figure 1-10 Citrullination
Citrullination is the deimination of arginine to citrulline by peptidylarginine deiminase. The substrate for this enzyme is peptidylarginine, the reaction does not occur on free arginine. Production of free citrulline however can occur as a byproduct of NOS or the urea cycle.

Five human PAD isoforms have been identified and their roles in pathology and chemokine interactions have been reviewed by Moelants *et al.*, 2012. The isoforms have differing tissue expression patterns, and substrate specificity is also exhibited. For example only PAD2 citrullinates beta actin, and only PAD4 modifies histone H3 (Darrah *et al.*, 2012). PAD are expressed by the epidermis, granulocytes, lymphocytes and some epithelial cells, with increased expression associated with a range of inflammatory diseases including rheumatoid arthritis and multiple sclerosis. Stress activated pathways involving p53 both regulate and are regulated by PAD4 citrullination (Tanikawa *et al.*, 2009), with autocitrullination further controlling enzyme activity (Méchin *et al.*, 2010).

All human PAD isoforms are calcium binding, and so calcium levels are crucial in regulating enzyme activity (Moelants *et al.*, 2012). Normal intracellular calcium levels are insufficient for function, but oxidative stress is known to cause an influx of cytoplasmic calcium, suggesting that PAD will be active in situations such as ischaemia-reperfusion and inflammation. Serum PAD has increased activity in rheumatoid arthritis patients (Basu *et al.*, 2011). Citrullination of multiple protein families has been identified including the structural proteins actin and cytokeratin. Histone citrullination alters chromatin remodelling and so effects protein

expression. Citrullination is also needed for neutrophil extracellular trap formation and so is heavily involved in immune regulation (Moelants *et al.*, 2012).

Chemokines can be citrullinated and, as with nitration, this is not a silent modification; it affects function. All modifications found so far show citrullination to be an anti-inflammatory modification, decreasing *in vivo* chemokine function. Citrullination of both CXCL10 and CXCL11 does not alter CXCR3 binding but does result in a decrease in calcium signalling, chemotactic activity and heparin binding (Loos *et al.*, 2008). Studies of CXCL12 show that PAD decreases function, with an increase in functional impairment correlated with an increased number of citrullinated arginines (Struyf *et al.*, 2009). Such modified chemokine may occur in for example, Crohn's disease, where co-expression of CXCL12 and PAD has been described (Struyf *et al.*, 2009). CXCL8 has increased CXCR1 binding once citrullinated on specific arginines but decreased heparin binding so does not induce neutrophil chemotaxis *in vivo* (Proost *et al.*, 2008). These modifications highlight important differences between *in vivo* and *in vitro* chemokine function.

Bacterial PAD have also been identified, produced by *P. gingivalis* (PPAD). Unlike the human isoforms these can modify both free and peptidyl-arginine and are not calcium dependent. Chemokines are potentially subject to modification by these enzymes but as yet it is unconfirmed (Moelants *et al.*, 2014). As well as modifying GAG binding, citrullination also has a role in preventing enzymatic cleavage. Citrullination of CXCL8 prevents cleavage by gingipains to more active forms of the chemokine, thus potentially dampening inflammation (Moelants *et al.*, 2014).

1.7 HYPOTHESIS

Chemokine production following cellular stress and concurrent modification of those chemokines represents a powerful paradigm for the regulation of inflammation. Not all modifications can occur to all chemokines and not all affect function. It is this heterogeneity which makes understanding chemokine biology within the inflammatory environment crucial and it may offer better therapeutic options.

1.8 SPECIFIC AIMS OF THIS STUDY

Chemokines play a crucial and complex role in inflammation. This study sets out to enhance the current understanding of chemokine regulation during inflammatory and stressful conditions. Three areas of study were focused on and work was carried out to:

- To determine how tissue stress effects the production of the chemokines CCL2, CCL5, CXCL8 and CXCL10

- To investigate which chemokines are subject to post-translational modification. Specifically if CCL2, CCL5 and CXCL8 undergo the oxidative stress associated modification, nitration, and if CCL5 citrullination occurs

- To characterise the functional effects of nitration, focussing on CCL2

2 General Materials and Methods

2.1 Laboratory Procedure

All experimental procedures were conducted according to the Control of Substances Hazardous to Health (COSHH) regulations. All laboratory work was carried out in compliance with the University Safety Policy. Laboratory procedures were performed in accordance with the Newcastle University publications 'Safe Working with Biological Hazards', and 'Safe Working with Chemicals in the Laboratory'. Tissue culture was carried out in compliance with the regulations for containment of class II pathogens.

Unless otherwise stated all reagents used are from Sigma.

2.2 Cell Culture

All cells were grown in a humidified incubator at 37°C with 5% CO_2 and tissue culture was carried out in a Class II containment cabinet. Cells were grown in 25, 75 and 175cm^2 tissue culture flasks or 6 well plates (Greiner Bio-One). Adherent cells were washed with phosphate buffered saline (PBS; Sigma) and detached by incubation with trypsin-EDTA (Sigma). Detached cells were then centrifuged (5min, 500xg), resuspended in tissue culture medium, counted by haemocytometer if necessary, and seeded into new flasks or plates. If cells were to be used for assays requiring immediate cell surface protein expression, cells were detached from the flask using EDTA-PBS (Sigma).

2.2.1 *Culture Media*

All media were supplemented with 2mM L-glutamine (Sigma), 10% foetal bovine serum (FBS; Biosera) and 100U/ml penicillin and 100µg/ml streptomycin (Sigma). Other supplements were added as required.

2.2.2 *Cell Storage*

Cells were detached as described above and resuspended in 1ml freezing media (10% dimethylsulphoxide (DMSO; Sigma) in FBS) in a cryovial. Cells were cooled at 1°C per minute in a "Mr Frosty" (Nalgene) overnight in a -80°C freezer to prevent

cryoinjury. Cells were transferred to liquid nitrogen for long term storage. Cells were retrieved from storage by rapid thawing in a 37°C water bath and washed with media to remove DMSO before seeding into flasks.

2.2.3 *Cell Counting and Viability*

To determine cell number and concentration, 10µl cell suspension was counted using an improved Neubauer chamber haemocytometer.

If cell viability needed to be assessed, cell suspension was mixed 1:1 with 0.4% trypan blue (Sigma) prior to counting. Trypan blue cannot pass through viable cell membranes, but if the cell is necrotic, trypan blue crosses the membrane, staining the cell blue. Live and dead cells can therefore be distinguished, counted by haemocytometer, and the percentage viability calculated.

2.2.4 *Cell Lines*

2.2.4.1 H69

The cholangiocyte cell line H69 was created by Grubman *et al.*, 1994 from human intrahepatic biliary epithelial cells immortalised by transfection with SV40 large T antigen. Cells were cultured in complete low glucose Dulbecco's Modified Eagle's medium (DMEM)/Nutrient Mixture F12 Ham (3:1; Sigma) supplemented with: 18mM adenine (Sigma), 2µM triiodothyronine (Sigma), 5.5µM adrenaline (Sigma), ITS-X supplement (10µg/ml insulin, 5.5µg/ml transferrin, 2µg/ml ethanolamine, 6.7ng/ml sodium selenite; Gibco, Invitrogen), 1µM hydrocortisone solution (Sigma).

2.2.4.2 HKC8

The renal epithelial cell line HKC8 was created by Racusen *et al.*, 1997 from adult human renal tubule epithelium and cells were immortalised using adenovirus 12-SV40. Cells were cultured in complete DMEM F12 Ham (Sigma).

2.2.4.3 EA.hy926

EA.hy926 were created by the fusion of human umbilical vein endothelial cells with the human lung carcinoma cell line A549 (Edgell *et al.*, 1983) and are used in

this study to model endothelium for *in vitro* transendothelial chemotaxis. Cells were cultured in complete DMEM (Sigma).

2.2.4.4 THP-1

THP-1 is a monocytic cell line derived from the peripheral blood of a 1 year old with acute monocytic leukemia (ATCC TIB-202). Cells were cultured in complete RPMI-1640 (Sigma).

2.2.4.5 HEK-CCR2b

Human embryonic kidney (HEK-293) cells have previously been transfected with the chemokine receptor CCR2b by our group (Wain *et al.*, 2002) using a vector encoding the human chemokine receptor CCR2b and neomycin phosphotransferase, giving G418 resistance. Cells were cultured in complete DMEM (Sigma) with 800µg/ml G418 (Calbiochem) to maintain selection of the transfectants.

2.2.4.6 CHO-CCR5

Chinese hamster ovary (CHO-K1) cells previously transfected with the human chemokine receptor CCR5 (Ali *et al.*, 2000) were cultured in complete DMEM F12 Ham (Sigma) with 800µg/ml G418.

2.2.4.7 Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood taken from healthy volunteers. Blood was thoroughly mixed with 1 unit heparin per ml blood to stop coagulation before dilution 1:1 with serum-free RPMI-1640 and carefully layering 10ml above 5ml Lympholyte-H (Cedarlane Laboratories). The blood was then separated using density gradient centrifugation (800xg, 20min). The interface containing PBMC was extracted and washed twice in serum-free RPMI-1640 (500xg, 5min) before counting. Cells were serum starved for 1hr prior to use in assays. CCR2 expression on T cells and monocytes was confirmed by flow cytometry (Figure 2-1).

Ethical approval to obtain blood from healthy volunteers was granted by the County Durham and Tees Valley Research Ethics Committee (12/NE/0121).

2.2.4.8 Neutrophils

Neutrophils were isolated from whole blood taken from healthy volunteers. All reagents were warmed to 37°C before use. 10ml blood was mixed with 1ml 3.8% sodium citrate (Sigma) as an anti-coagulant. The blood was then centrifuged for 20min at 200xg, brake rate 0, and the plasma layer removed. 2.5ml of 6% dextran T-500 in 0.9% NaCl (Pharmacosmos) was added, the mix made up to the original blood volume with 0.9% saline solution and cells left to sediment for 30min. The leukocyte rich upper layer was removed and made up to 50ml with 0.9% saline solution and centrifuged at 200xg for 5min, brake rate 1. The pellet was then re-suspended in 2.5ml 55% percoll plus (GE Healthcare) and layered on top of layers of 70% and 81% percoll plus before centrifugation at 700xg for 20mins, brake rate zero. Neutrophils were then collected from between the 70% and 81% layers, and washed in Hank's Balanced Salt Solution (modified with $NaHCO_3$, without phenol red, calcium chloride or magnesium sulphate; Sigma) with centrifugation at 200xg for 5min before use in assays. This work was performed under my supervision by the BSc project students P Milligan and F Tak.

2.2.5 *Characterisation of Cell Lines*

Flow cytometry was used to establish which chemokine receptors are present on the epithelial cell lines (Figure 2-2 and Figure 2-3). This was performed to see if the epithelial cells were capable of chemokine scavenging. The antibodies used were all from R&D Systems, and cells were stained according to the manufacturer's instructions, with appropriate isotype controls (see Table 2).

H69 cells have previously been validated as epithelial cells using the markers E-cadherin, cytokeratin-19 and zona occludens-1 (Barker, 2011).

2.2.6 *Mycoplasma testing*

Mycoplasma can alter cell responses but is not visible by light microscope. To ensure cells were not infected, cells were tested using MycoAlert™ mycoplasma detection kit (Lonza) in accordance with the manufacturer's instructions.

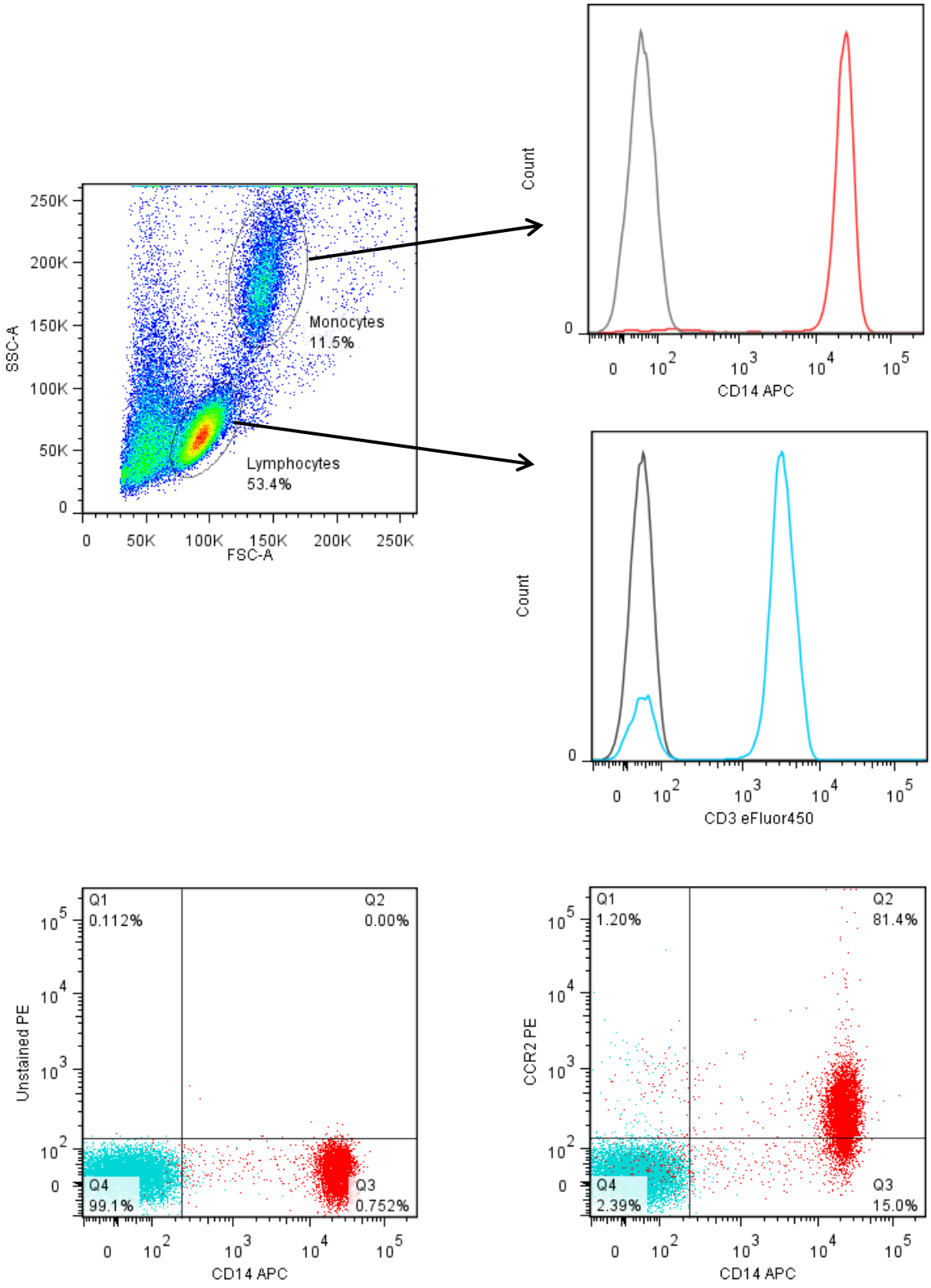


Figure 2-1 Characterisation of CCR2 expression on monocytes and T cells.
Upper panels show identification of monocytes and T cells using the markers CD14 (red) and CD3 (blue) respectively.
Lower panels show unstained control (left) and CCR2 staining (right) on monocytes and T cells.
Representative of three experiments.

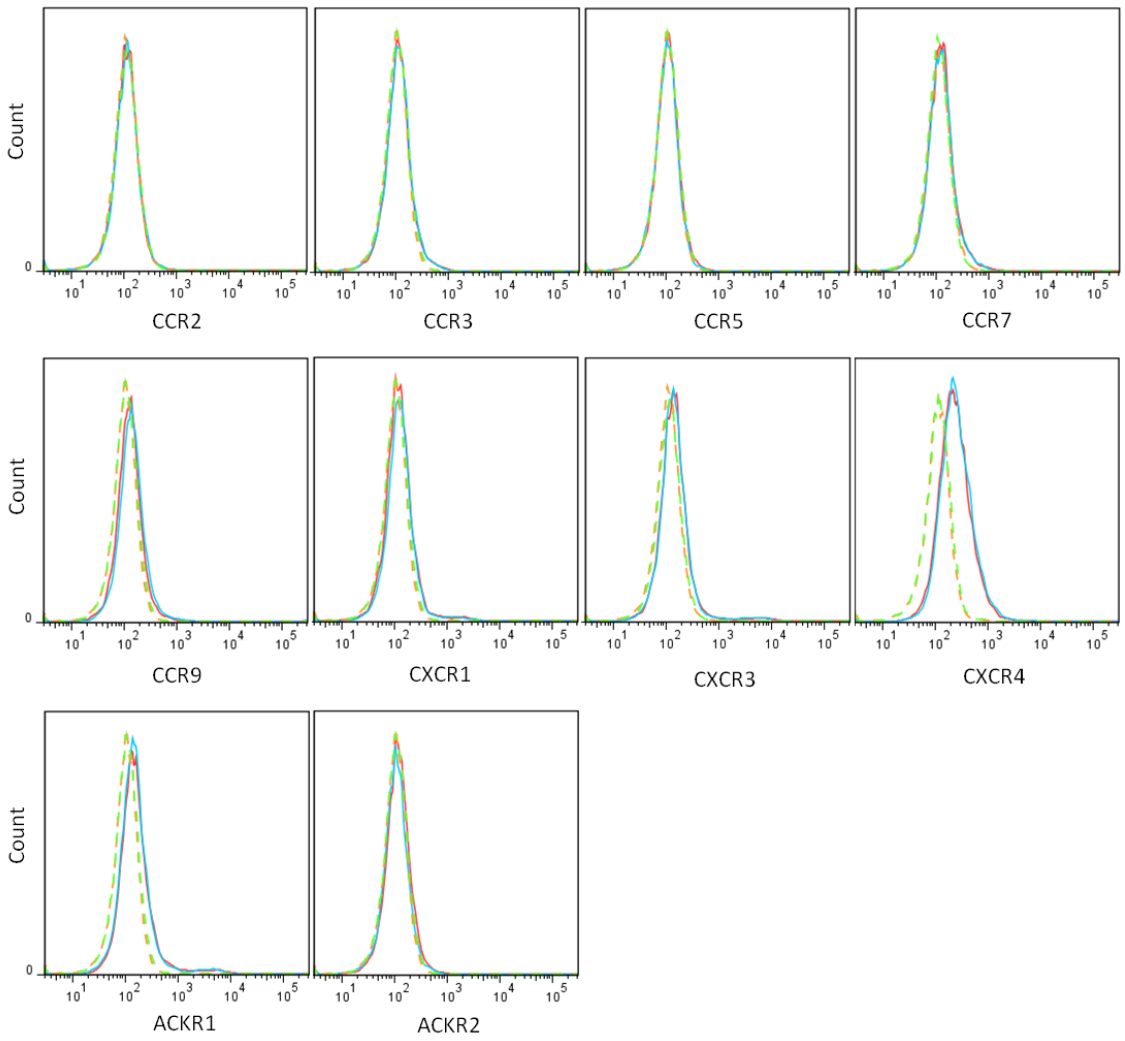


Figure 2-2 Chemokine receptor expression on H69 cells
Representative flow cytometry staining of H69 cells for range of chemokine receptors (solid lines) and isotype controls (dashed lines). n=2

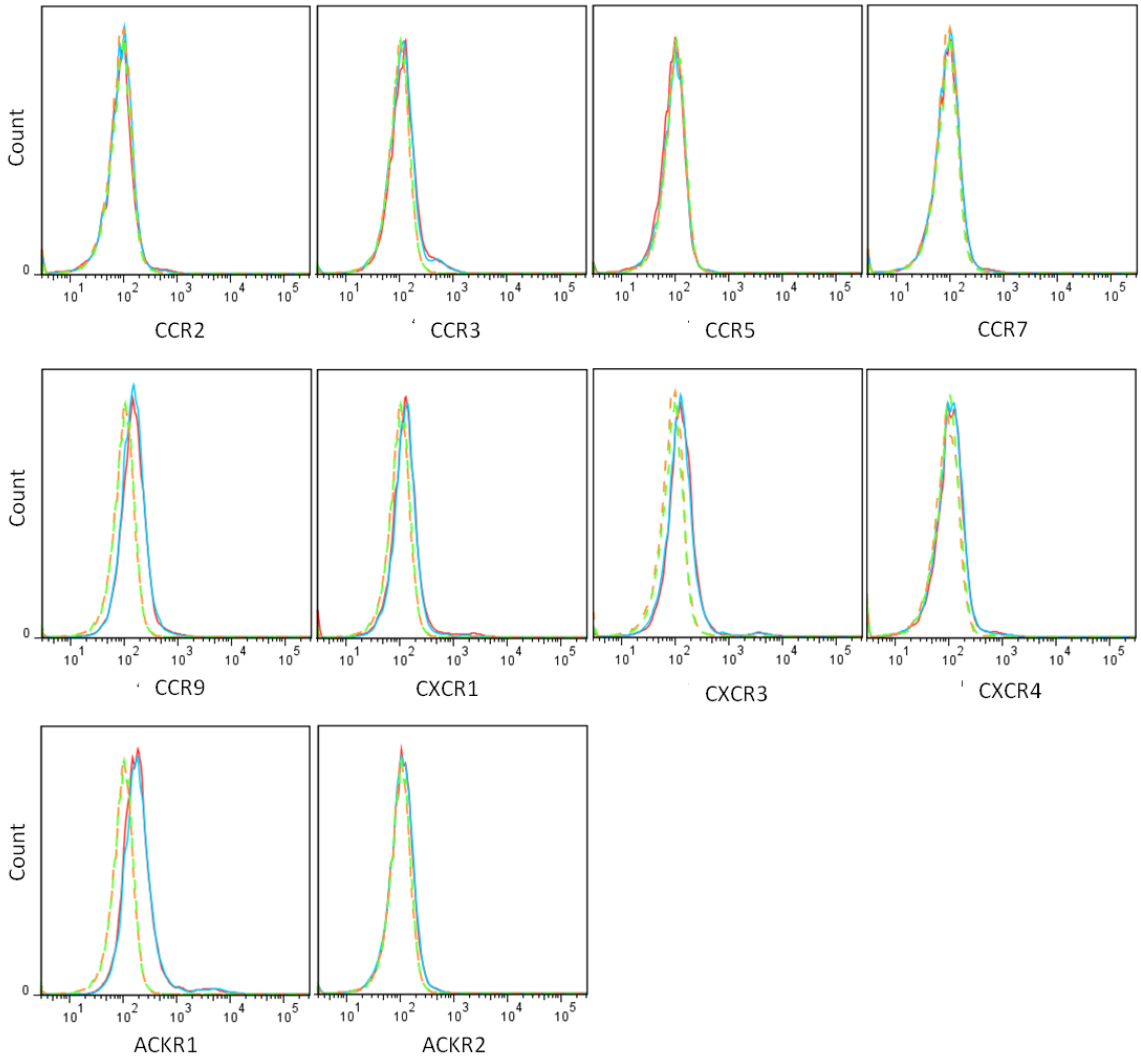


Figure 2-3 Chemokine receptor expression on HKC8 cells
Representative flow cytometry staining of HKC8 cells for range of chemokine receptors (solid lines) and isotype controls (dashed lines). n=2

2.3 Flow Cytometry

2.3.1 *General Principles*

Flow cytometry is a laser-based technique which uses principles of light scattering and, in many cases, emission from fluorochromes, to gain information on single cells as they pass through a laser in a fluid stream.

Light is reflected and refracted by cells and their internal structures. This light is measured, detected, and converted into electrical pulses by optical detectors. This can give information on the physical characteristics of the cells. Light scattered in the forward direction of the laser beam is focused by a lens and converted into a parameter known as Forward Scatter (FSC). This gives information about the size and shape of the cell. Light scattered perpendicular to the plane of the beam is called Side Scatter (SSC) and is proportional to the granularity of the cell. The combination of these two parameters can help identify cell types.

In addition to information on physical characteristics, cells can be labelled with dyes or fluorochrome-conjugated antibodies which allow detection of specific cell-surface markers. When fluorochromes are stimulated at a particular wavelength they are excited but then return to their original unexcited state by emission of lower energy and longer wavelength light. This emitted light is detected, split in to specific colours by wavelength and digitised, as with FSC and SSC. Ideally, fluorochromes should be selected so that their emission and excitation wavelengths have sufficient spectral separation to allow fluorescence to be detected individually. If more than one fluorochrome is used per tube and this is not the case, there is a chance that the signal of one fluorochrome will be artificially increased by photons from the periphery of another fluorochrome's spectrum. This is further complicated as fluorochromes emit a spectrum that will activate different detectors to different extents if they do not emit at a single wavelength. If needed, electronic compensation algorithms can be set up at the beginning of the experiment.

In this study flow cytometry has been used to determine the presence of cell-surface receptors, using fluorochrome-conjugated antibodies, and to count cells as

a ratio to beads following chemotaxis, using the FSC and SSC characteristics of cells and beads (see section 2.8.1.1b).

2.3.2 *Staining of Cell-Surface Antigens*

Cells were removed from culture, counted and resuspended in 2% FBS/PBS. 250,000 cells in 50µl were stained per tube with the antibody concentration according to the manufacturer's instructions (see Table 2). Unstained and appropriate isotype controls were also performed.

Antigen	Clone	Isotype	Label	Manufacturer
hCXCR1	42705	mIgG2a	FITC	R&D
hCXCR3	49801	mIgG1	PE	R&D
hCXCR4	44717	mIgG2b	APC	R&D
hCCR2	48607	mIgG2b	PE	R&D
hCCR3	61828	rIgG2a	FITC	R&D
hCCR5	CTC5	mIgG2b	FITC	R&D
hCCR7	150503	mIgG2a	PE	R&D
hCCR9	112509	mIgG2a	FITC	R&D
hDARC	358307	mIgG2a	FITC	R&D
hD6	196124	rIgG2a	FITC	R&D
hCD3	OKT3	mIgG2a	eFluor 450	eBioscience
hCD14	61D3	mIgG1	APC	eBioscience
mCD3	17A2	rIgG2b	eFluor 450	eBioscience
mCD45	30-F11	rIgG2b	PerCP-Cyanine5.5	eBioscience

Table 2 List of antibodies used in flow cytometry

2.3.3 *Instrument and Analysis*

All flow experiments were performed using BD FACSCanto II flow cytometers and data recorded using BD FACSDiva software. Data were analysed using FlowJo software v.7.6.4 (Treestar).

2.4 Real-time Quantitative Reverse Transcription Polymerase Chain Reaction

In order to decrease the risk of sample contamination, all reagents used were RNase free, only sterile filter tips were used and all tubes were autoclaved and/or treated with UV light exposure.

2.4.1 *RNA Isolation*

RNA was isolated using TRI-Reagent (Sigma) as per the manufacturer's instructions, and my previous work (Barker, 2011), based on the method developed by Chomczynski (Chomczynski and Sacchi, 1987). All work was carried out on ice and centrifugation was at 4°C. Treated cells were lysed directly on the plate using 1ml TRI-Reagent, pipetted to form a homogeneous lysate and allowed to stand for 5min to allow nucleoprotein complex dissociation. 200µl chloroform (Sigma) was then added prior to vigorous shaking for 15sec and allowed to stand at room temperature (RT) for 15min. The lysate was then separated into three phases by centrifugation (12000xg, 15min). The upper colourless phase containing

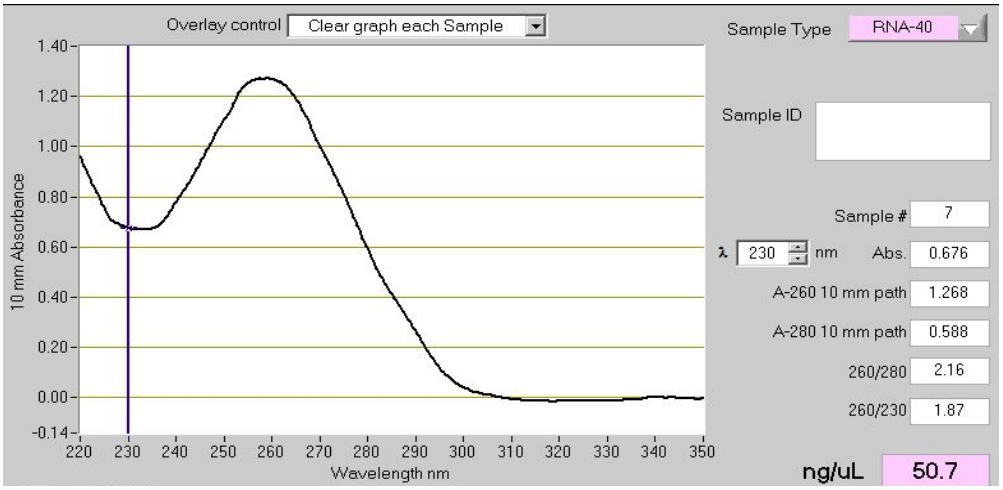


Figure 2-4 Representative plot from Nanodrop
RNA quality was analysed using a Nanodrop Spectrophotometer. 260/280 and 260/230 ratios can be determined as well as RNA concentration.

the RNA was transferred to a new tube and 0.5ml 2-propanol (Sigma) added. After standing for 10min the RNA precipitate was pelleted by centrifugation (12000xg, 10min). The pellet was washed using 75% ethanol in DEPC-treated water, vortexed and centrifuged at 7500xg for 5min. The pellet was air dried before resuspending in 50µl DEPC treated water. The RNA was analysed using a

NanoDrop ND-1000 Spectrophotometer to determine concentration and purity (see Figure 2-4).

2.4.1.1 Gel electrophoresis

Gel electrophoresis was used to check the degradation state of the isolated RNA. 2µl RNA with orange G loading buffer was run on a 1% agrose gel (50ml tris-acetate-EDTA (TAE) buffer, 0.5g agrose and 0.5µg/ml ethidium bromide; TAE buffer contains 40mM Tris, 20mM acetic acid and 1mM EDTA at pH8.0; all Sigma) at 90V for approximately 45min. RNA was then visualised and imaged using an AlphaImager (Alpha Innotech Corporation) to demonstrate intact ribosomal RNA subunits, as shown in Figure 2-5.

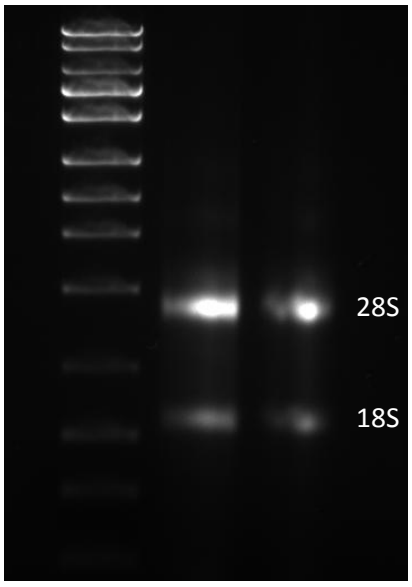


Figure 2-5 Confirmation of RNA integrity.
Following RNA isolation using TRI-Reagent, RNA was run on a 1% agrose gel. The 2 bands show the RNA from the 18S and 28S ribosomal subunits, rRNA being the most abundant RNA and so the most apparent bands.

2.4.1 *First Strand Complementary DNA Synthesis*

Complementary DNA (cDNA) was made from the RNA suspension using an AffinityScript Multiple Temperature cDNA synthesis kit (Aligent Technologies) or Tetro cDNA Synthesis Kit (Bioline) and a thermocycler (T100™ Thermal Cycler, Bio-Rad) according to the manufacturer's instructions (Aligent – 5min at 65°C, 10min at 25°C, 60min at 50°C and 15min at 70°C. Bioline - 30min at 45°C and 5min at 85°C). 2µg RNA was used per reaction and following synthesis short term storage of cDNA samples was at 4°C until PCR amplification.

2.4.2 *TAQMAN REAL-TIME PCR*

2.4.2.1 General Principles

Polymerase chain reaction (PCR) is a technique used to amplify a specific gene from a cDNA template, using primers specific to the gene of interest: the more abundant the starting template, the more product will be created. Conventional PCR is semi-quantitative as levels of product can be visualised by gel electrophoresis and compared; real-time PCR (qPCR) is quantitative. Both techniques require comparison to a housekeeping gene, for example GAPDH, which serves as an endogenous control.

PCR is performed in a thermocycler which repeatedly heats samples to a range of temperatures allowing the reaction to occur. First, an initialisation step is needed to activate the polymerase, and then a series of steps are repeated, normally for forty cycles. Cycles are as follows: denaturing step - causes disruption of hydrogen bonds, creating single stranded DNA, annealing step - annealing of the primers to the single stranded template, elongation step – the optimal temperature for polymerase activity, allowing synthesis of a new strand of DNA. There is exponential amplification in which the amount of product doubles, followed by levelling off and plateauing in which no more product is formed due to exhaustion of reagents.

qPCR allows the detection of PCR products during their formation by the generation of a fluorescent signal. In this study Taqman probes were used, which have a high energy fluorescing dye (reporter; 6-carboxyfluorescein) at the 5’ end and a low energy quencher (non fluorescence quencher-minor groove binder; NFQ-MGB) at the 3’ end of the probe, as shown in Figure 2-6. If the probe is intact, when the reporter is excited by light it does not fluoresce as energy is transferred to the quenching molecule (fluorescence resonance energy transfer). The probe anneals to specific sequences between the forward and reverse primers and as the polymerase extends the primers, the 5’ exonuclease of the enzyme cleaves the probe, separating the reporter and quencher. This allows the reporter to fluoresce, and fluorescence increases with every cycle, proportional to the accumulation of

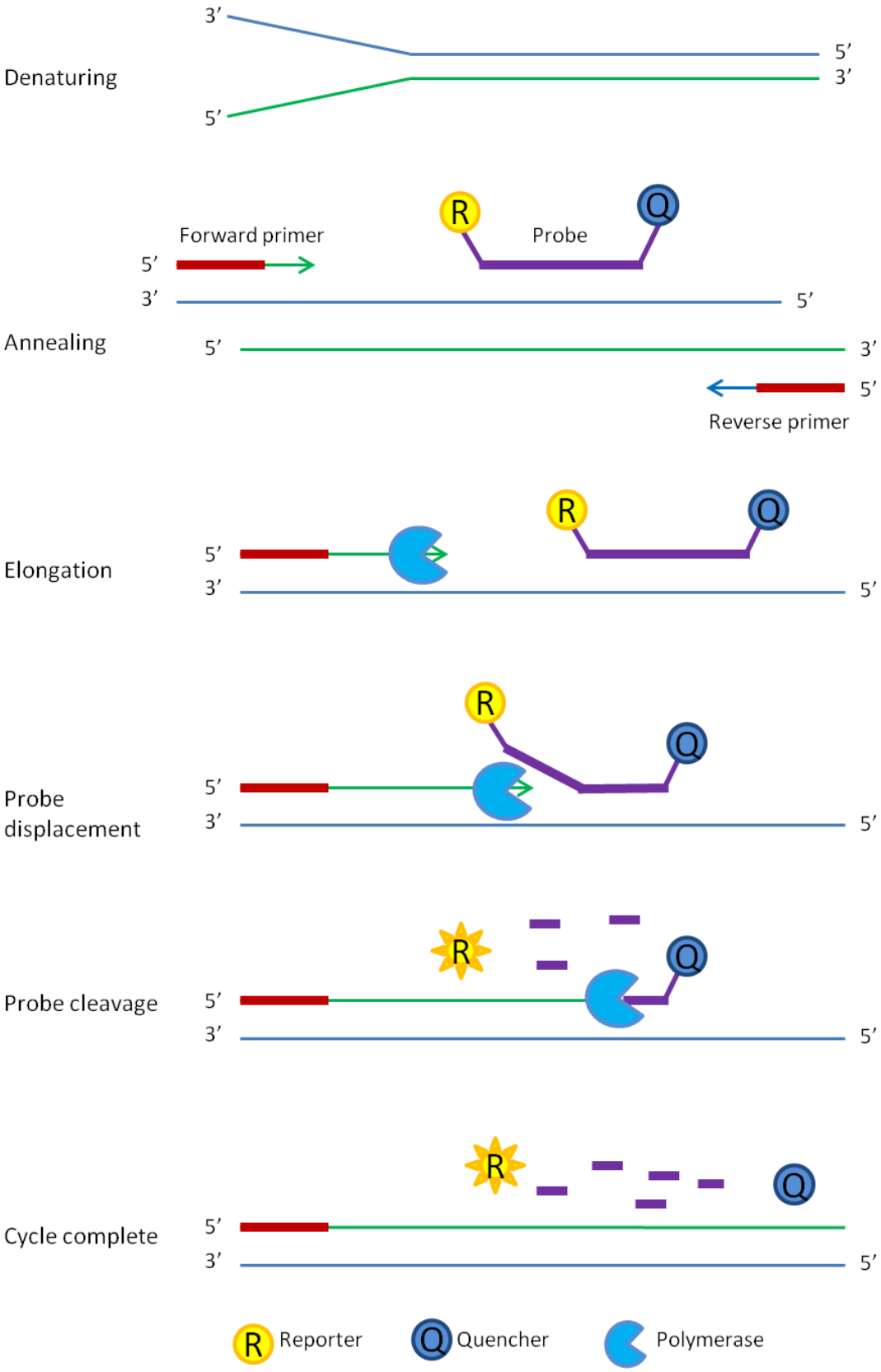


Figure 2-6 Taqman qPCR schematic
Steps of qPCR - initial denaturation of the cDNA followed by annealing of PCR primers and probe to complementary regions of DNA. The polymerase then extends the strand using the cDNA template, and the exonuclease activity displaces and cleaves the probe. This allows the reporter and quencher to be separated resulting in reporter fluorescence. The increase in fluorescence is directly proportional to the amount of PCR product.

PCR products. An important parameter for quantification is the threshold cycle (C_t), this is the cycle number at which the fluorescence level passes a fixed threshold, set within the lower third of the exponential phase of increasing fluorescence (see Figure 2-7). The lower the C_t, the higher the initial amount of template.

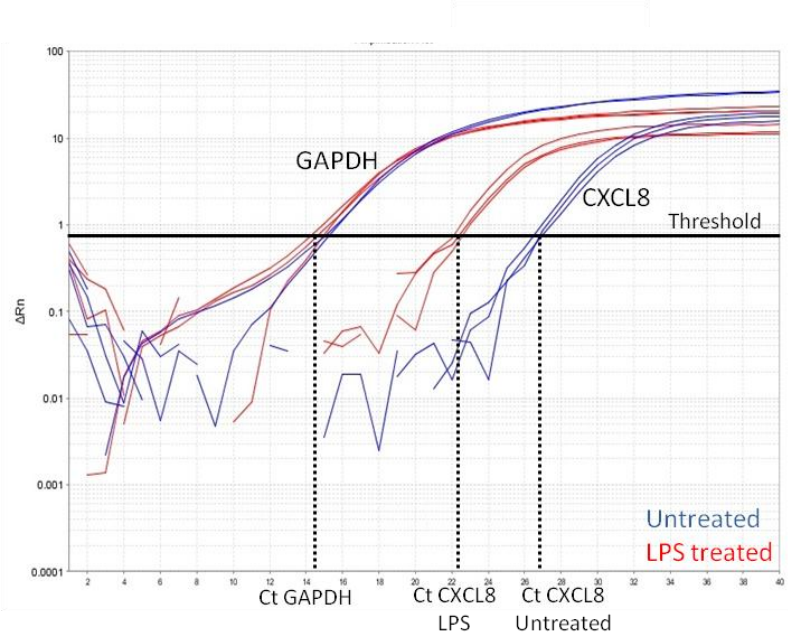


Figure 2-7 Real-time PCR amplification plot
The threshold is placed in the lower third of the linear phase of the amplification curve, establishing the threshold cycle (Ct) for each sample. This allows calculation of the fold increase relative to the housekeeping gene GAPDH using ddCt. The plot shows amplification of cDNA from H69 cells both untreated and following treatment with the positive control LPS. The lower the Ct value, the higher the starting level of gene specific mRNA and so CXCL8 expression is increased following LPS treatment.

2.4.2.2 Method

Real-time PCR (qPCR) was performed to quantify the alteration in mRNA expression of various chemokines and enzymes following cell treatment. 20µl reactions were performed in MicroAmp Optical 96 well reaction plates (Applied Biosystems). Each reaction contained: 1µl cDNA, 8µl sterile water, 1µl TaqMan primer-probes (shown in Table 3) and 10µl 2xBrilliant II QPCR Master Mix (Aligent Technologies) or SensiFast Probe Hi-ROX Mix (Bioline). Reactions were carried out in triplicate in a StepOnePlus real-time PCR machine (Applied Biosystems).

In this study two protocols were used for qPCR, a normal and a fast cycle (times in brackets). In order to activate the *Taq* polymerase, the reaction was first heated to

95°C for 10min (20sec). Then a step of 95°C for 15sec (1sec) followed by 60°C for 1min (20sec) was repeated 40 times.

Target	Assay ID
CCL2	Hs00234140_m1
CCL5	Hs00174575_m1
CXCL8	Hs00174103_m1
CXCL10	Hs00171042_m1
CD26	Hs00175210_m1
PADI2	Hs00247108_m1
PADI4	Hs00202612_m1
GAPDH	Hs02758991_g1

Table 3 List of primers used in real-time PCR
All are TaqMan® Gene Expression Assays from Applied Biosystems with FAM dye.

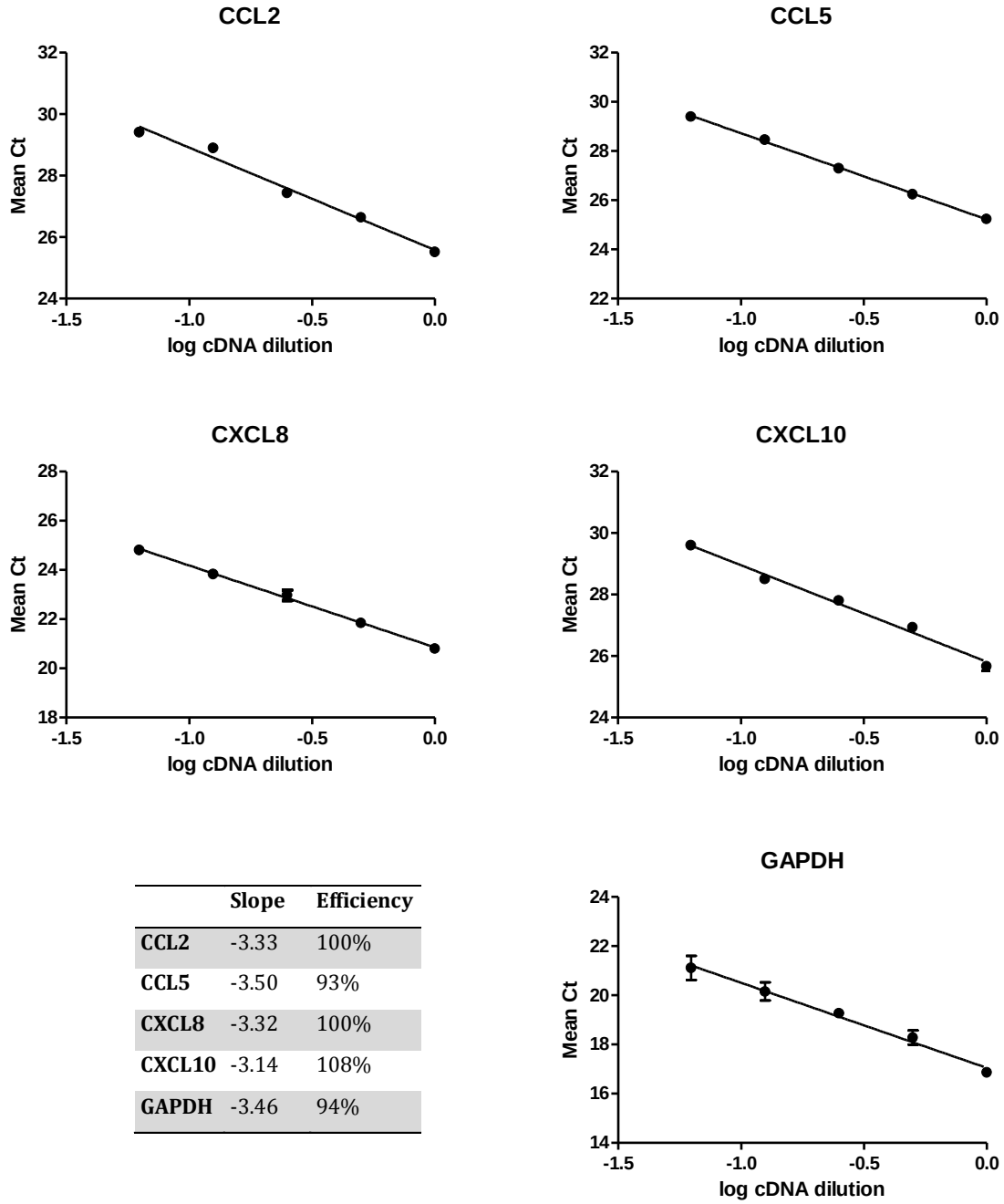
Prior to reactions primers were validated (see Figure 2-9). The house-keeping gene used was GAPDH (Que *et al.*, 1999). ΔΔCt value, error and significance were calculated as described by Yuan *et al.* (Yuan *et al.*, 2006) and shown in Figure 2-8.

$$\Delta Ct = Mean\ gene\ of\ interest\ Ct - mean\ housekeeping\ gene\ Ct$$

$$\Delta\Delta Ct = treated\ sample\ \Delta Ct - baseline\ sample\ \Delta Ct$$

$$Fold\ change = 2^{-\Delta\Delta Ct}$$

Figure 2-8 Equations for fold change calculation in real-time PCR



	Slope	Efficiency
CCL2	-3.33	100%
CCL5	-3.50	93%
CXCL8	-3.32	100%
CXCL10	-3.14	108%
GAPDH	-3.46	94%

Figure 2-9 Validation of TaqMan primers for realtime PCR.
A serial dilution of sample template cDNA gave increasing threshold cycle (Ct) values with decreasing cDNA levels. The table shows the gradient of each line and calculated reaction efficiency. Plotted is mean Ct ± SEM

2.5 PROTEIN DETERMINATION BY IMMUNOASSAY

Levels of chemokine were generally quantified using enzyme linked immunosorbent assay (ELISA).

2.5.1 *ENZYME-LINKED IMMUNOSORBENT ASSAY*

ELISA is a widely used technique to determine protein levels in a sample. It is an antibody based technique in which the final antibody is conjugated to an enzyme, usually horse radish peroxidase (HRP), and the final step involves adding the enzyme's substrate in a buffer, which will develop colour if the enzyme is present. This level of colour change can then be quantified by spectrophotometry.

Two forms of ELISA have been used in this study – sandwich and direct. In a sandwich ELISA an antibody against the protein of interest is coated on to a 96 well microtitre plate. The sample is then added, followed by a second antibody against the protein. This is then detected by an enzyme conjugated antibody and a colour change reaction. Direct ELISAs are detected in the same way but the protein of interest is directly bound to the plate. In both cases, quantification is achieved by comparison to a standard curve.

2.5.1.1 General ELISA protocol

For sandwich ELISAs, a 96-well plate (Immulon HBX or Costar) was coated with 100µl monoclonal antibody in PBS overnight at 4°C and then blocked with PBS containing protein (casein or BSA) for 1-2hr. This is to reduce non-specific binding. Plates were washed three times between every step with 150µl 0.05% Tween/PBS and antibodies diluted in block buffer. 100µl sample was then incubated for 1-2hr prior to incubation with a second protein specific antibody, normally polyclonal. If this second antibody is biotinylated it can be detected using streptavidin-HRP. If not a HRP conjugated antibody against the polyclonal can be used. The ELISA is then developed using one of two colour change reactions: tetramethylbenzidine (TMB) or o-phenylenediamine (OPD). TMB (Sigma) was dissolved in 250µl DMSO, added to 25ml TMB buffer (0.1M sodium acetate, pH adjusted to 4.9 with 0.1M citrate) and 3.7µl H_2O_2, the colour change reaction was then stopped with 50µl 1M H_2SO_4 and measured at 450nm. For OPD, 100µl OPD was added to 12.5ml citrate

buffer (0.1M citric acid, pH5) with 5µl H_2O_2. The reaction was again stopped with 50µl 1M H_2SO_4 but colour change measured at 490nm. Plates were read using an Opsys MR plate reader (DYNEX Technologies) or a Synergy plate reader (BioTek). For some assays wavelength correction at 595nm was also performed.

For direct ELISAs the detection protocol was the same but sample was bound to the plate in carbonate buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH9.6) overnight prior to blocking so a capture antibody is not required.

2.6 PROTEIN NITRATION

The basic nitration protocol was received from Prof. Antonella Viola, University of Milan, and further optimisation performed previously (Barker, 2011).

Chemokine nitration was achieved by adding peroxynitrite (Cayman Chemical) at a final concentration of 1mM, to 1µM chemokine in deionised water (dH_2O) and incubating at 37°C for up to 10min. Chemokine was freshly nitrated for each experiment and all chemokines purchased from Almac unless otherwise stated. Peptides were nitrated by the same method.

Peroxynitrite concentration was established according to the manufacturer's instructions. Stock peroxynitrite was diluted 40-fold with 0.3M NaOH and absorbance at 302nm measured using a Nanodrop. The concentration was then calculated using the peroxynitrite extinction coefficient ($1670M^{-1}cm^{-1}$).

2.7 PROTEIN LEVEL DETERMINATION

Protein concentration was determined using a Pierce BCA protein assay kit (Thermo Scientific) as per kit instructions, using the microplate procedure. The bicinchoninic acid (BCA) assay is a commonly used method which combines the biuret reaction with colourimetric detection using BCA. Proteins in an alkaline solution can reduce Cu^{2+} to Cu^{1+} and BCA chelation by Cu^{1+} results in a colour change from green to purple which can be measured at 562nm (nearest wavelength available on the plate reader used is 595nm).

Due to the oxidative potential of peroxynitrite (see Figure 2-10), for accurate quantification of samples containing peroxynitrite, dialysis was needed. Samples

were dialysed against 500ml PBS overnight using Slide-A-Lyzer MINI Dialyse Units, 2000 molecular weight cut-off (MWCO) (Thermo Scientific).

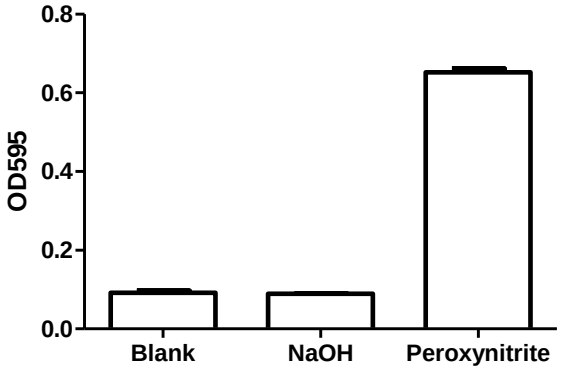


Figure 2-10 BCA assay showing peroxynitrite can give false positives
A BCA assay (Thermo Scientific) was performed using water, peroxynitrite, or NaOH (the peroxynitrite carrier) in the absence of protein to show that the oxidative potential of peroxynitrite can give false positives.

2.8 CHEMOTAXIS

Chemotaxis assays allow evaluation of the ability of chemokines to induce the chemotaxis of cells. In this study several forms of chemotaxis assay were used. Standard bare membrane assays determine the level of response to a chemokine, mainly dependent on chemokine-receptor interactions. As a more physiological assay, transendothelial chemotaxis was performed. For this chemokine-GAG interactions are also required to enable cells to migrate through the endothelial monolayer. To measure the number of cells recruited *in vivo*, a murine air-pouch model was used.

2.8.1 *IN VITRO*

In vitro chemotaxis assays were carried out using a transwell system as shown in Figure 2-11. Prior to chemotaxis, 24 well companion plates (BD Falcon) were blocked with 1ml 1% BSA/RPMI per well for 1hr to prevent chemokine binding and therefore lowering the concentration of available chemokine. FBS is not used as a blocking agent in chemotaxis as it contains bovine chemokines which may affect results (Struyf *et al.*, 2001; De Buck *et al.*, 2013). After this, 800µl chemokine in 1% BSA/RPMI was added to each well and a cell culture insert (BD Falcon) containing cells in 0.5ml 1% BSA/RPMI carefully lowered into each well. Wells

containing 1% BSA/RPMI only were performed as a negative control. The plate was then incubated at 37°C and cells counted. See Table 4 for individual conditions for each cell type.

Some cell types need fibronectin coated filters to encourage adherence to the filter for counting. For such assays the underside of filters was coated with 80µl 2.5µg/ml fibronectin (Sigma) for 30min. The excess fibronectin was then removed and filters allowed to dry for a further 30min.

Cell type	Filter size	Fibronectin	Cell number	Migration time	Counting method
PBMC	3µm	No	500 000	90min	Flow cytometry Filter staining
Neutrophil	3µm	No	500 000	90min	Haemocytometer
HEK-CCR2b	8µm	Yes	200 000	6hr	Filter staining
CHO-CCR5	8µm	Yes	100 000	5hr	Filter staining

Table 4 Chemotaxis conditions for different cell types
Cells require different conditions dependent on cell size and motility.

2.8.1.1 Transendothelial chemotaxis

In order for more physiological conditions to be created *in vitro*, transendothelial chemotaxis assays were performed. This was as above except 72hr prior to the assay, $5x10^4$ EA.hy-926 cells were cultured in 0.5ml complete RPMI-1640 in cell culture inserts. Media was only placed in the insert, not in the well underneath, to discourage cells from growing through the filter, forming a 'double' monolayer and making final cell counting more difficult.

2.8.1.1 Counting migrated cells

a) Filter staining

Cells which adhered to the lower side of the filter were manually counted by microscopy. The upper side of filters were gently wiped with a cotton bud to remove non-migrated cells and then filters were fixed in methanol at -20°C (1hr to overnight). After fixation filters are washed in dH_2O and stained using

haematoxylin (Sigma) for 10min. Tap water was then used to blue the stain before dehydration with 50%, 75%, 90% and 100% ethanol. After air drying, filters were mounted with DPX mountant (Sigma) and migrated cells counted (5 high power fields per filter), see Figure 2-11.

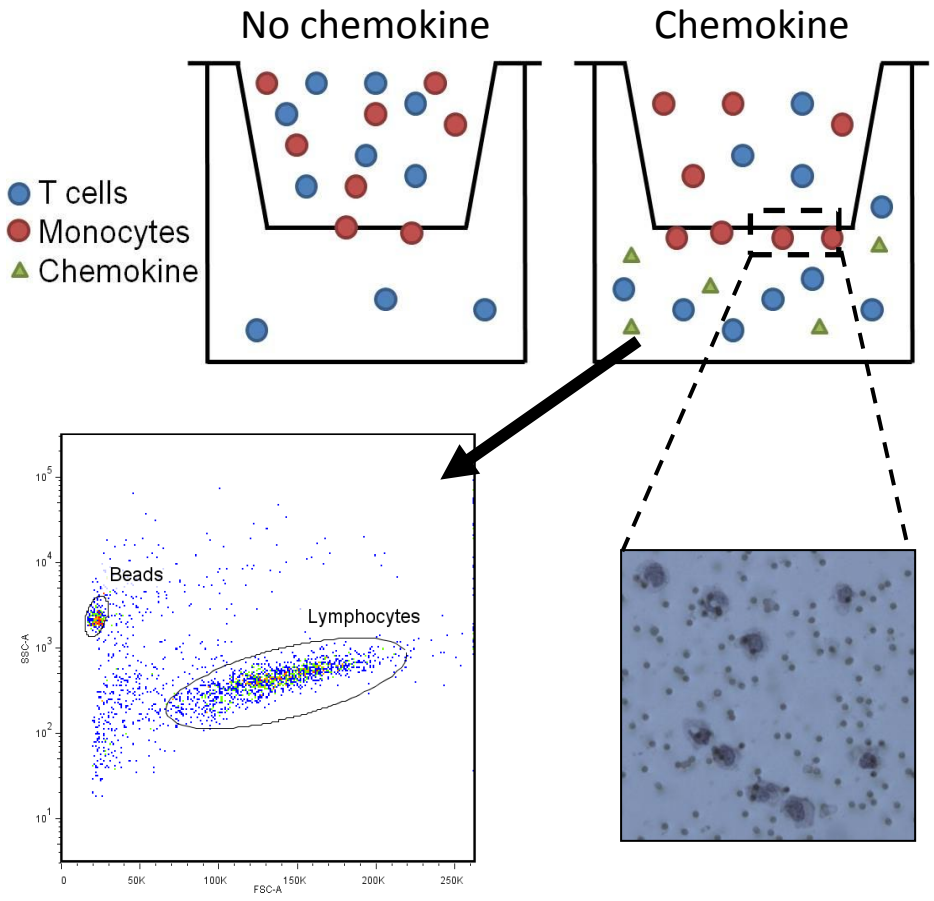


Figure 2-11 Schematic of a chemotaxis experiment.
Cells are placed in a filter above a well with or without chemokine. After incubation at 37°C for 90min, T cells have migrated to the lower well where they can be counted by flow cytometry as a ratio to a known number of counting beads. Migrated monocytes are adhered to the underside of the filter and are counted using a microscope.

b) Flow cytometry

Cells which fully migrated through the filter into the well below were counted using flow cytometry. Well contents were transferred into appropriate tubes (BD Falcon), centrifuged at 500xg for 5min, and cell pellet resuspended in 50µl 1% BSA/RPMI. A known number of, normally 2000, counting beads (UltraRainbow calibration particles, Spherotech Inc) was then added and flow cytometery used to determine the number of migrated cells as a ratio to beads, see Figure 2-11.

Neutrophils migrated into the lower chamber were counted by haemocytometer under my supervision by the BSc project students P Milligan and F Tak.

2.8.2 *IN VIVO*

In vivo function of chemokines was assayed using an air pouch model. The generation of air pouches was described previously by (Ali *et al.*, 2005b) and outlined in Figure 2-12, in full compliance with UK Home Office regulations for animal experimentation (licence PPL60/3786). Air pouches were created by injecting 3ml sterile air subcutaneously into the back of each animal under general anaesthetic (isofluorane). Pouches were then topped up with 1ml air injections on days 2, 4, and 5. This produced stable fluid-filled pouches (Figure 2-13). On day 6, each pouch was injected with 1ml chemoattractant or PBS negative control. After 24hr recruited cells were recovered by lavaging the pouch twice with 0.75ml 3mM EDTA/PBS. Cells were then analysed by flow cytometry and counted by haemocytometer.

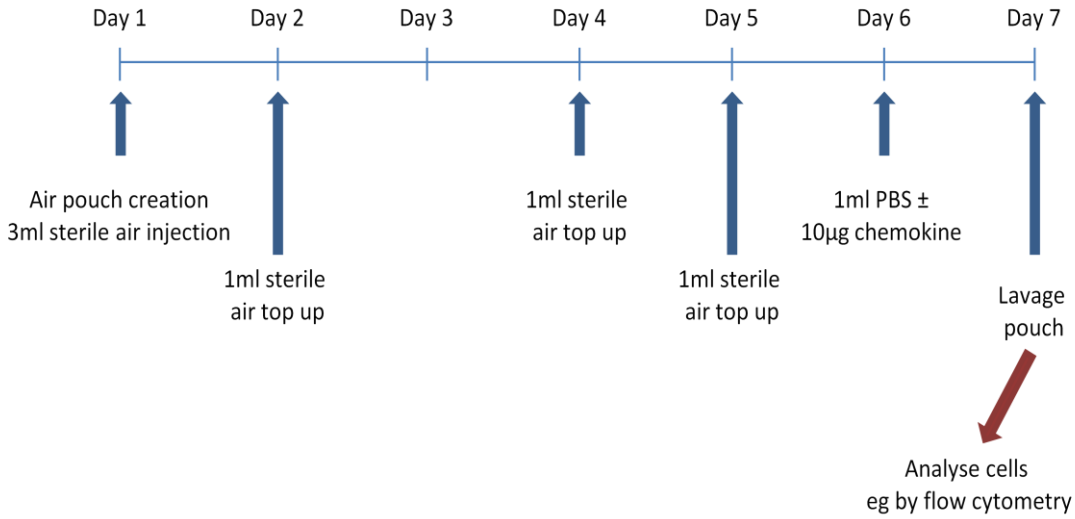


Figure 2-12 Schematic of the air pouch *in vivo* chemotaxis model

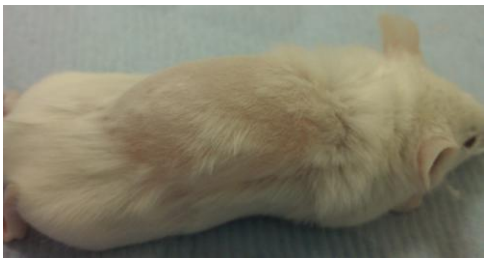


Figure 2-13 Air pouch
Shown is a murine air pouch. On day 7 the pouch was lavaged to recover migrated cells, for this the back was shaved to allow clear access to the pouch.

2.9 MASS SPECTROMETRY

Mass spectrometry (MS) measures the mass-to-charge ratio (m/z) of molecules, allowing calculation of the mass of the sample. Firstly the sample is ionised, in the simplest situation by adding or removing protons. In certain techniques for example electrospray ionisation (ESI), larger molecules, especially peptides and proteins can gain or lose numerous protons forming a variety of charged states. A protein with many lysine and arginine residues can easily gain extra protons, ranging from a single to tens of protons, and in positive mode mass spectrometry this adds one extra Dalton mass as well as charge, creating a large number of possible m/z values.

The cloud of ionised molecules in the mass spectrometer is separated by m/z. In a simple machine the cloud of charged molecules is deflected round a bend and hits a detector. Ions of the same mass-to-charge ratio will undergo the same amount of deflection and hit the detector at the same time. For example a molecule of 500 mass units with one proton attached and thus a +1 charge would have an m/z of 500 but a molecule of 1000 mass units with two protons attached would also have an m/z of 500. Similarly, a mass difference of 45Da would be seen as an m/z increase of 22.5 for +2 ions and 15 for 3+. For analysis of more complex spectra, deconvolution algorithms can be applied to establish the monoisotopic masses of the proteins or peptides in the sample.

ESI coupled to both MS and tandem MS (ESI-MS/MS) are the methods used in this study for the detection of post-translational modification. It has been used for establishing the mass of whole proteins, synthesised peptides and tryptic digests of proteins. The latter allows determination of which residues specifically are modified and not just the overall alteration in mass following post-translational modification. Specific details are given in the relevant chapters.

2.10 STATISTICAL ANALYSIS

Statistical analysis was carried out using GraphPad Prism 5.0. Unless otherwise stated, all graphed data depicts the mean value of three experiments, each performed in triplicate. ANOVA and Student's t-test (abbreviated to t-test throughout) are the statistical analyses used.

Error bars on graphs illustrate the standard error of the mean. Dotted lines on graphs represent the negative control. P values on graphs are denoted by asterisks as follows: *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$.

2.11 IMAGES

Chemical structures were drawn using ChemBioDraw Ultra 2012 (CambridgeSoft, PerkinElmer).

Three dimensional protein structure images were created using Jmol.

3 ALTERATION OF CHEMOKINE EXPRESSION DURING STRESS

3.1 INTRODUCTION

The immune system has evolved to defend against numerous types of infection and malignancy. As a result there is a consequent need to elicit different responses based on the pathological stimulus in question. For this reason different cell activating stimuli will result in different 'activated' phenotypes.

As discussed in section 1.3, chemokines have different and specific patterns of regulation. The chemokines involved in normal cell trafficking and tissue homeostasis, such as CXCL12, are constitutively expressed, whereas proinflammatory chemokines can be induced. Many of these chemokines are induced by cytokines including IL-1β or TNFα, and it was specific upregulation in response to IFNγ which lead to the discovery of the chemokines CXCL9 and CXCL10 (Luster *et al.*, 1985; Liao *et al.*, 1995). The expression of many proinflammatory chemokines involves the activation of numerous signalling pathways. These can be different for each stimulus, transcription factor and cell type, as well as the added complexity of cross talk between these pathways. This intricate system allows the fine tuning required for the signal and tissue specific biological responses needed.

These different chemokine responses can be seen *in vivo* by considering specific chemokine involvements in disease models (Segerer *et al.*, 2000). For example in models of kidney disease, CCL2 and CCL5 seem to be consistently involved whilst other chemokines play a role depending on the specific disease. Within the inflammatory environment certain chemokines can be produced by both the cells of the affected organ and infiltrating leukocytes, further complicating the picture. In the kidney CCL2 and CCL5 are generally thought to originate from the mesangial cells and infiltrating monocytes, whereas CX3CL1, another chemokine with some involvement in kidney disease, is predominantly bound to endothelial cells in the glomerulus (Anders *et al.*, 2001; Panzer *et al.*, 2006). In rats with cresentic glomerulonephritis, CCL2 is selectively expressed in the glomeruli and not the tubulointerstitium (Panzer *et al.*, 2001). The effects of these chemokines also differ. Early studies showed CCL2 and CCL5 neutralising antibodies decreased monocyte

influx in a model of cresentic glomerulonephritis but only CCL2 had a role in the development of interstitial fibrosis (Lloyd *et al.*, 1997). It is not just CC chemokines which have involvement in kidney diseases, CXCL9, but not CXCL10, is involved in the murine model, nephrotoxic serum nephritis (Menke *et al.*, 2008).

In the liver different chemokines have been found to be important, again highlighting both the different chemokines produced during inflammation and the differential chemokine expression within each organ. It is thought that CCR5 has a role in the recruitment of leukocytes to the portal tracts, and CXCR3 to the parenchyma as reviewed by Oo *et al.*, 2010. The sources of CXCR3 ligands in liver inflammation include hepatocytes, stellate cells, sinusoidal endothelial cells and infiltrating leukocytes. These ligands are increased on hepatic endothelium during chronic hepatitis, and levels correlate with disease outcome (Curbishley *et al.*, 2005). As found in kidney inflammation, CX3CL1 can be involved in human liver disease; expression increases in biliary epithelial cells during primary biliary cirrhosis (PBC) (Isse *et al.*, 2005). CC chemokines also have a role in liver inflammation. Plasma and hepatic levels of CCL2 correlate with alcoholic liver disease (ALD) severity, neutrophil infiltration and CXCL8 levels, however they do not correlate with steatosis (Degre *et al.*, 2012). Sections from another inflammatory liver disease have been examined in this chapter, primary sclerosing cholangitis (PSC). Both PSC and PBC have increased levels of CCL26 but CCL11 was only found to be increased in PSC (Landi *et al.*, 2014). The exact role of chemokines in inflammation is complex. In some mouse models of liver inflammation $CCR5^{+}$ lymphocyte infiltration is characteristic, however $CCR5^{-/-}$ mice are more susceptible to Con A-induced hepatitis showing that this receptor is not simply pro- or anti-inflammatory (Ajuebor *et al.*, 2005; Moreno *et al.*, 2005). Con A induces liver specific inflammation which is driven by hepatic expression of IFNγ and mediated by activated T cells. In this model CCL3 blockade reduces disease, by reducing the infiltration of $CCR1^{+}$ T cells (Ajuebor *et al.*, 2004).

During transplantation and inflammation the production of reactive species, both ROS and RNS, is a major source of stress as discussed in sections 1.2 and 1.4.1. These are produced following organ reperfusion, by mitochondria and oxidases in epithelial and endothelial cells, and by infiltrating leukocytes capable of oxidative

burst. As well as causing tissue damage, oxidative stress can also stimulate proinflammatory factors including chemokines, and is a major factor in their production in the situations discussed above.

So far in this chapter, only transcriptional regulation of chemokines during inflammation has been described. However this is only one method for chemokine regulation during inflammation, as discussed earlier in sections 1.3.1 and 1.6. Post-translational modifications are an important mechanism for modifying chemokine function, potentially causing abrogation, potentiation or alteration of function. In some cases these modifications can have wider effects, for example antagonists can be formed, decreasing the activity of wild type chemokines also present (Proost *et al.*, 1998b). If there is loss of GAG binding function with unaffected receptor activating potential, an anti-inflammatory agonist could be formed (O'Boyle *et al.*, 2009), the biological implications of which are discussed later in this chapter.

A key question is whether chemokine modification is cell intrinsic or extrinsic; can cells modify the chemokines they produce or are other modifiers needed? Data in this chapter shows that, at least in some circumstances, cells are capable of intrinsic modification. All of this complex regulation feeds together to allow the tight control of inflammation integral to the delicate control of leukocyte behaviour.

3.1.1 *SPECIFIC AIMS*

- Establish how chemokine gene expression and protein production alter in response to stress
- Determine whether these chemokines are functional and if there is potential for alteration of function by post-translational modification during such stress
- Demonstrate the presence of chemokine modification in an inflammatory environment

3.2 SPECIFIC MATERIALS AND METHODS

3.2.1 *CELL TREATMENT*

H69 and HKC8 cells were grown in 6 well plates (Greiner Bio One) or T25 tissue culture flasks (Greiner Bio One) for cell treatment for RNA isolation or collection of media for analysis of chemokine production. Treatments for qPCR analysis were as follows: 1µg/ml LPS-EB (*E. coli 0111:B4*; Invivogen), 6hr; 12.5-200ng/ml PMA, 6hr; 200µM hydrogen peroxide (Sigma), 6hr with media changed after 2hr; 600µM peroxynitrite (Cayman Chemicals), 6hr (all previously optimised Barker, 2011 or Brain, 2010). For serum starvation cells were grown in their normal media and then changed to serum-free media overnight prior to treatment. For cell treatment for ELISA analysis cells were treated for 24 or 48hr and a range of concentrations for each treatment used.

For creation of a poly-chemokine stimulus for chemotaxis assays (section 3.2.3), H69 cells were treated with 1µg/ml LPS-EB for 6hr. Media was then removed, cells washed with PBS and new media added. Media was collected 18hr later and used in assays.

For the purification of chemokine from cell treated media, 6 well plates were used to produce media for the ELISAs to determine the preferred cell line and treatment. For purification, 2 litre of HKC8 treated media was produced in T125 flasks (Greiner Bio One).

3.2.2 *PROTEIN DETERMINATION BY IMMUNOASSAY*

3.2.2.1 ELISAs

For the measurement of chemokine levels following cell stress various ELISAs were performed, all using the basic protocol previously outlined (section 2.5.1.1). CCL5 and CXCL8 were detected using Duosets (R&D Systems) according to the manufacturer's protocol and for CCL2 an ELISA kit from Peprotech was used. Figure 3-1 shows the standard curves from these ELISAs used for quantification. Samples were assayed at 2 different concentrations, neat and 1/10 diluted in block buffer. CXCL12 levels were also determined using a Duoset (R&D Systems).

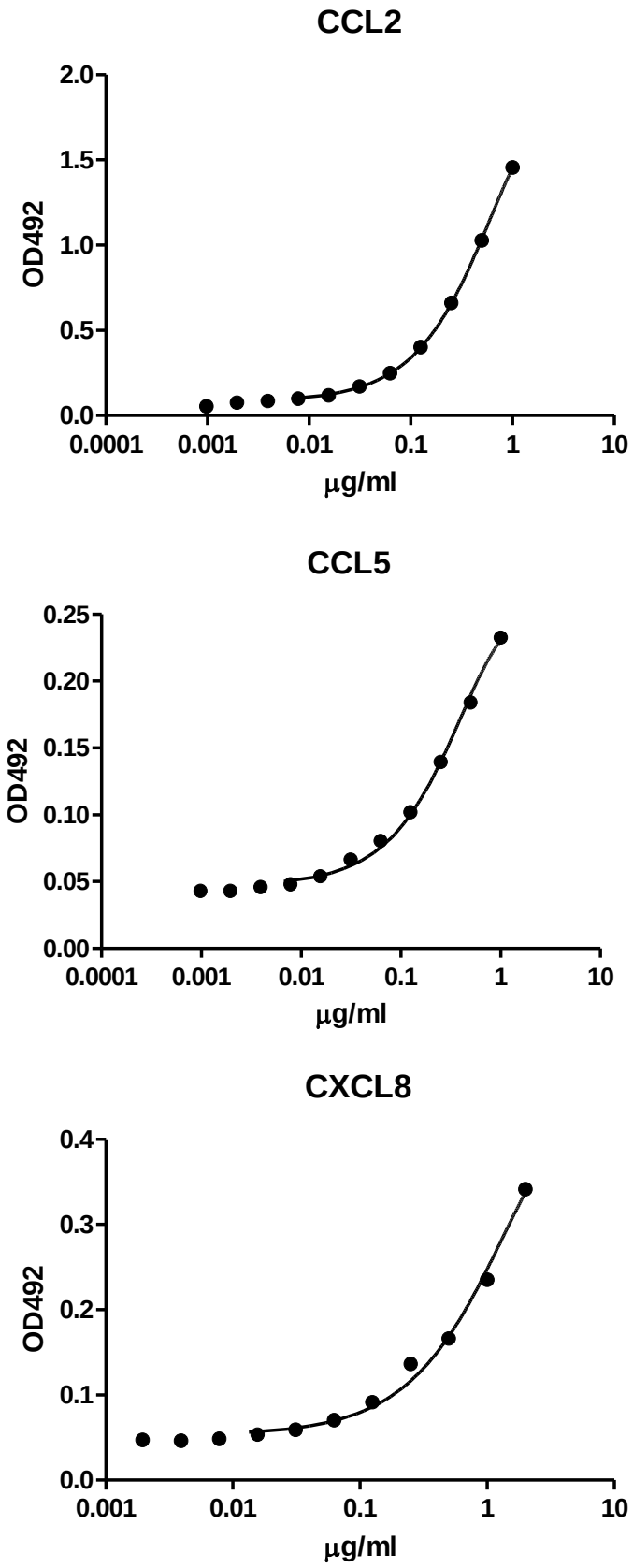


Figure 3-1 Elisa standard curves
Example standard curves from chemokine ELISAs, performed in duplicate. CCL2 ELISA kit (Peprotech), CCL5 and CXCL8 Duoset (R&D Systems)

3.2.2.2 MesoScale Discovery

Determination of the levels of CCL2, CCL4, CCL5, CCL11, CCL13, CCL17, CXCL8 and CXCL10 for chemotaxis assays was achieved using MSD® kits (Mesoscale Discovery, MD) as per the manufacturer's instructions. A very similar method to an ELISA, Instead of the enzymatic reaction in ELISAs, the detection antibody in an MSD is coupled to an electrochemoluminescent label ('sulfo-tag') which emits light at a 620nm when an electric charge is applied to the carbon electrode in each well. This light is detected by a charge-coupled device (CCD) camera within the MSD Sector analyser and then protein concentrations calculated from the standard curve using MSD Workbench software (MesoScale).

3.2.3 *CHEMOTAXIS*

3.2.3.1 *In vitro* chemotaxis

Transendothelial chemotaxis was performed as described in section 2.8.1.1. T cell migration in response to chemokines produced by LPS stimulated H69 was assayed. 800µl H69 conditioned media per well and 100nM PS372424 (Ligand Pharmaceuticals), a small molecule CXCR3 agonist (Stroke *et al.*, 2006; Nedjai *et al.*, 2012), or 2µg/ml CXCR3 neutralizing antibody (clone 49801, R&D Systems) was added to appropriate filters immediately prior to lowering into well. The assay was incubated for 90min and migrated cells counted by flow cytometry (section 2.8.1.1).

3.2.3.2 *In vivo* air pouch

Air pouch was created as in section 2.8.2 but the mice used were humanised as described by O'Boyle *et al.* 2012. Female NOD.Cg-*Prkdc*^{scid} *Il2rgtm1*$^{Wjl/SzJ}$ mice (Charles River; 8 week old) were given 10^7 human PBMC in 0.5ml PBS by intraperitoneal injection. After 21 days air pouches were generated and six days after creation of air pouches, each pouch was injected with 1ml LPS treated H69 media (see section 3.2.1). Animals received intravenously 0.1ml vehicle control, a CXCR3 agonist PS372424 (1µM in blood), or CXCR3 neutralizing antibody (25µg). The pouch was lavaged and human cells counted by flow cytometry. The *in vivo* model was established by Dr G O'Boyle and Mr C Fox, Newcastle University.

3.2.3.3 Determining level of LPS

Quantifying the level of LPS in media used in the *in vivo* chemotaxis assay was done using HEK-Blue™-hTLR4 cells (InvivoGen) as per the manufacturer's instructions and was performed by Dr Jeremy Palmer, Newcastle University. HEK-Blue™-hTLR4 cells are a stably transfected HEK-293 cell line which expresses the LPS receptor complex TLR4, MD-2 and CD14. A secreted embryonic alkaline phosphatase (SEAP) reporter gene was co-transfected and placed under the control of an NFκB promoter. Stimulation with LPS activates NFκB inducing SEAP production and catalysis of a colour change reaction in the detection medium. This colour change is then quantified against a standard curve constructed using *E. coli* K12 LPS (Invivogen) by measuring absorbance at 620nm.

3.2.4 *CHEMOKINE PURIFICATION*

Natural chemokine was purified from HKC8 conditioned media. This was achieved using three types of chromatography for increasing purity, with ELISAs and silver stain SDS-PAGE performed between each purification step to identify positive fractions and determine purity.

All purification work, with the exception of production of the original sample, was carried out in the Laboratory of Molecular Immunology, Rega Institute, Katholieke Universiteit, Leuven, Belgium with the help of Prof. Paul Proost and technicians Noëmie Pörtner and Lien Leutenez. NanoLC-MS was performed at the Gasthuisberg campus, Katholieke Universiteit, Leuven.

3.2.4.1 Sample preparation

Two litres of conditioned media from HKC8 cells which had been serum starved and treated with 5μM peroxynitrite for 48hr was produced.

Prior to purification, the sample was centrifuged and 0.45μm filtered under vacuum to remove particulate. It was then diluted 1 in 3 with 50mM Tris to lower the salt concentration of the sample to approximately that of the loading buffer, ensuring protein binding to the column.

3.2.4.2 Affinity Chromatography

Affinity chromatography separates proteins based on reversible interactions between the target proteins and a specific ligand attached to the chromatography matrix, in this case heparin. Unbound protein is then washed out and conditions changed to elute the proteins of interest which are bound to the column. For heparin the ionic strength is increased to disrupt the charge interaction between heparin and chemokines.

Sample was loaded at 3ml/min on to a 20ml heparin-sepharose column (GE Healthcare) prior to eluting using a linear gradient (50mM-2M NaCl). 5-10ml fractions were collected; all steps were carried out at 4°C using an AKTA purifier (GE Healthcare). Eluent used: 50mM Tris, 50mM NaCl, pH7.4, protein eluted using: 50mM Tris, 2M NaCl, pH7.4.

3.2.4.3 Ion Exchange Chromatography

Ion-exchange chromatography (IEX) separates proteins by differences in surface charge: positively charged chemokines reversibly bind the negatively charged cation exchanger, methyl sulfonate (Mono S). As pH alters the surface charge of proteins, IEX is performed at a pH significantly below the pI to ensure protein binding to Mono S.

Following affinity chromatography fractions were still contaminated with many proteins and so IEX was performed to further purify samples. Fractions positive for the chemokine of interest were pooled and dialysed against 2l 50mM formic acid, pH4, 4°C with agitation in 3500 MWCO dialysis tubing (Spectrum). This was for both desalting and buffer exchange. Dialysis is needed as fractions are eluted from heparin-sepharose by increasing ionic strength, but for IEX samples need to be loaded at low ionic strength. The pI of many chemokines is around 9, so samples need to be at pH4, significantly below the pI to ensure binding to Mono S.

Dialysed sample was 0.45µm filtered before loading in 50mM formic acid, pH4 onto a 1ml Mono S column (5/5-GL; GE Healthcare). Samples were then eluted using a 1hr linear gradient increasing to 1M NaCl in 50mM formic acid. 1ml

fractions were collected and all steps were carried out at 4°C using an AKTA purifier (GE Healthcare).

3.2.4.4 Reversed Phase - High Performance Liquid Chromatography

As a final purification step a third chromatography method was used. Positive fractions from IEX were purified by reversed phase – high performance liquid chromatography (RP-HPLC). RP-HPLC separates proteins by hydrophobicity, proteins stick to the column in a highly aqueous mobile phase and are eluted using a highly organic mobile phase, in this case the organic solvent acetonitrile. The stationary phase of RP-HPLC columns is made of hydrophobic alkyl chains. The chain length used in this study was C8 as it is suitable for smaller proteins and peptides.

Positive fractions following IEX were pooled and injected on to a 2.1x220mm Brownlee C8 Aquapore RP-300 column (PerkinElmer) in 0.1% TFA and run using an Aglient 1100 HPLC system. Samples were then eluted at 0.4ml/min using a three stage acetonitrile gradient: started in 0.1% TFA, increasing to 20% acetonitrile after 10min, then a flatter gradient, to increase chemokine separation, increasing to 40% acetonitrile after 85min. Column effluent was collected in 400µl fractions.

3.2.4.5 Fraction Identification

a) Gel Electrophoresis

To visualise protein purity following chromatography, fractions were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and silver stained. SDS-PAGE is a technique which uses an electrical current to drive proteins though a gel with the rate of movement being influenced by the size, shape and charge of the molecule. SDS is an anionic detergent which in combination with reducing agents such as β-mercaptoethanol can denature proteins, forming linear structures with a negative charge proportional to length. This allows proteins to be separated by size and an estimation of molecular weight made by comparison to a protein ladder.

Large gels were made as shown in Table 5 using the Biorad Protean system. Running and spacer gels were poured one above the other and left to set, with the spacer covered with a layer of butanol to prevent oxidation. Butanol was then washed off, the stacking gel poured and left to set with a 25 well comb.

20µl of each fraction were heated in sample buffer at 95°C for 5min to denature proteins and add a negative charge. Sample buffer was at a final concentration of: 2% SDS, 6% glycerol, 1% β-mercaptoethanol, 0.005% brilliant blue G, 25mM Tris HCl pH6.8. Gels were run at 96V for 16hr with anode buffer (0.2M Tris base, pH8.9) and cathode buffer (20mM Tris base, 20mM tricine, 0.7mM SDS, pH8.2).

b) Silver staining

Gels were fixed for 1hr in 10% ethanol, 2.5% acetic acid, 1.25% sulfosalicylic acid and washed 3 times in 20% ethanol prior to incubation with ammonium hydroxide solution (163mM NH_4OH, 14.7mM NaOH, 20% ethanol, 10% $AgNO_3$, in degassed dH_2O) for 1hr. Gels were washed in dH_2O and 20% ethanol and developed for 5-10min in 20% ethanol, 0.01% citric acid, 0.037% formaldehyde. When the bands had developed to the required density, gels were placed in stop solution (20% ethanol, 0.5% acetic acid) for 1min and washed in dH_2O for 30mins. Gels were then shrunk using 50% ethanol overnight for imaging and storage at 4°C.

	Running gel	**Spacer gel**	**Stacking gel**
Acrylamide	9.75ml (5%)	7.50 ml (3.3%)	1.25 ml (5%)
Gel buffer (3M Tris base, 10mM SDS pH8.45)	10.00ml	10.00ml	2.50ml
Glycerol	3.20ml	-	-
dH_2O	7.05ml	12.50ml	6.25ml
10% APS	200µl	200µl	100µl
TEMED*	20µl	20µl	10µl

Table 5 Components for large SDS-PAGE gels
* TEMED - Tetramethylethylenediamine

c) ELISA

ELISA were performed on fractions to determine which fractions contained the chemokines of interest. These were performed as described in section 2.5.1.1 with some modifications. The block buffer used was 0.1% casein/0.05% Tween/PBS, all subsequent incubation steps were carried out at 37°C. The final development was using TMB. Antibodies used are shown in Table 6.

	Coating antibody	**Secondary antibody**	**Detection**
CCL2	Mouse anti-hCCL2 (R&D clone 23007) 1/300	Biotinylated goat anti-hCCL2 (R&D #279) 1/1000	Streptavidin-HRP (R&D)
CCL5	Mouse anti-hCCL5 (R&D Duoset)	Biotinylated goat anti-CCL5 (R&D Duoset)	Streptavidin-HRP (R&D)
CXCL8	Polyclonal anti-hCXCL8 (Rega#4576) 1/300	Mouse anti-hCXCL8 1/2000	Goat anti-mouse HRP 1/2500

Table 6 Antibodies used in ELISAs for fraction identification

d) Mass spectrometry

HPLC fractions containing the highest concentrations of chemokine were analysed by nanoLC-MS using a Thermo Scientific Q Exactive mass spectrometer. Due to low chemokine concentration the nano column was needed to concentrate the sample sufficiently for identification.

3.2.1 *TISSUE STAINING*

3.2.1.1 Tissue Sections

Formalin fixed paraffin embedded (FFPE) 4μm tissue sections were used for immunohistochemistry (IHC). Biopsies from human liver with a range of inflammatory conditions, or time-zero biopsies from liver transplants post reperfusion were stained. Blocks from a mouse model of kidney ischaemia-reperfusion injury were kindly donated by Prof N. Sheerin's group, Newcastle University.

Ethical approval was obtained from a local regional ethics committee concerning the use of patient samples. Samples represented excess liver tissue that had been taken for diagnostic purposes; diagnosis and staging of disease in each sample was identified by means of a number allocated by the Cellular Pathology department. No patient identifiable information was used during the project. Approval reference REC 06/Q0905/150 Amendment 1; Date of amendment 09/01/2007 By the Newcastle and North Tyneside Local Research Ethics Committee 1.

The kidney IRI animal work was carried out under Home Office license PPL60/3786. Tissue from a murine ischaemia-reperfusion injury model (De Vries *et al.*, 2003) was used for IHC. Briefly, the left kidney was clamped for up to 45min and then both kidneys harvested 24hr post reperfusion, the right being used as the contralateral control. Surgical work was performed by Mr Christopher Fox, Newcastle Comparative Biology Centre.

3.2.1.2 Immunohistochemical Staining

Sections were dewaxed in xylene for 10min and rehydrated through 100%, 95%, 70% ethanol to running water. Endogenous peroxidase activity was then blocked using 3ml H_2O_2 in 200ml methanol for 10min and sections washed in water followed by TBS (pH7.6; Tris-buffered saline). This is crucial as antigen detection is by a peroxidase based methods and so endogenous enzyme may lead to non-specific background staining. The formalin used to fix tissue sections can cause protein crosslinking and prevent the epitope being accessed by antibodies, for this reason antigen retrieval is needed. This was carried out by pressure cooking slides in citrate buffer (pH6) for 1min at pressure then cooled rapidly in running water and washed in TBS. Biotin block (Vector Laboratories) was then performed according to the manufacturer's instructions. This is especially important when staining liver tissue which has a high level of endogenous biotin. If this is not blocked then high background may occur as biotinylated antibodies are used in detection. Sections were then blocked for 1hr with 20% normal swine serum, incubated with primary antibody, biotinylated secondary antibody and then developed with TBS washes between each step. Vectastain ABC(Px) kit (Vector Laboratories) was then used as per manufacturer's instructions prior to development with DAB (2.5ml DAB, 2µl H_2O_2) for 1min. Sections were finally

counterstained in haematoxylin Sigma), blued in Scott's tap water and dehydrated through 70% to 99% ethanol, then into xylene before mounting using DPX.

For 3-nitrotyrosine staining the primary antibody used was rabbit anti-nitrotyrosine polyclonal (1/500, 1hr; Millipore, 06-284) as previously optimised (Barker, 2011). For CCL2 the antibody used was rabbit anti-CCL2 polyclonal (1/500, 1hr; Abcam, ab9669), previously optimised by Mrs B Innes. No primary antibody controls were also performed.

3.2.1.3 Haematoxylin and eosin staining

Sections were dewaxed and rehydrated as described in section 3.2.1.2. Slides were then stained with haematoxylin (Sigma) for 1min, rinsed, blued in Scott's tap water for 30sec, rinsed and finally stained with eosin (1min; Sigma). Slides were then dehydrated and mounted as described in section 3.2.1.2. H&E staining was performed by Mrs B Innes.

3.3 RESULTS

3.3.1 *ALTERATION IN CHEMOKINE TRANSCRIPTION BY STRESSED EPITHELIUM*

To establish the different responses to stress of liver and kidney epithelium, appropriate cell lines were stressed with peroxynitrite, hydrogen peroxide, PMA or LPS, and the chemokines produced measured. Before measuring chemokine levels, cell viability following stress was assessed and none of the treatments were found to be cytotoxic (Figure 3-2).

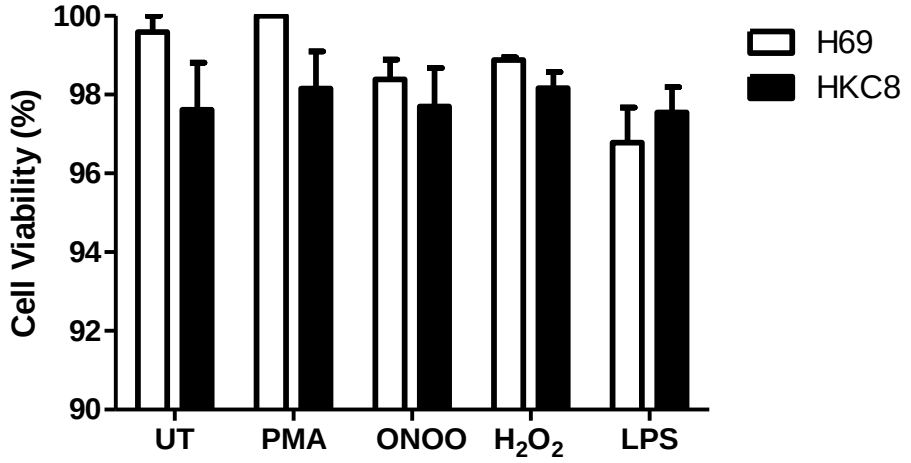


Figure 3-2 Cell viability following stress
H69 and HKC8 cells were untreated (UT) or treated with 600μM peroxynitrite (ONOO), 200μM H_2O_2, 1μg/ml LPS or 20ng/ml PMA for 6hr, as for subsequent real-time PCR analysis. Cell viability was then determined using Trypan Blue. n=3

TaqMan qPCR was used to examine transcriptional changes in the inflammatory and senescence associated chemokines CCL2, CCL5, CXCL8 and CXCL10 following cell stress. Results were normalised to GAPDH and fold increase calculated by ddCt, compared to untreated cells. Figure 3-3 shows that PMA, hydrogen peroxide and LPS all increase CCL2 transcription in H69, hydrogen peroxide giving the greatest mean fold increase of 8. Only PMA however significantly increases CCL2 levels in the kidney epithelium. The kidney cannot be stimulated to produce increased CCL5 under any of the conditions examined and the liver cells only produce increased CCL5 in response to LPS. The CXC chemokines CXCL8 and CXCL10 showed a greater potential for induction in response to stress (Figure 3-4) with PMA stimulating a 412 fold increase in CXCL8 mRNA levels in HKC8 cells and

39 fold in H69. CXCL10 had more specific induction, only being upregulated by LPS in H69 and PMA in HKC8.

The effect of serum starvation, another form of cellular stress, on chemokine induction was also assessed. Figure 3-5 and Figure 3-6 show serum starvation stimulates chemokine production, with the largest effect being on CXCL10 mRNA levels in kidney cells. The combined stresses of PMA and serum starvation were also studied as shown in Figure 3-7 and Figure 3-8. The effect of PMA on chemokine production is far greater on serum starved cells than untreated (Figure 3-3 and Figure 3-4), giving a greater fold increase compared to untreated and the starting levels of chemokine production is also higher than cells in sera.

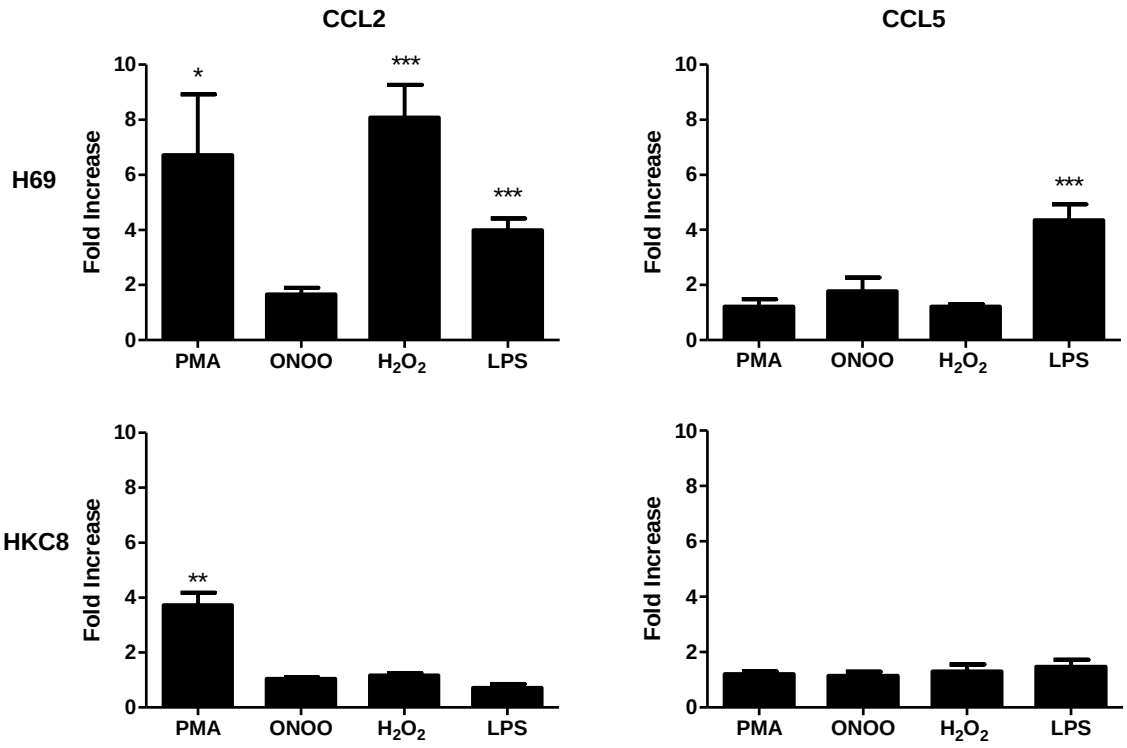


Figure 3-3 Induction of CCL2 and CCL5 in H69 and HKC8 cells following stress
Cells were treated with 600μM peroxynitrite (ONOO), 200μM H_2O_2, 1μg/ml LPS or 20ng/ml PMA for 6hr. Following RNA isolation using TRI-Reagent and cDNA synthesis, TaqMan qPCR was performed. Fold increase was calculated using ddCt relative to untreated cells, normalised to GAPDH. Shown is the mean fold change of four experiments, each performed in triplicate. Statistics denote a larger than 3 fold change which is significantly different to 1 (t-test).

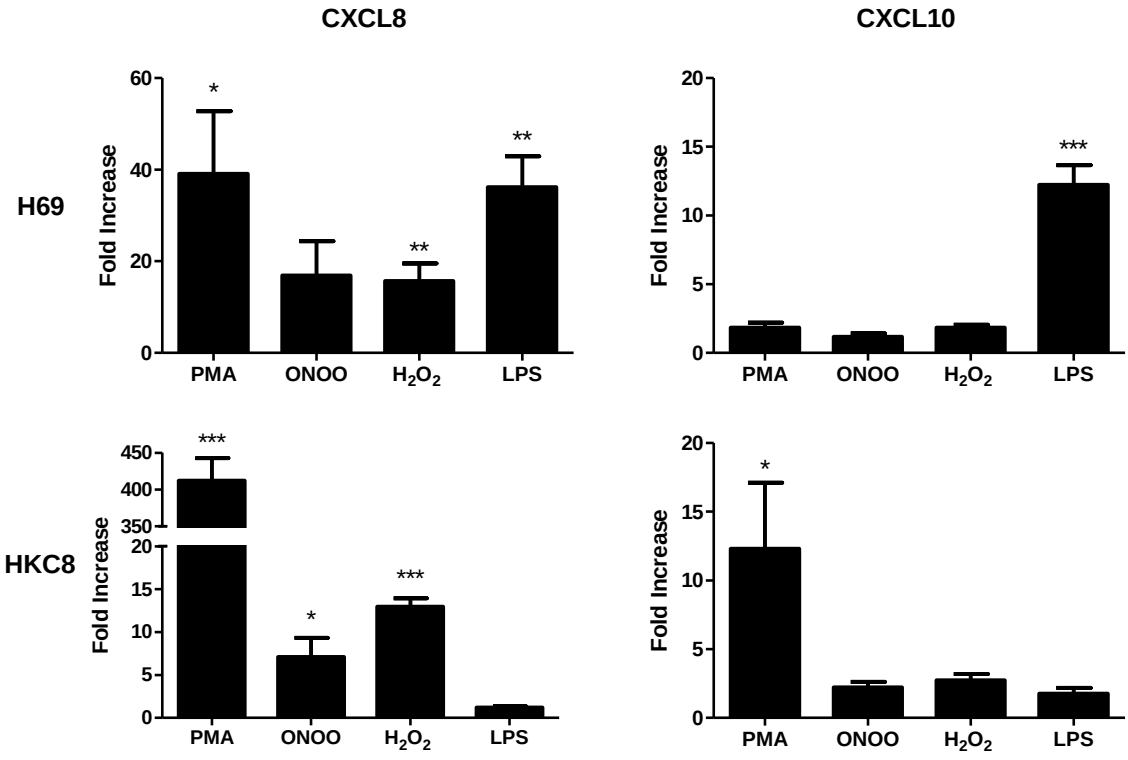


Figure 3-4 Induction of CXCL8 and CXCL10 in H69 and HKC8 cells following stress
Cells were treated with 600μM peroxynitrite (ONOO), 200μM H_2O_2, 1μg/ml LPS or 20ng/ml PMA for 6hr. Following RNA isolation using TRI-Reagent and cDNA synthesis, TaqMan qPCR was performed. Fold increase was calculated using ddCt relative to untreated cells, normalised to GAPDH. Shown is the mean fold change of four experiments, each performed in triplicate. Statistics denote a larger than 3 fold change which is significantly different to 1 (one sample t-test).

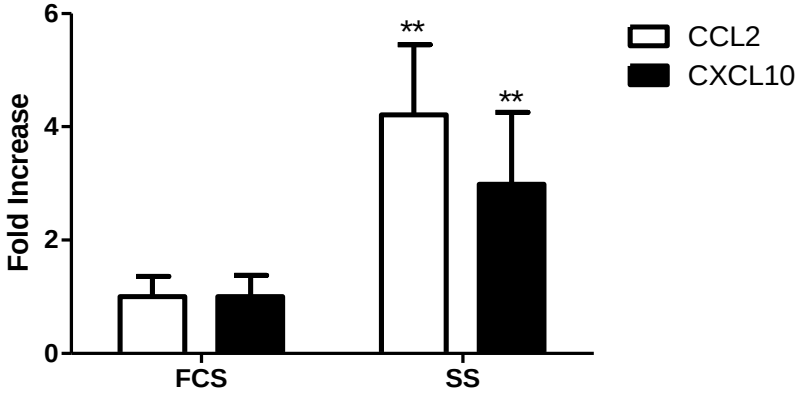


Figure 3-5 Induction of CCL2 and CXCL10 in H69 cells following serum starvation
Cells were subject to serum starvation overnight. TaqMan qPCR was performed and fold increase calculated using ddCt relative to cells grown in serum, normalised to GAPDH. FBS-cells grown in 10% serum, SS-serum starved cells. Results are from a representative experiment of three experiments, each performed in triplicate.

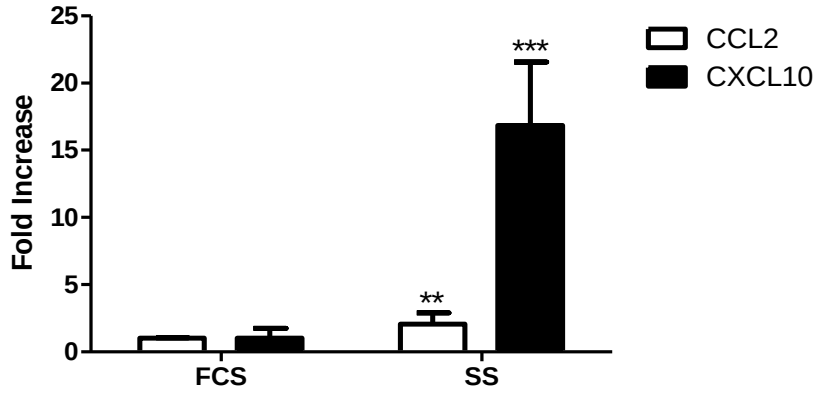


Figure 3-6 Induction of CCL2 and CXCL10 in HKC8 cells following serum starvation
Cells were subject to serum starvation overnight. TaqMan qPCR was performed and fold increase calculated using ddCt relative to cells grown in serum, normalised to GAPDH. FBS-cells grown in 10% serum, SS-serum starved cells. Results are from a representative experiment of three experiments, each performed in triplicate.

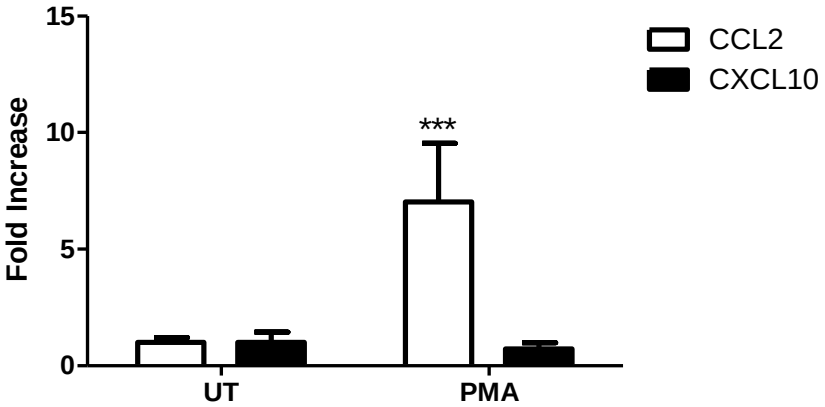


Figure 3-7 Induction of CCL2 and CXCL10 in serum starved H69 cells following PMA treatment
Cells were treated with 20ng/ml PMA for 6hr following overnight serum starvation. TaqMan qPCR was performed and fold increase calculated using ddCt relative to untreated cells (UT), normalised to GAPDH. Results are from a representative experiment of three experiments, each performed in triplicate.

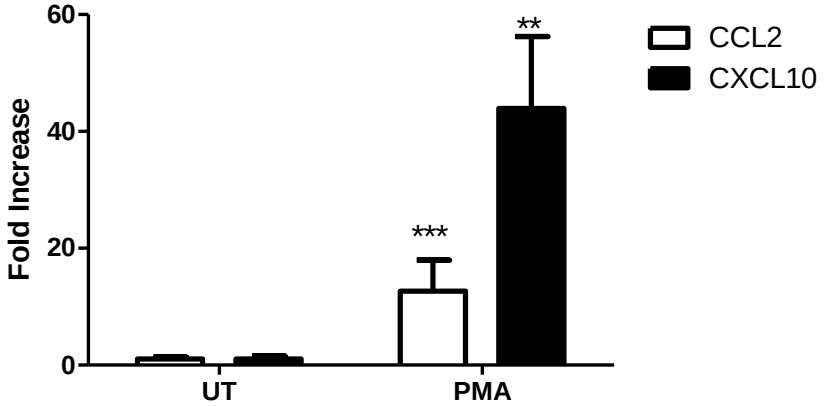


Figure 3-8 Induction of CCL2 and CXCL10 in serum starved HKC8 cells following PMA treatment
Cells were treated with 20ng/ml PMA for 6hr following overnight serum starvation. TaqMan qPCR was performed and fold increase calculated using ddCt relative to untreated cells (UT), normalised to GAPDH. Results are from a representative experiment of three experiments, each performed in triplicate.

3.3.2 *CHEMOKINES PRODUCED ARE FUNCTIONAL*

The qPCR data shows that stressed epithelium is able to upregulate transcription but to establish any potential physiological consequences the function of the chemokines needs to be determined. LPS treated H69 cells were chosen as the example treatment for this study as it was the only treatment which increased the transcription of all four chemokines evaluated in section 3.3.1. Cells were treated with LPS for 6hr, washed, fresh media added, and cells incubated for a further 18 hours. This short LPS treatment was necessary as the aim was to produce a poly-chemokine mix without high levels of LPS. Residual LPS could potentially be a confounding variable especially in subsequent *in vivo* assays. HEK-Blue-hTLR4 cells were used to determine LPS levels and Figure 3-9 shows very low residual levels in the 'LPS wash' treated media.

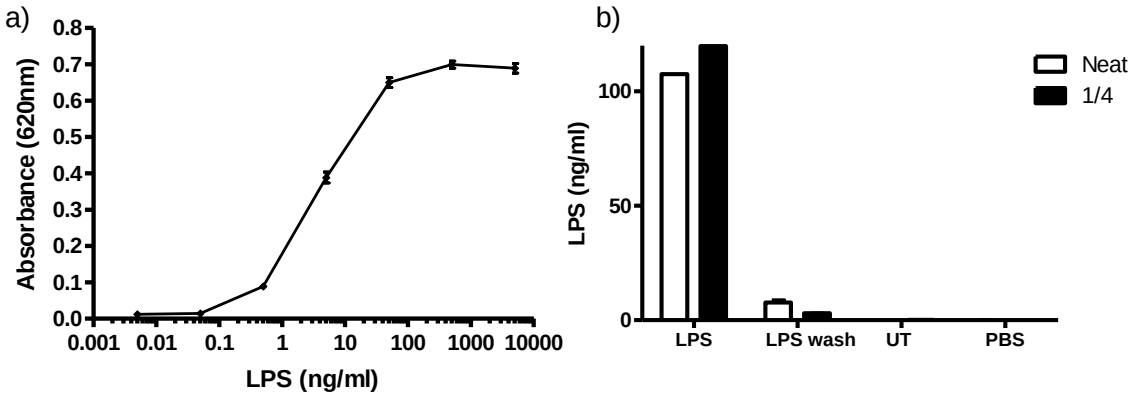


Figure 3-9 Quantification of LPS in H69 supernatant using HEK-Blue-hTLR4 cells
a) Standard curve constructed using *E. coli* K12 LPS. In the presence of LPS, secreted embryonic alkaline phosphatase (SEAP) causes a colour change reaction which can be detected by measuring absorbance at 620nm
b) Concentration of LPS in samples. Samples were used neat and diluted 1 in 4. LPS – H69 cells treated with 1µg/ml LPS for 24hr, LPS wash – cells treated with LPS for 6hr, media changed and 18hr later media was collected, UT – media collected from untreated H69 cells.

To determine the chemokine concentration present in the media, ELISA and MSD were used (Figure 3-10). Serum starved cells were also tested in this assay. The data shows a range of chemokines are present in the LPS treated media. Both *in vitro* and *in vivo* chemotaxis assays were then performed to establish if these chemokines have chemotactic potential. Transendothelial chemotaxis was used to

assess the ability of T cells to migrate in response to the chemokines produced by H69 cells (Figure 3-11). The migration of these cells could be blocked using the small molecule CXCR3 agonist PS372424 but not by a CXCR3 blocking antibody. Supporting results were found in an *in vivo* air-pouch model (Figure 3-12).

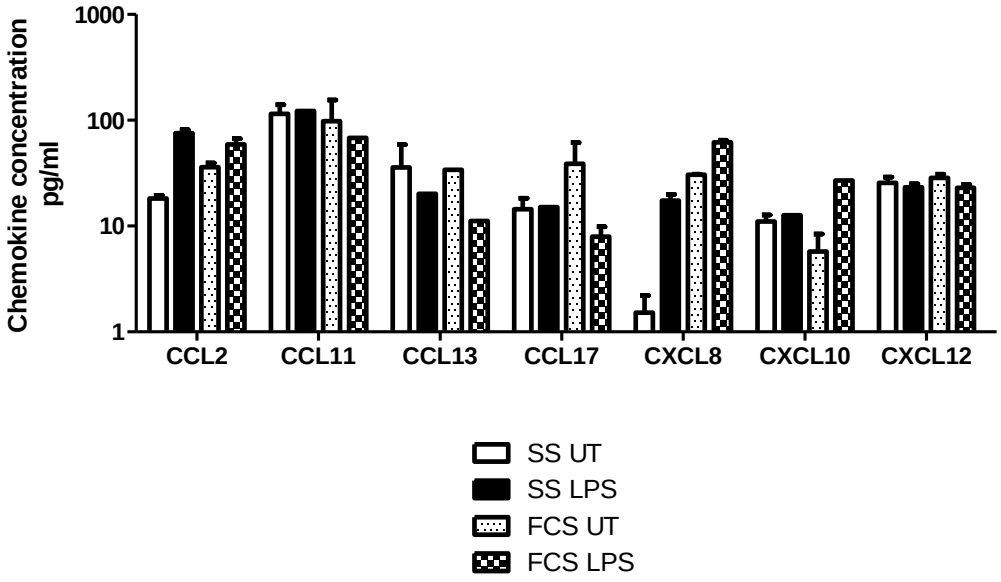


Figure 3-10 Chemokine production following LPS treatment of H69 cells
Cells were treated with 1µg/ml LPS for 6hr, media changed and 18hr later media collected. ELISA or MSD was used to quantify chemokine protein levels in this media. SS-cells were serum starved overnight, FBS-cells were grown in serum-containing media, UT-untreated cells. n=1

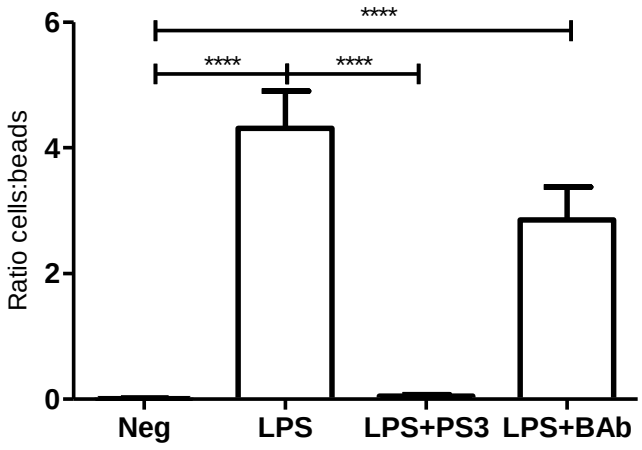


Figure 3-11 LPS treated H69 supernatant is chemotactic *in vitro*
Transendothelial chemotaxis was performed to evaluate T cell migration in response to the poly-chemokine stimulus produced by LPS treated H69 cells. T cells from PBMC were placed in a 3µm filter above a well containing media, ± 100nM PS372424, ± 2µg/ml CXCR3 blocking antibody (BAb), and incubated at 37°C for 90mins. Negative control is non cell treated media. Migrated cells were counted by flow cytometry as a ratio of cells to counting beads. Statistics – unpaired t-test - all $p<0.0001$, n=3 in triplicate.

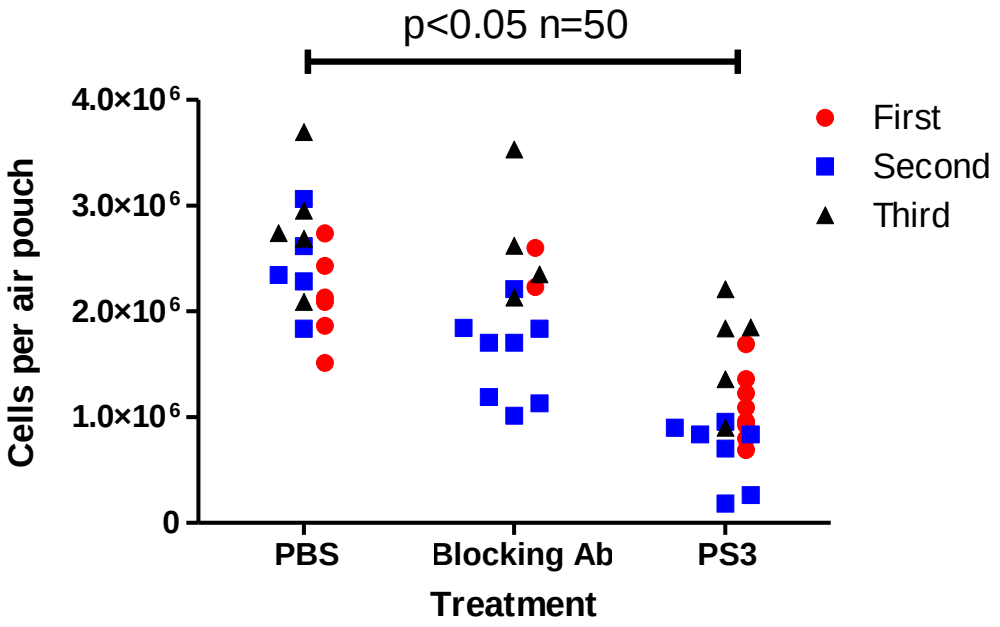


Figure 3-12 LPS treated H69 supernatant is chemotactic *in vivo*
Human cell migration into air pouches of humanised SCID mice 24hr following intrapouch administration of LPS-treated H69 supernatant and intravenous administration of PBS, 1μM PS372424 or 25μg CXCR3 blocking antibody (BAb). Shown is three independent experiments, each with >5mice per group.

3.3.3 *CHEMOKINES PRODUCED MAYBE MODIFIED*

As well as assessing the functional potential of chemokines produced by stressed epithelial cells, it was also determined if these chemokines had undergone modification. To do this chemokines needed to be purified and analysed by mass spectrometry.

CCL2, CCL5 and CXCL8 ELISAs were used to determine a suitable cell type and treatment to use to produce the large amounts of conditioned media for this analysis (Figure 3-13 - Figure 3-18). However each ELISA was only performed once (in duplicate) and so robust conclusions cannot be drawn as to how each treatment effects chemokine production. For CXCL8, exact concentrations could not be reached for some samples as even diluted samples were above the top standard (Figure 3-18). 5μM peroxynitrite with serum starvation of HKC8 for 48hr was chosen as the treatment as it gave the highest levels of a range of chemokines and reducing the amount of serum present is of technical benefit for later purification.

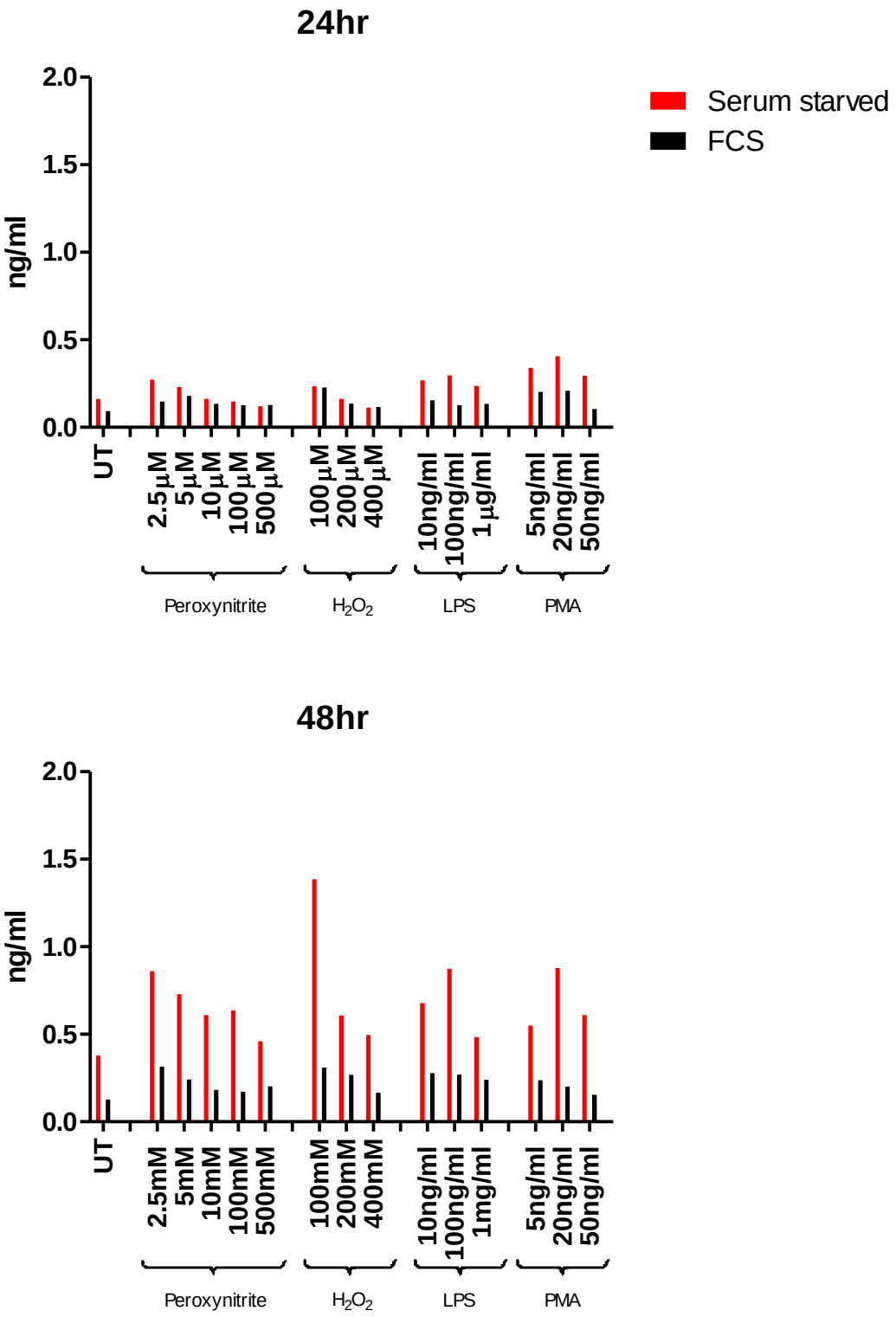


Figure 3-13 CCL2 production by H69 cells
H69 cells were treated with a range of concentrations of peroxynitrite, hydrogen peroxide, LPS and PMA for 24 and 48hr. CCL2 levels in supernatant was measured by CCL2 ELISA (Peprotech)

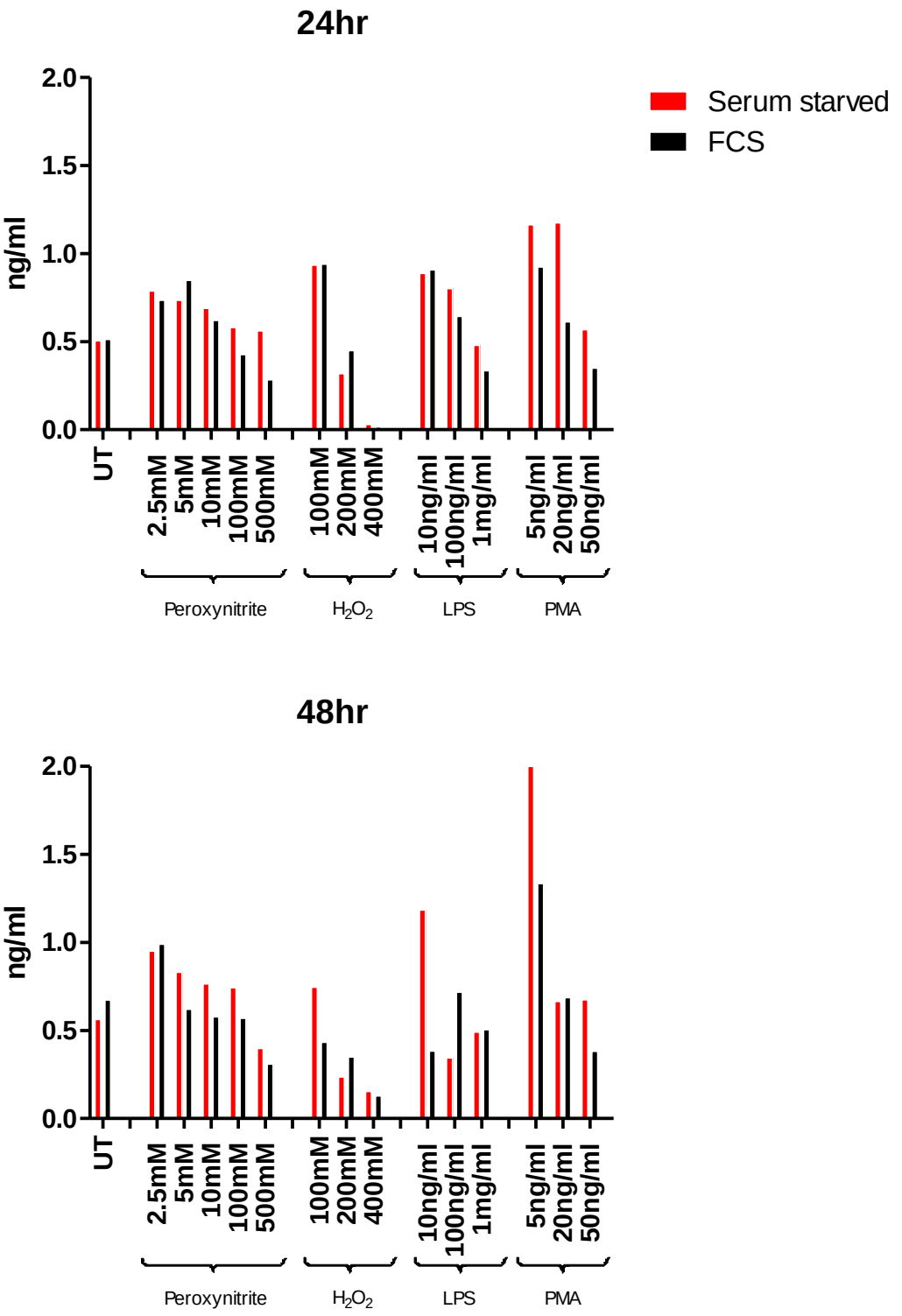


Figure 3-14 CCL2 production by HKC8 cells
HKC8 cells were treated with a range of concentrations of peroxynitrite, hydrogen peroxide, LPS and PMA for 24 and 48hr. CCL2 levels in supernatant was measured by CCL2 ELISA (Peprotech)

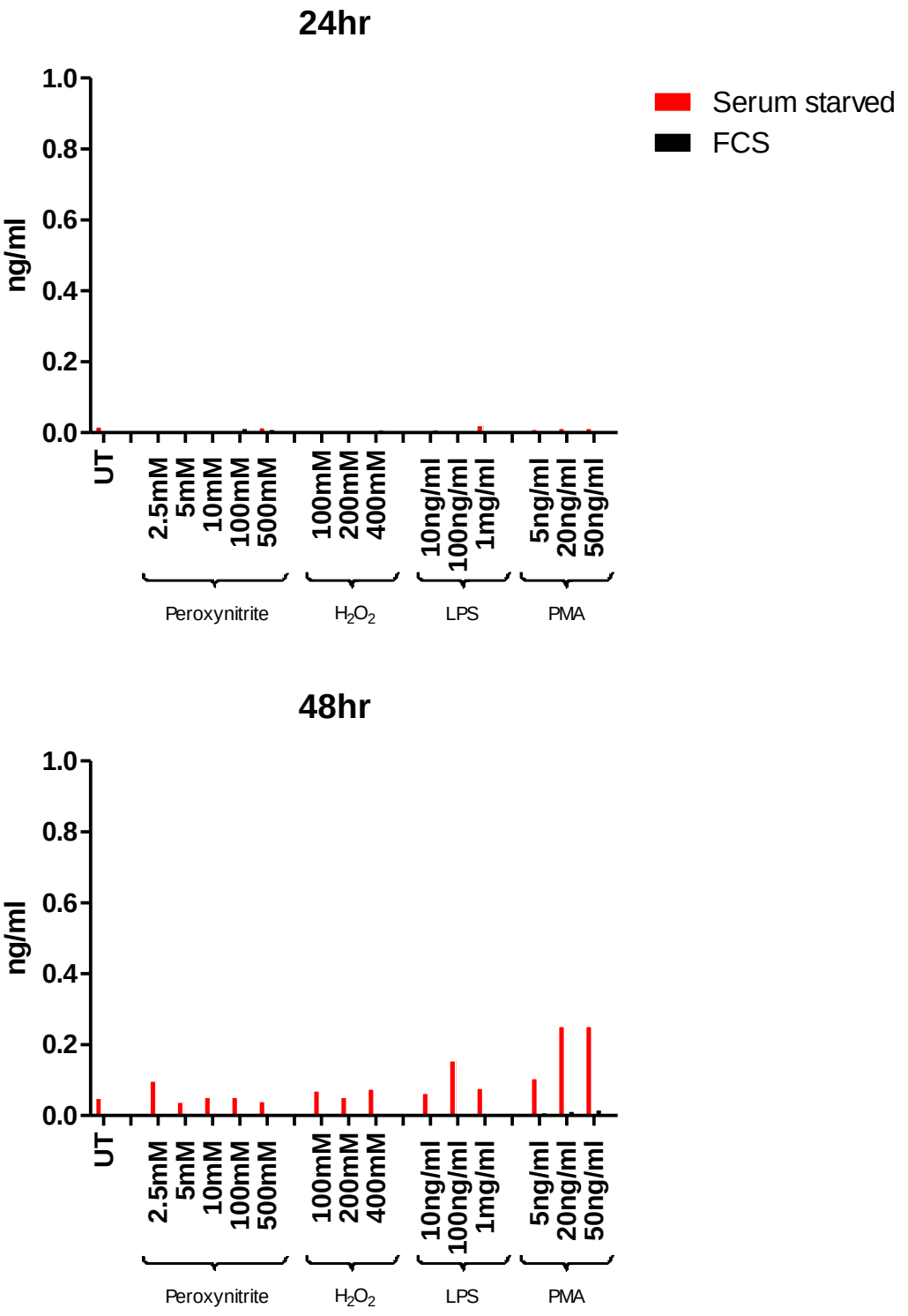


Figure 3-15 CCL5 production by H69 cells
H69 cells were treated with a range of concentrations of peroxynitrite, hydrogen peroxide, LPS and PMA for 24 and 48hr. CCL5 levels in supernatant were determined by CCL5 ELISA (Duoset, R&D Systems) using supernatant neat and diluted 1:10 with PBS.

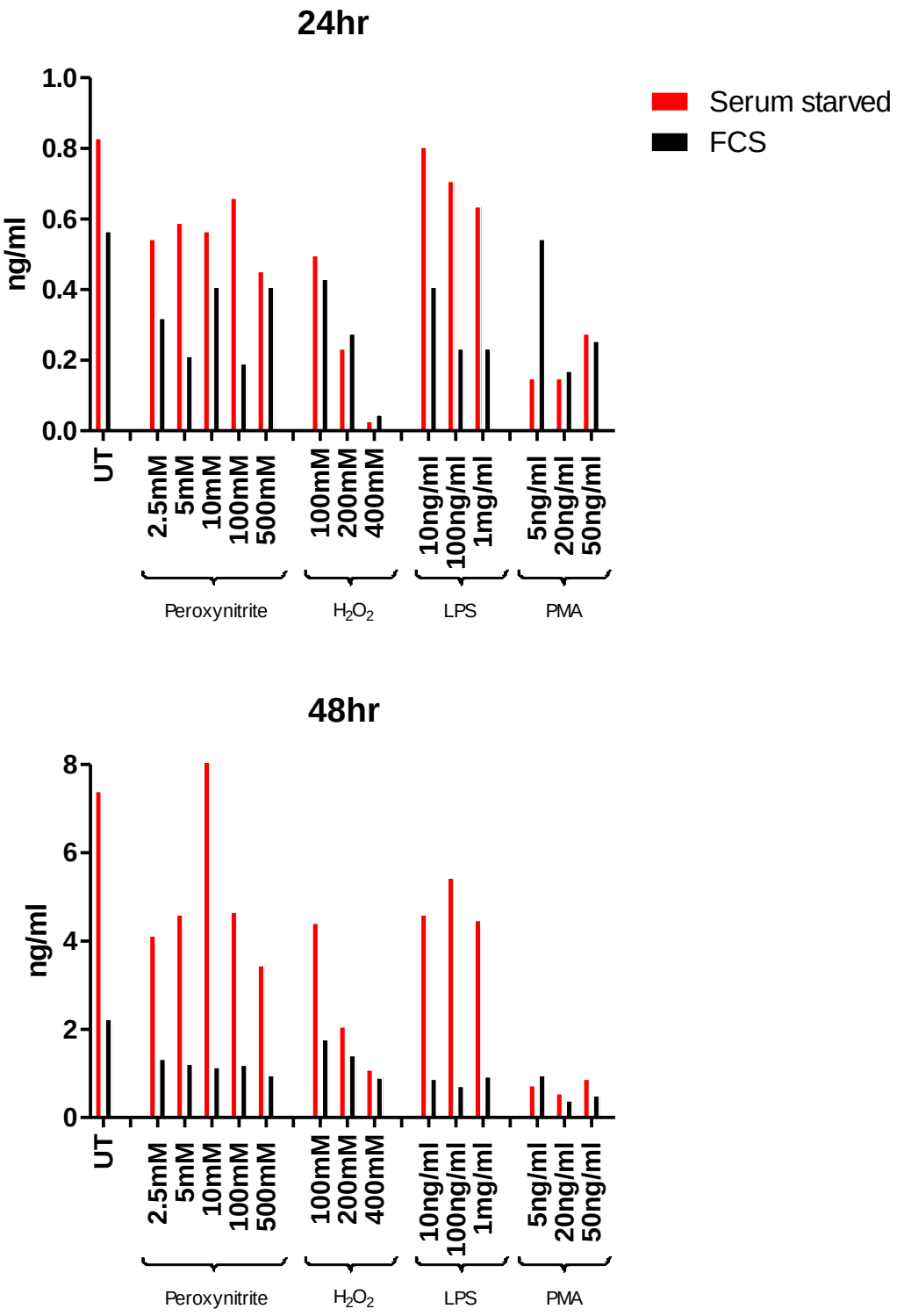


Figure 3-16 CCL5 production by HKC8 cells
HKC8 cells were treated with a range of concentrations of peroxynitrite, hydrogen peroxide, LPS and PMA for 24 and 48hr. CCL5 levels in supernatant were determined by CCL5 ELISA (Duoset, R&D Systems) using supernatant neat and diluted 1:10 with PBS.

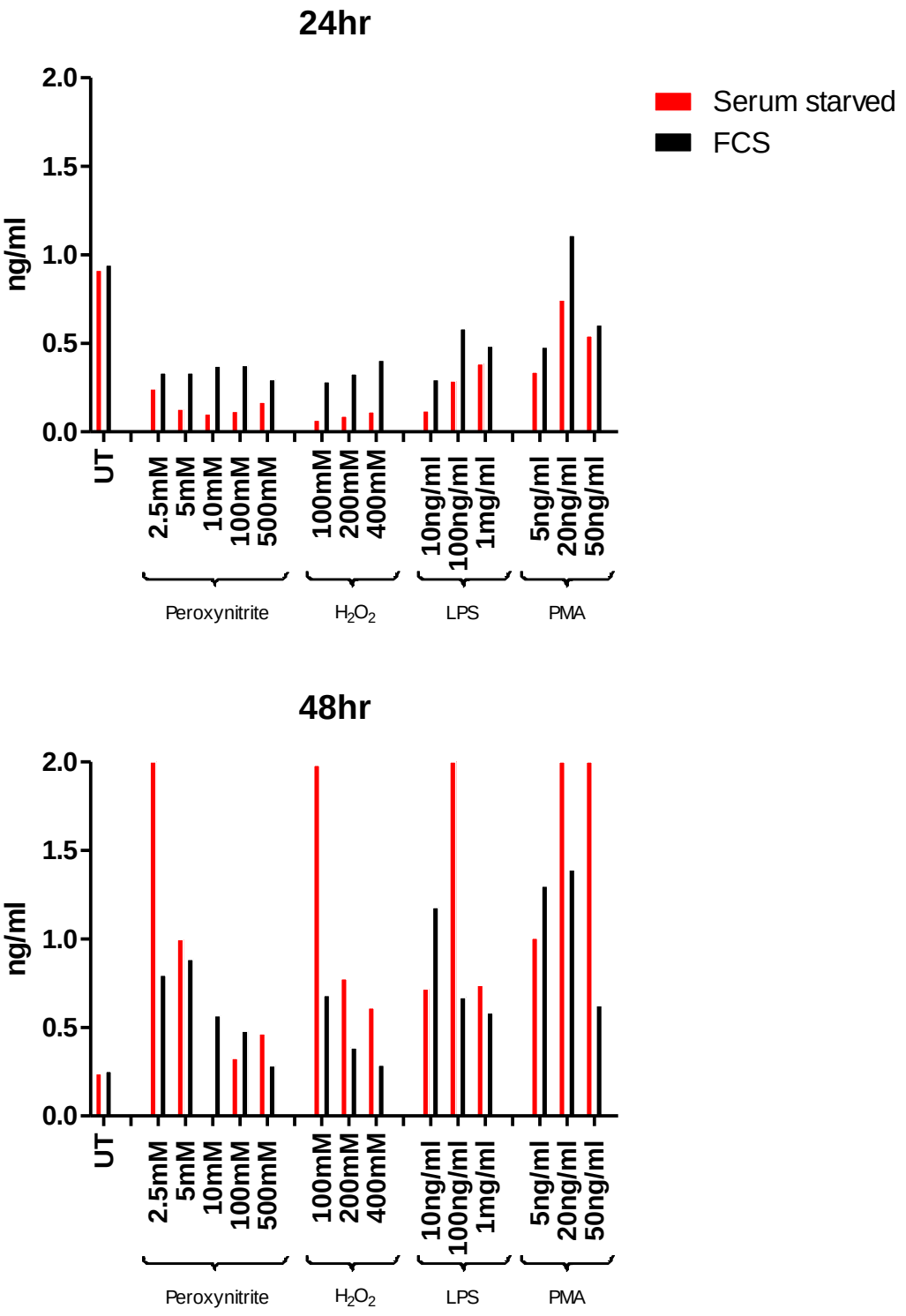


Figure 3-17 CXCL8 production by H69 cells
H69 cells were treated with a range of concentrations of peroxynitrite, hydrogen peroxide, LPS and PMA for 24 and 48hr. CXCL8 levels in supernatant were determined by CXCL8 ELISA (Duoset, R&D Systems) using supernatant neat and diluted 1:10 with PBS.

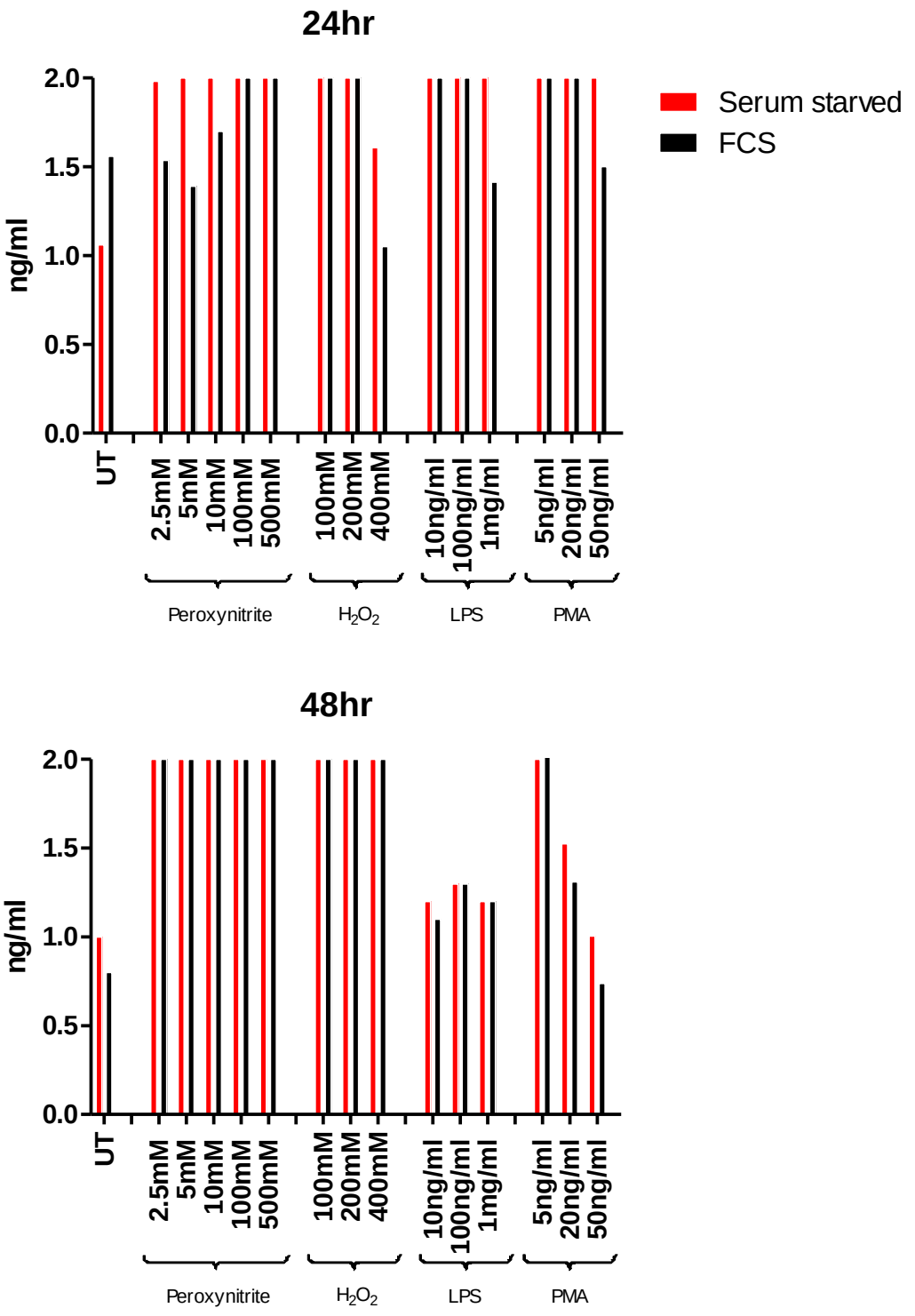


Figure 3-18 CXCL8 production by HKC8 cells
HKC8 cells were treated with a range of concentrations of peroxynitrite, hydrogen peroxide, LPS and PMA for 24 and 48hr. CXCL8 levels in supernatant were determined by CXCL8 ELISA (Duoset, R&D Systems) using supernatant neat and diluted 1:10 with PBS.

To isolate the chemokines produced by stressed HKC8 cells filtered media was first purified by heparin-sepharose affinity chromatography. ELISAs were used to determine positive fractions and the concentrations of the chemokines CCL2, CCL5 and CXCL8 (Figure 3-19 and Figure 3-20a). The CCL5 positive fractions were then further purified by ion-exchange chromatography and HPLC as shown in Figure 3-20b. Chemokine concentrations were again determined by ELISA, and silver stain SDS-PAGE used to determine the level of contamination of other proteins remaining within the fractions. Chemokines are around 10kDa but due to low concentrations cannot positively be identified on the gels. The final stage of purification was HPLC (Figure 3-20c). Positive fractions were analysed by mass spectrometry to determine is any of the CCL5 present in the media had been subject to post-translational modification. Figure 3-21 demonstrates the presence of truncated CCL5 as the molecular weight shows first two amino acids have been cleaved.

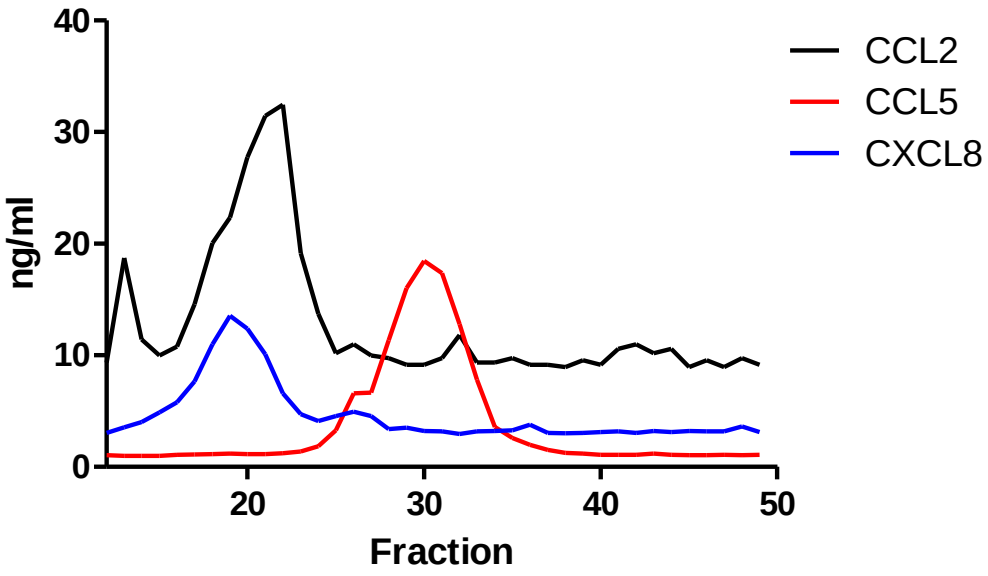


Figure 3-19 Purification of chemokines from HKC8 treated media
ELISA data showing heparin-sepharose fractions containing CCL2, CCL5 and CXCL8 isolated from HKC8 media.

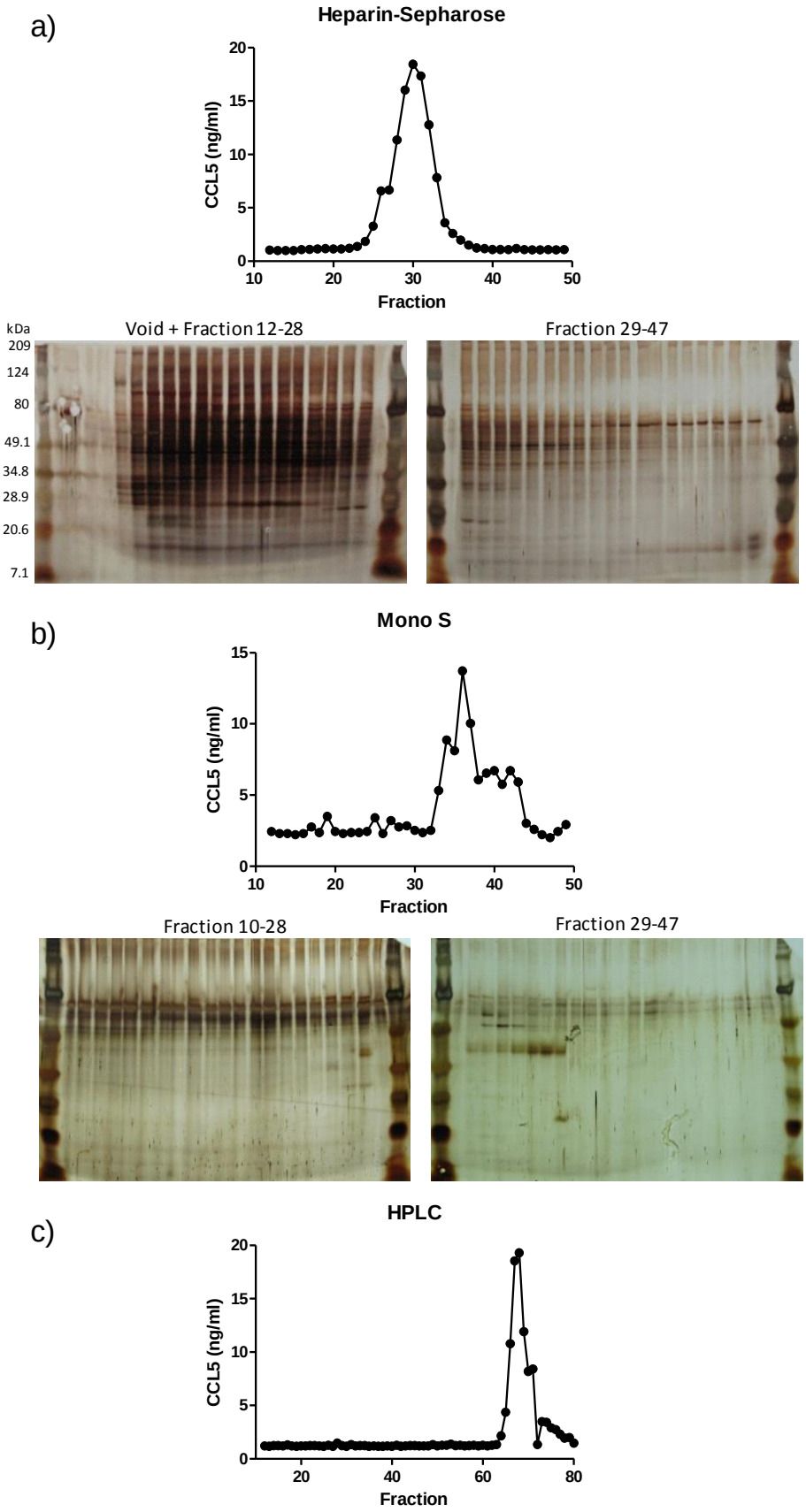
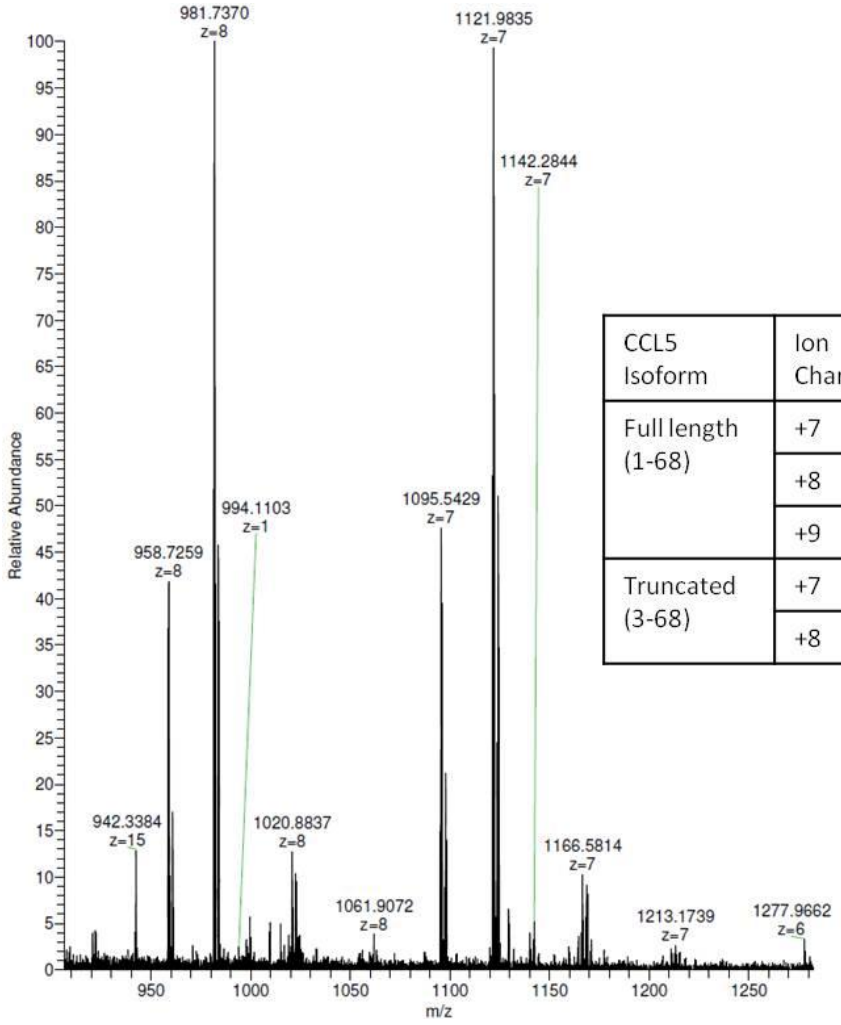


Figure 3-20 Purification of CCL5 from HKC8 treated media
ELISA data and SDS-PAGE images for each step of purification. a) Heparin-sepharose, b) Ion exchange (Mono S), c) HPLC.



CCL5 Isoform	Ion Charge	m/z	MW
Full length (1-68)	+7	1121.98	7846
	+8	981.74	
	+9	872.77	
Truncated (3-68)	+7	1095.54	7661
	+8	958.73	

Figure 3-21 Identification by mass spectrometry of CCL5 isoforms produced by HKC8 cells
Example MS plot from CCL5 positive HPLC fractions (Fraction 67) and table explaining the CCL5 isoforms found. m/z – mass:charge ratio, MW – molecular weight.

Previous studies have found the enzyme CD26 to be responsible for cleaving the first two amino acids of chemokines including CCL5. For this reason the presence of CD26 was analysed using Taqman qPCR and both HKC8 and H69 cells were found to express the enzyme (Figure 3-22). This data suggests that in this situation CD26 could be the enzyme responsible for the CCL5 modification but other proteases cannot be ruled out.

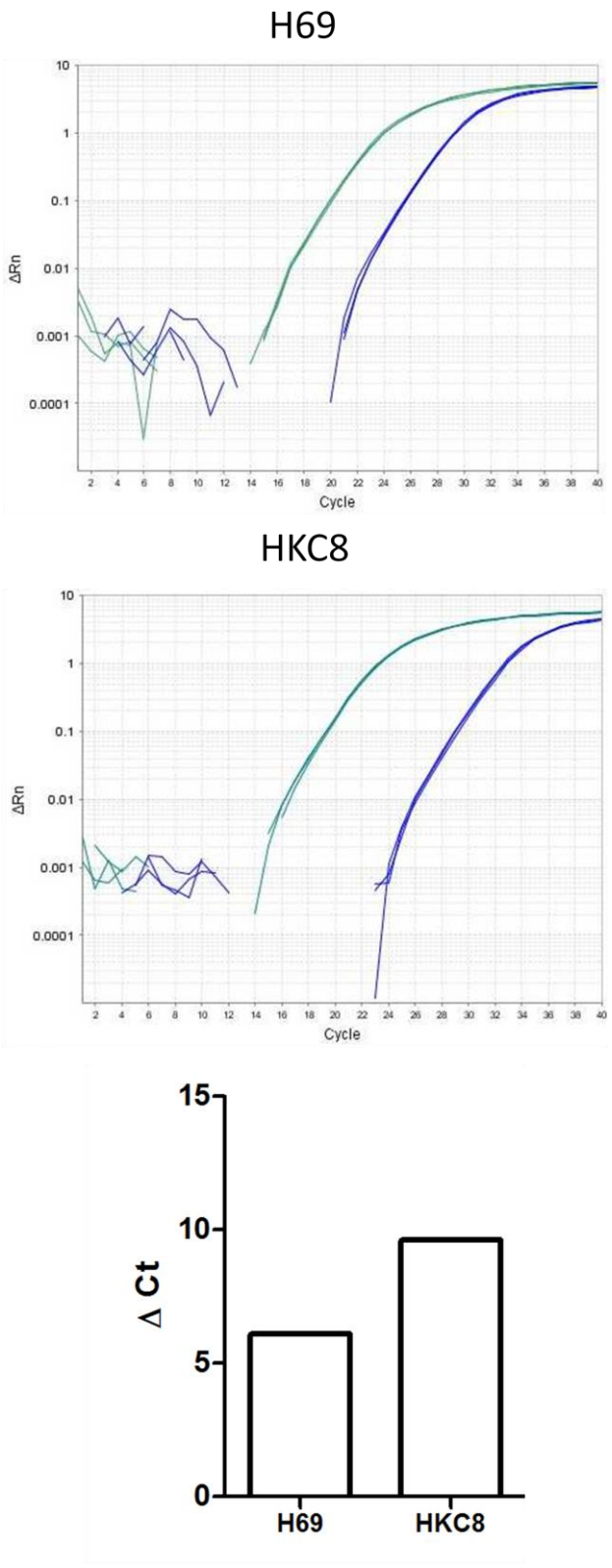


Figure 3-22 CD26 expression in H69 and HKC8 cells
Upper - Real-time PCR amplification plots showing presence of CD26 in H69 and HKC8 cells Blue - CD26, Green – GAPDH. Lower - delta Ct values relative to GAPDH. n=2

3.3.4 *OXIDATIVE STRESS IN VIVO*

The previous work in this chapter has been examining the effects of oxidative stress on chemokine production *in vitro*. This section aims to give evidence of chemokines and cellular stress *in vivo* in inflammatory and transplant situations.

3-nitrotyrosine is a marker of the presence of reactive nitrogen species. Sections from a murine model of kidney ischaemia-reperfusion injury were stained for 3-NT and an increased level of staining was found in the ischaemic kidneys compared to control, but little difference between 35 and 45min ischaemia time (Figure 3-23). H&E staining shows loss of tubular cells, exudate and interstitial oedema with IRI, confirming that higher levels of tissue damage occur in association with increased levels of 3-NT.

Figure 3-24 shows 3-NT levels in human liver biopsies. The upper four panels are from inflammatory liver disease and the lower two are time zero biopsies taken immediately post-transplant and after reperfusion. These images show high levels of peroxynitrite are associated with the inflammatory infiltrate and surrounding tissue has also been subject to protein modification by peroxynitrite although to a lesser extent.

CCL2 is also expressed in the situations in which peroxynitrite is present (Figure 3-25). Sections from patients with similar diseases to those stained in Figure 3-24 were stained for CCL2 and results show low level expression in liver tissue with increased levels in inflammatory infiltrate, especially in the cirrhosis patients.

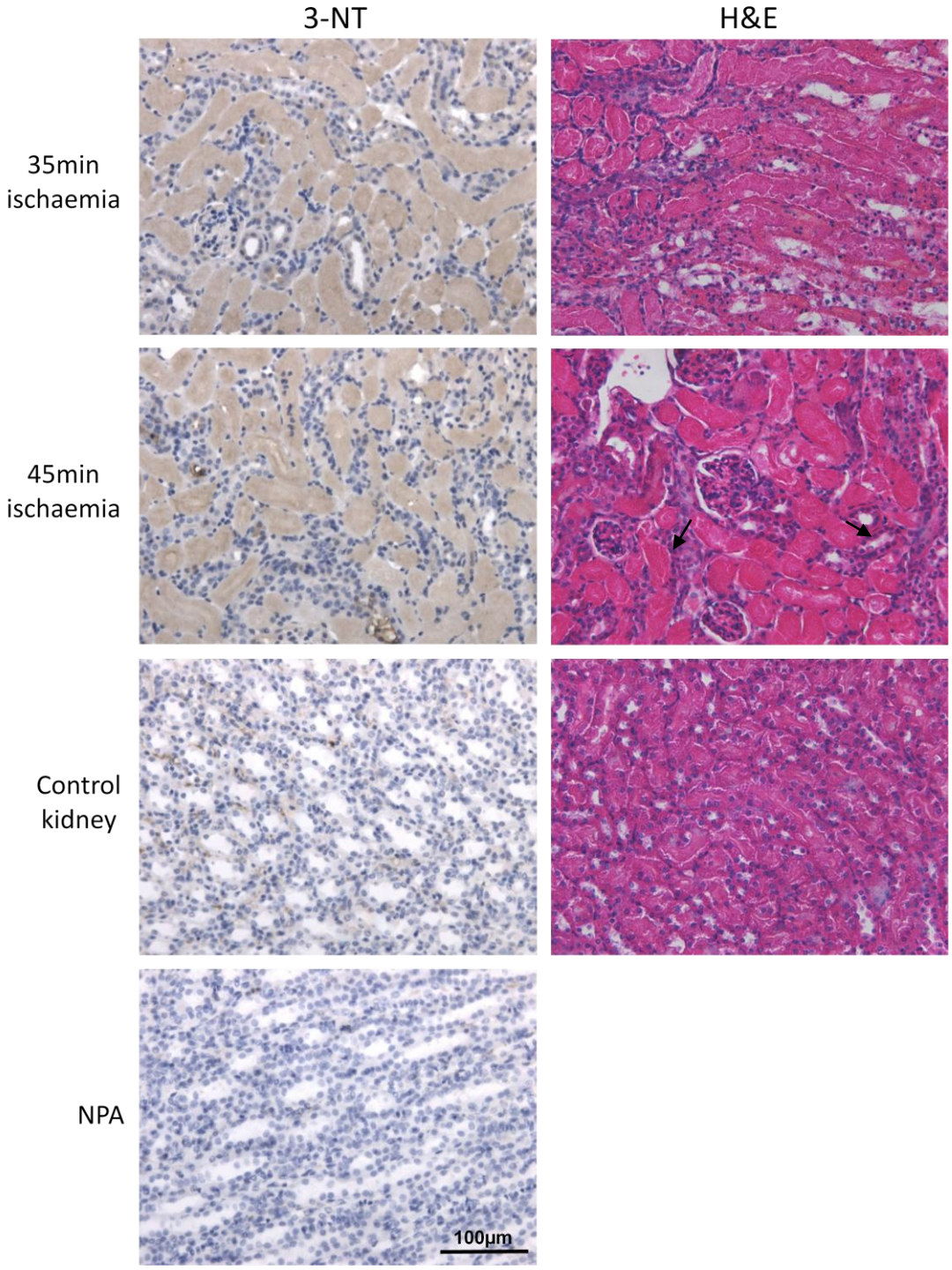


Figure 3-23 Effect of ischaemia-reperfusion injury on murine kidney
Kidney sections from a murine model of ischaemia-reperfusion were stained by immunohistochemistry for 3-nitrotyrosine (3-NT; left) or H&E (right). The left kidney was ischaemic for 35 or 45min and organs harvested 24hr post reperfusion, the right kidney was used as a control. 3-nitrotyrosine staining was developed by DAB (brown) and counterstained with haematoxylin (blue). No primary antibody control (NPA) is shown. Four mice were tested for each time point and a representative image shown. Arrows highlight example areas of damage, for example tubular shrinkage. Original magnification x20.

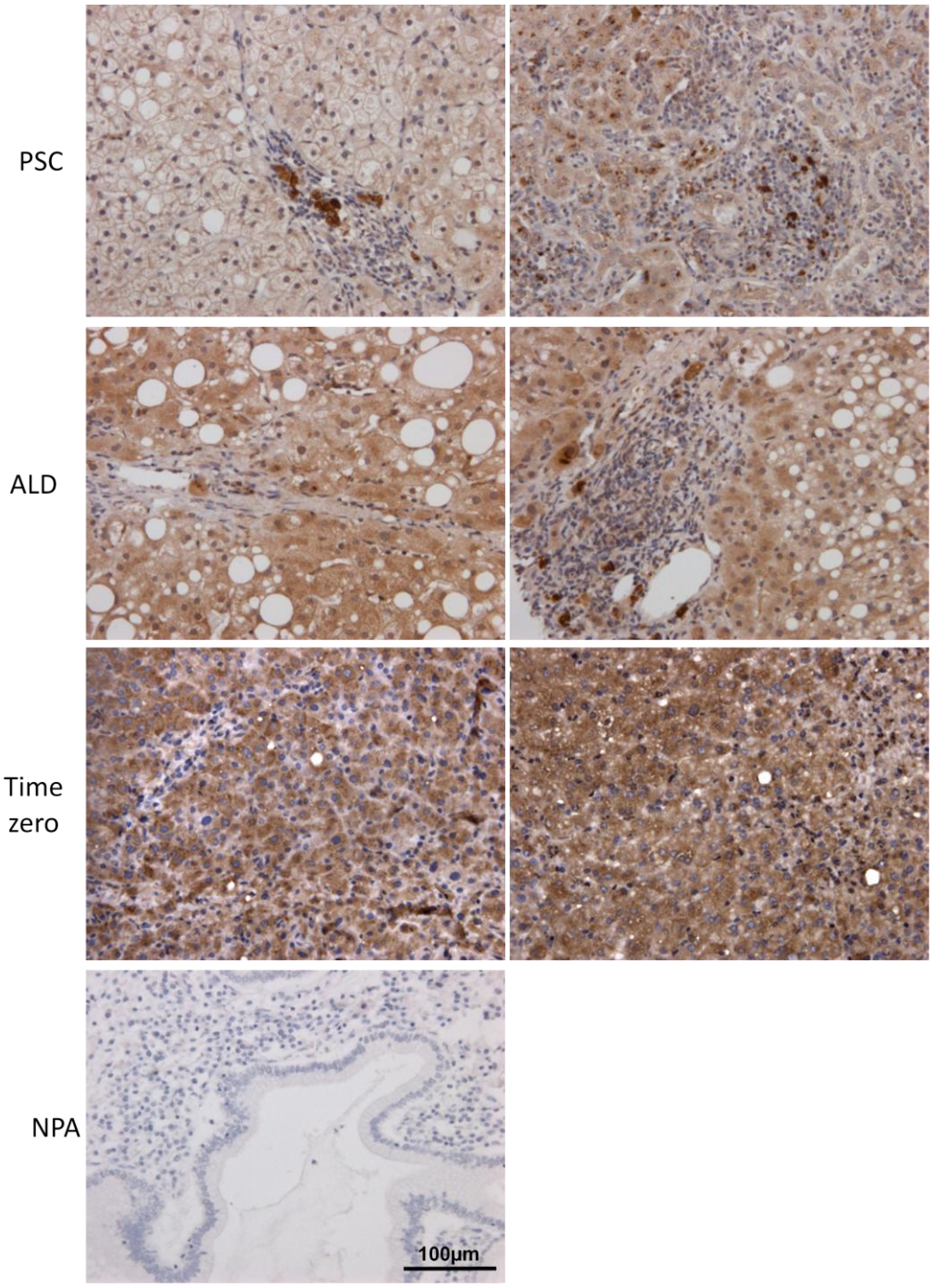


Figure 3-24 Presence of 3-nitrotyrosine in inflammatory liver disease.
4µm sections from human liver biopsies were stained for 3-nitrotyrosine by immunohistochemistry. The signal was developed by DAB (brown) and counterstained with haematoxylin (blue). The biopsies were from patients with primary sclerosing cholangitis (PSC), alcoholic liver disease (ALD) or time zero biopsies from newly reperfused transplants. No primary antibody control (NPA) is shown. Six disease samples and five time zero biopsies were stained and representative images are shown. Original magnification x20.

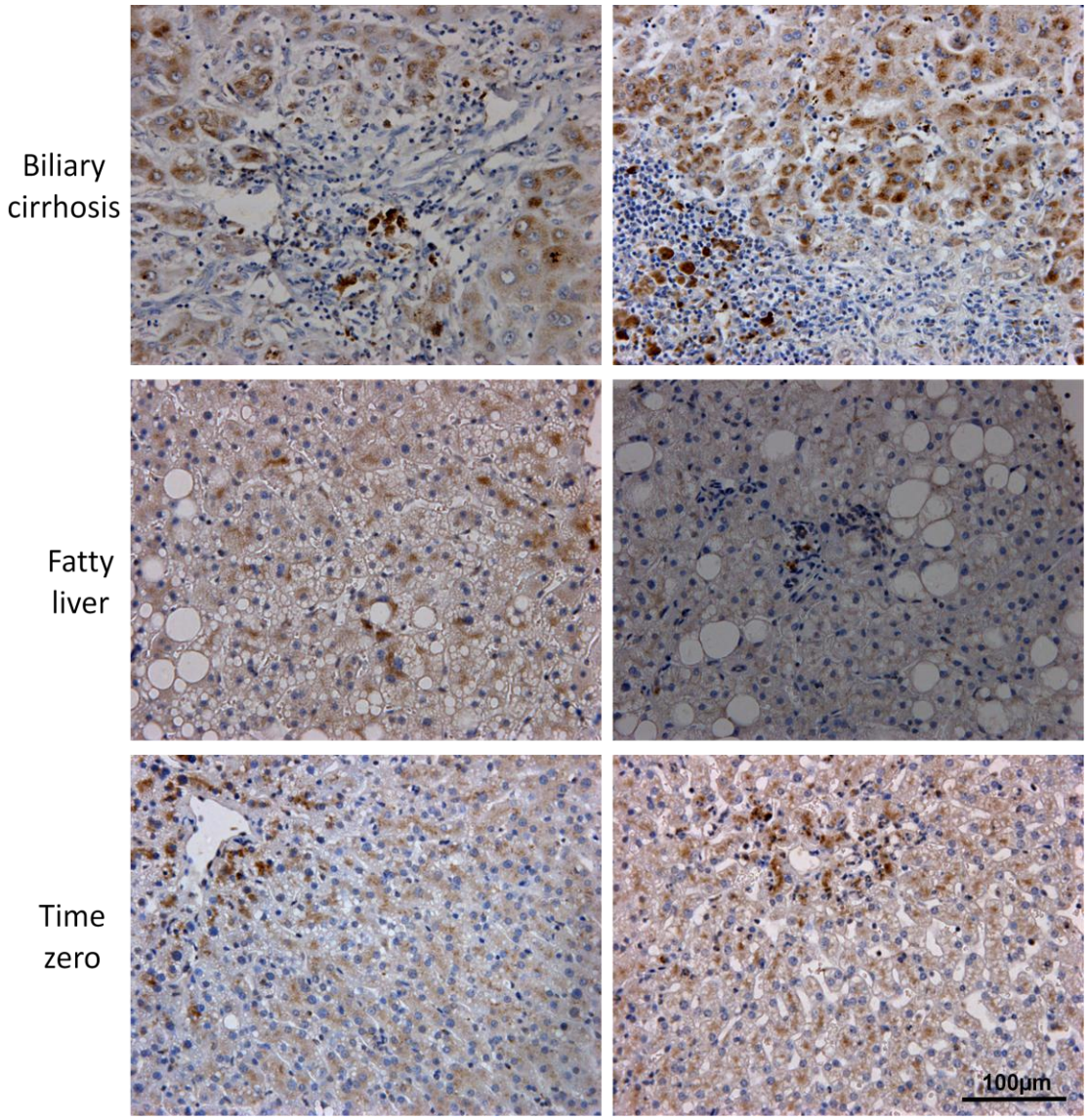


Figure 3-25 Presence of CCL2 in inflammatory liver disease.
4µm sections from human liver biopsies were stained for CCL2 by immunohistochemistry. The signal was developed by DAB (brown) and counterstained with haematoxylin (blue). The biopsies were from patients with biliary cirrhosis, fatty liver, or time zero biopsies from newly reperfused transplants. Six disease samples and three time zero biopsies were stained and representative images are shown. Original magnification x20.

3.4 DISCUSSION

The data in this chapter highlights the intricate regulation of the chemokine system in response to stress, a complexity which has evolved to allow the immune system to elicit different responses based on the pathological stimulus present and the time course of the damage. Such different 'activated' phenotypes are demonstrated by the PCR data in this chapter; there is not a binary response to stress. This study extends from my previous work demonstrating that oxidative stress increases CCL2 and CXCL8 transcription in H69 cells (Barker, 2011) to look at a wider range of stresses and a second tissue type, kidney epithelium. Epithelium was examined in this study rather than other cell types linked to oxidative stress and inflammation, such as macrophages or stromal cells. It was chosen as the focus due to its involvement in IRI and chemokine production and also the potential of these cells to be modified by long term stress. They can undergo EMT or become senescent, secreting more chemokines and spreading senescence, causing further functional loss of the tissue. Immunoassays were also performed to look at the alteration of chemokine levels during stress but this was only a single 'snapshot' due to the expense of MSD analysis and so robust conclusions cannot be drawn from these results. Initial findings however support the notion of cell, chemokine and stress specific responses.

All the proinflammatory chemokines tested by qPCR were induced by at least one form of cellular stress, with CXCL8 having the greatest capacity for induction and CCL5 the lowest at the time point studied, in terms of both level of induction and breadth of inducers. CCL5 is thought to have a larger role in the amplification than the initiation of the immune response (Deckers *et al.*, 1998) so a low level of CCL5 induction at this early time point is not surprising. CXCL10, initially discovered as an IFN inducible gene can also be stimulated by other stresses. PCR data shows LPS can upregulate its production in liver cells and previous studies, although in murine macrophages, demonstrate this is through a MyD88 independent pathway involving both NF-κB and IFN-regulatory factor 3 (IRF-3) (Kawai *et al.*, 2001). There is little, if no previous evidence for PMA stimulating CXCL10 production and defining the mechanism is beyond the scope of this study. PMA is a stimulator of the NF-κB pathway so this is a potential mechanism for CXCL10 induction (Mut *et al.*, 2010).

A notable absence in response to stress is that of renal epithelia to LPS. However, HKC8 have previously been shown to lack TLR4 mRNA (Bland *et al.*, 2001) and so the lack of chemokine induction is unsurprising. This finding highlights the limitations of work involving cell lines, or at least the extent of the conclusions which can be drawn. This lack of stress response is cell line specific and not the case for kidney epithelium as a whole. The opposite is actually the case as TLR4 is important in the production of proinflammatory chemokines in kidney IRI (Wu *et al.*, 2007). Determining the extent to which the findings in this study are purely cell line dependant or a cell type specific response is important.

The concentrations of the stress inducers used in this work are those previously optimised to give a chemokine response in the H69 cell line. Whether this level of stress is physiological is uncertain. Peroxynitrite cannot be detected *in vivo* (Szabo *et al.*, 2007), as discussed in section 1.4.1.1, so the exact maximal concentration reached at a site of inflammation cannot be determined. Carbon dioxide and other molecules present can also affect the form and therefore effects of the reactive species. Metal ions are crucial for hydrogen peroxide chemistry and so it is not just the concentration of the reactive species that affects reactivity (Halliwell *et al.*, 2000). PMA is not a physiological inducer of stress but as discussed in section 1.4.2 is an activator of PKC which is involved in numerous stress responses *in vivo*. These are all potential limitations of this study but it is an *in vitro* study using immortalised cell lines designed to model the differential stress responses rather than accurately represent possible *in vivo* conditions.

Serum starvation increases the expression of both CCL2 and CXCL10, and along with PMA can synergistically increase the levels of both chemokines (cell line dependent). Different stresses acting synergistically to further increase chemokine production has been found in numerous other studies both *in vitro* and *in vivo* (Yasumoto *et al.*, 1992; Dent *et al.*, 2014) . The exact effects of serum starvation are undefined but induction of NF-κB has been found in some cell types (Kohno *et al.*, 2012). Effects could also be due to decreased antioxidant levels, therefore increased oxidative stress. Selenium is required for the antioxidants thioredoxin reductase and glutathione peroxidise and in a study evaluating the importance of differing selenium concentrations between batches of FBS, FBS with the highest

selenium levels gave the smallest increase in luciferase under control of an oxidative stress gene (Karlenius, 2011). Most cultures are thought to be selenium deficient despite the presence of serum. A complete absence of FBS and therefore selenium will presumably have a profound effect on antioxidant activity.

The data discussed so far looks at the transcriptional regulation of chemokines during stress. To confirm this translated into functional chemokines, chemotaxis was performed. The mix of chemokines produced by LPS stimulated liver epithelial cells can elicit the migration of T cells both *in vitro* and *in vivo*. The use of both a CXCR3 blocking antibody and the small-molecule CXCR3 agonist PS372424 in these assays confirm the protein data of chemokine levels following LPS stimulation that a range of chemokines are produced. For these assays both human T cells and humanised mice were used as a Q196E amino acid change between the human and mice CXCR3 means the agonist PS372424 has no effect on the murine receptor (Nedjai *et al.*, 2012). The blocking antibody gives some reduction in chemotaxis although this is not significant suggesting that although some CXCR3 ligands are present, the blockade of chemokine function can be readily compensated for by other chemokines present in the poly-chemokine mixture. The agonist causes complete loss of migration, fitting with previous data from our group (O'Boyle *et al.*, 2012). The agonist acts by similar mechanisms to non-GAG binding chemokine mutants which can activate the chemokine receptor but no directional cue is given as a GAG-bound gradient is absent (Ali *et al.*, 2005b; O'Boyle *et al.*, 2009). This receptor activation causes receptor internalisation, and so the cell is unable to respond to chemokine due to lack of cell surface receptor until surface receptor is regenerated. PS372424 administered i.v. can desensitise cells, preventing migration towards chemoattractant in the air pouch. This is particularly significant in the case of CXCR3 as this chemokine receptor requires *de novo* synthesis and is not simply recycled like other chemokine receptors (Meiser *et al.*, 2008). The agonist is also capable of desensitising multiple chemokine receptors (O'Boyle *et al.*, 2012) and so loss of response to other chemokines present is also achieved. Despite being counterintuitive, this explains the complete abrogation of transendothelial T cell migration by the CXCR3 agonist.

The focus of the next two chapters will be the post-translational modification of chemokines during stress, another mechanism for the fine tuning of chemokine responses. This chapter gives evidence of both the production and modification of CCL5 by HKC8 cells. The majority of CCL5 produced was in its wild type form but a truncated form, missing the two N-terminal amino acids, was also detected. This is a well characterised chemokine modification by CD26 (Oravecz *et al.*, 1997; Proost *et al.*, 1998a; Schols *et al.*, 1998; Struyf *et al.*, 1998a; Iwata *et al.*, 1999; Lim *et al.*, 2005) and so no further functional studies have been carried out. The modification skews CCL5 towards a T cell chemoattractant by preventing CCR1 and CCR3 binding but not altering signalling through CCR5. This is one of few examples of non-immune cell mediated chemokine cleavage (Struyf *et al.*, 1998a) and gives direct evidence that cells are able to alter the chemokines they produce, an intrinsic mechanism for chemokine regulation. Although it cannot be ruled out that other proteases are responsible for the CCL5 processing in this situation, evidence from previous studies and data provided here showing production of CD26 by HKC8, builds a strong case that it is the enzyme responsible.

As well as demonstrating the effects of cellular stress on chemokines, *in vivo* evidence of chemokines in an oxidative stress rich environment has been verified in this chapter. Inflammation and IRI are circumstances in which oxidative stress is known to be pathological. The biopsy staining carried out shows 3-NT, a marker of the reactive nitrogen species peroxynitrite, is markedly increased following ischaemia-reperfusion and is also at high levels in the inflammatory infiltrate of multiple inflammatory liver diseases. CCL2 is also present in these liver diseases. Although its unknown from this data if the chemokine presence is directly due to upregulation by oxidative stress, or if the source of the chemokine is liver cells or infiltrating immune cells, it provides evidence for concurrent protein modification by peroxynitrite in the presence of CCL2 in physiological setting which is important for the biological relevance of data in subsequent chapters.

Tissue staining has also shown oxidative stress in time-zero biopsies, emphasising that even at the very beginning of the life of a transplant the organ has been subject to a large amount of oxidative stress. It is therefore likely to be damaged and have increased chemokine levels. Not only is this important in terms of

transplant biology but highlights a potential caveat in previous studies which used similar biopsies as 'normal' controls (Rygiel *et al.*, 2010). This tissue has already undergone a number of processes which could be predicted to affect its ability to accurately represent normal tissue for example potential brain death of the donor (de Vries *et al.*, 2011) and IRI. What can be classed as a normal control, especially when studying acute effects of oxidative stress is a complex argument, especially if it is for a human study. The human tissue staining carried out in this study has no 'normal' comparison, instead animal models were used as the best alternative. Liver biopsies are a high risk procedure and so are not carried out unless deemed clinically necessary, for this reason no healthy tissue in available. Cancer resection biopsies contain areas of tissue distinct from the tumour. These could be argued to be healthy but due to the effects of the tumour microenvironment and previous evidence of the paracrine effects of this on spreading damage and senescence (Coppe *et al.*, 2008; Leibovich-Rivkin *et al.*, 2013) this cannot be guaranteed to be 'normal'.

Overall this chapter highlights the complexity of regulation within the chemokine system. *In vivo*, chemokines are present at sites of inflammation and oxidative stress. Numerous stresses can alter both the transcription and production of chemokines in a tissue, stress and chemokine specific manner. The chemokines produced are functional but there is evidence of post-translational modification suggesting that the chemokines present may not all have their predicted function. These data show that a high level of regulation is necessary due to the numerous distinct situations to which chemokines and the immune system have to respond with minimal damage to the surrounding unaffected tissue.

4 CHEMOKINE POST-TRANSLATIONAL MODIFICATIONS

4.1 INTRODUCTION

Post-translational modifications of chemokines have previously been shown to diminish or potentiate the biology of said ligands. This occurs in a highly variable manner and so it is difficult to extrapolate a generalised paradigm. For this reason a careful ligand specific study is required. As discussed in section 3.3.1, oxidative stresses can alter the production of proinflammatory chemokines, and it is apparent that reactive species present in such stresses can directly modify proteins. This chapter sets out to examine the potential for chemokine modification within such inflammatory environments.

Establishing which chemokines can be modified, and by which mechanisms is important in understanding the complexity within the chemokine system. As highlighted in section 1.3.1 there are many levels of regulation to finely tune the chemokine response and ultimately the body's response to infection and injury. It is during these times of infection and inflammation that chemokines are both most abundant and most studied, however these are also the situations in which there is a higher potential for modification.

Previous studies of chemokine modification have already been discussed in detail in section 1.6, and evidence of truncation found in section 3.3.3. Briefly, chemokines can be enzymatically truncated, both enhancing and abrogating their chemotactic potential, depending on the chemokine, enzyme and cleavage site. This chapter focuses on identifying targets of residue modifications, namely nitration and citrullination. Citrullination decreases the function of certain chemokines, with more substantial effects being seen in *in vivo* assays than *in vitro*, due to profound alteration in GAG binding potential (Loos *et al.*, 2008; Proost *et al.*, 2008; Struyf *et al.*, 2009). Nitration has so far been less studied but has also been reported as a down regulator of chemokine function (Molon *et al.*, 2011). Establishing which chemokines can be modified by such mechanisms is crucial. Examining how these modifications interact together, and determining if some are inhibitory of others, are additional vital findings for fully understanding *in vivo* chemokine function but are outside the scope of this study.

Although multiple studies have examined the functional effects of chemokine modification, few have determined if modification can effect detection. Nitration is currently the only modification shown to prevent detection by commercially available antibodies however all modifications which alter function will have important implications for studies looking at chemokine levels. All protein modifications have the potential to alter epitope and therefore change which antibodies can detect the protein. Studies using such antibodies may not be seeing all chemokine present in the situation.

An increasing number of studies look at chemokines as biomarkers but until it is known which forms of chemokine are being detected, and therefore their function, accurate conclusions as to how these chemokines are involved in disease cannot be made. Until very recently links between chemokine modification and clinical significance had not been made. Counterintuitive findings that increased CXCL10 levels in hepatitis C patients were associated with worsened prognosis have now been explained with the discovery that significant amounts of the CXCL10 present in such cases is in a truncated, antagonistic form (Riva *et al.*, 2014). This highlights the importance of establishing which chemokine forms are present in each situation; studies that have only measured the amount of chemokine in tissue may not reflect the true biological potency.

As discussed in section 1.3, the prototypical chemokines chosen to be the focus of this study are CCL2, CCL5 and CXCL8, with CCL2 being studied in most depth. These are all chemokines known to be important during both inflammation and transplantation for monocyte, T cell and neutrophil recruitment respectively. The sequences of these three chemokines are shown in Figure 4-1 with some of the potential targets of both nitration and citrullination highlighted.

4.1.1 *SPECIFIC AIMS*

This chapter aimed to investigate post-translational modifications which can occur to chemokines. Specifically, experiments were carried out to:

- Determine if chemokines are subject to nitration, focussing on CCL2
- Establish methods for detection of nitrated chemokines
- Examine the potential for CCL5 citrullination

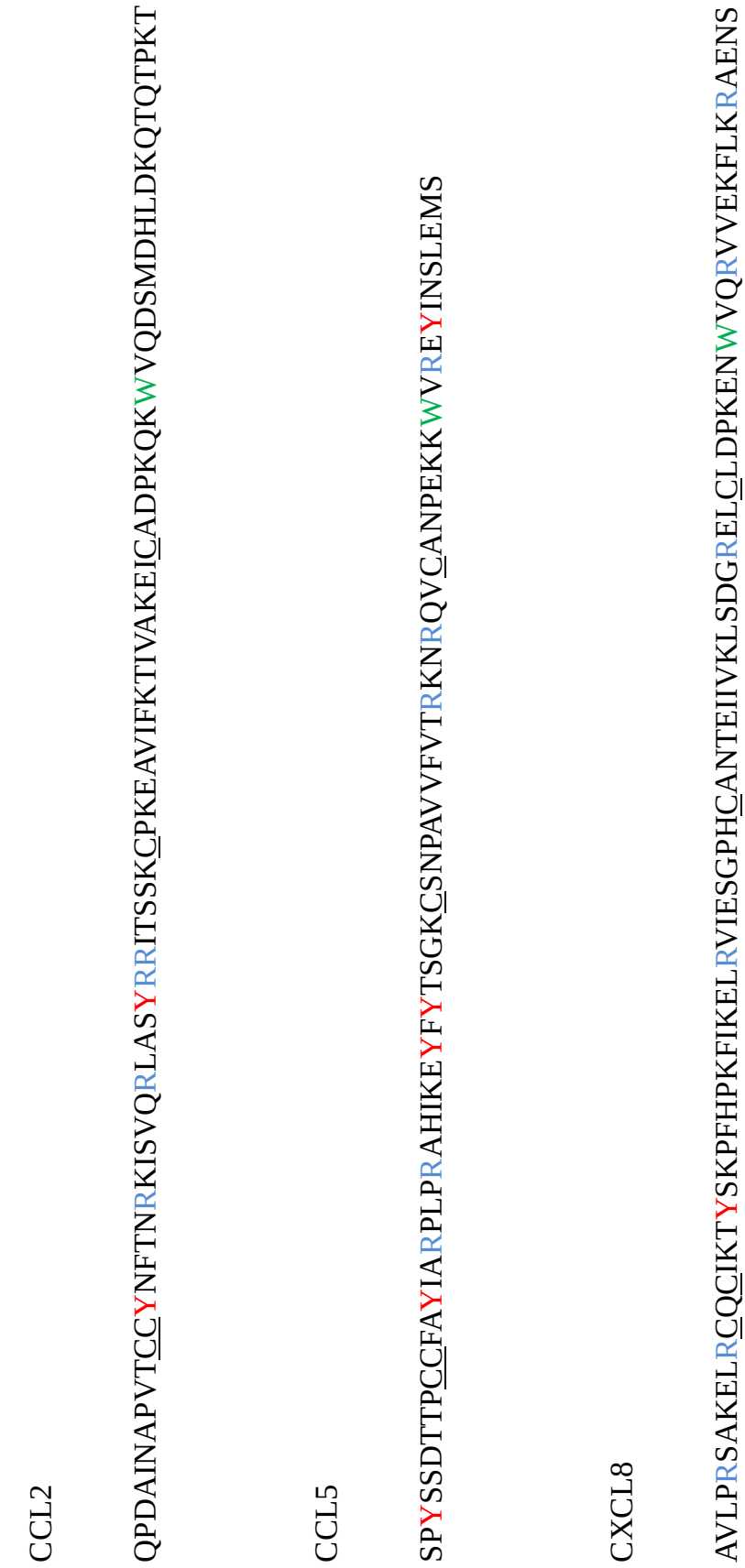


Figure 4-1 Amino acid sequences for CCL2, CCL5 and CXCL8
Sequences for the three chemokines examined. Highlighted are cysteine residues involved in disulphide bonds (underlined), some potential targets for modification by peroxynitrite (tyrosine – red, tryptophan – green) and potential targets for citrullination (arginine – blue). Sequences from (Uniprot).

4.2 SPECIFIC MATERIALS AND METHODS

4.2.1 *ELISAS*

Several ELISAs were used in this chapter using the basic method outlined in section 2.5.1.1.

For direct ELISAs, 10ng protein was bound to the plate. 3-nitrotyrosine detection was with anti-nitrotyrosine (1/1000, 2hr; Millipore, 06-284) and anti-rabbit-HRP (1/5000, 2hr, Sigma). CXCL8 and CCL5 detection was using components of R&D duosets and CCL2 detection was with R&D Systems antibodies MAB2791 and AB-279-NA.

A specific nCCL5 ELISA was developed. Plates were coated with rabbit anti-nitrotyrosine polyclonal antibody (5µg/ml) and the detection antibody used was the biotinylated goat anti-CCL5 from an R&D Duoset. Development was with TMB.

4.2.2 *LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY*

The exact residues modified by peroxynitrite in CCL2 were identified by mass spectrometry. Trypsin digest was performed prior to LC-MS/MS and analysed using Mascot (Matrix Science) as in Maassen and Hennig, 2011. MS was carried out by Dr Joe Gray, Pinnacle Laboratory, Newcastle University.

4.2.3 *PEPTIDE SYNTHESIS*

4.2.3.1 General principles

Peptide synthesis involves the formation of an amide bond between the carboxyl group and the amino group on adjacent amino acids in the peptide sequence. Solid-phase peptide synthesis (SPPS) is now the normal laboratory method for chemical peptide synthesis and was used in this study (Merrifield, 1963). Contrary to the biosynthesis of peptides, chemical synthesis is from the C-terminal. In this method of synthesis the growing peptide chain is immobilised by covalent attachment to a resin, this allows unused amino acids and by-products of the reaction to be washed away easily. In this study rink amide resin was used to create a C-terminal amide, similar to if the peptide was within a protein.

In order to stop unwanted reactions occurring, protecting groups are necessary on both the side chains and the amino terminus of each amino acid, for example tBu and Fmoc respectively. Peptides are synthesised by repeated cycles of coupling the next amino acid to the end of the immobilised chain, washing away free amino acid, deprotecting the N-terminus of the newly attached amino acid to prepare it for addition of subsequent amino acids, and a second wash step. Washing is important as each amino acid is added in major excess to increase yield. It is not until the synthesis is complete that side chains are deprotected using strong acid.

4.2.3.2 Method

a) Reagents

All reagents were purchased from Sigma unless otherwise stated. Peptide synthesis grade dimethylformamide (DMF) was purchased from AGTC Bioproducts. All resins and amino acid derivatives were purchased from Novabiochem. PyBOP® was purchased from Mimotopes and Apollo Scientific. Fritted polypropylene reaction vessels were used for all reactions and resin swelling, with DMF as the reaction solvent. For peptide couplings PyBOP was used as the activator. Amino acid side chain functionality was protected as follows: Fmoc-Arg(Pbf)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(*t*Bu)-OH. The resin used was high loading Rink Amide (100-200 mesh) with a specific loading of 0.71g/mol. Pre-swelling of the resin was carried out in dichloromethane (DCM) for a minimum of 1hr, followed by washing with DMF. The resin was washed in DCM and then shrunk in diethyl ether before TFA cleavage. Mass spectra were collected on a Waters TQD mass spectrometer (LC-MS/MS).

All peptide synthesis and related mass spectrometry was carried out in the Department of Chemistry, Durham University, UK with the help of Dr Steven Cobb, Mr Alex Hudson and Mr Sam Lear.

b) Synthesis

Fragments of CCL2 containing possible nitration sites were synthesised (AS<u>Y</u>RR and QK<u>W</u>VQ). Automated SPPS was carried out on a CEM Liberty1 single-channel microwave peptide synthesiser equipped with a Discover microwave unit. All

reactions were carried out using the 30ml polytetrafluoroethylene reaction vessel, with microwave heating and agitation by bubbling nitrogen. Couplings were carried out using Fmoc-protected amino acid (5 equivalents), PyBOP (10 equivalents) and N,N-Diisopropylethylamine (DIPEA; 20equiv, 2M solution in N-Methyl-2-pyrrolidone). The reaction vessel was drained after each cycle and fresh reagents were added. Microwave-assisted couplings at 0.1mmol scale were carried out for 10min at 75°C and 25W power. The Fmoc group was removed by a single treatment with a piperidine solution (20% v/v in DMF) at 75°C (45W) for 3min.

c) Cleavage

Peptide-resin was treated with 1.90ml TFA, 50µl dH_2O and 0.05ml triisopropylsilyl for 3hr at RT. The resin was then removed by filtration the filtrate was evaporated under reduced pressure. 20ml ice-cold diethyl ether was added and the suspension was centrifuged. After centrifugation the supernatant was removed. Ice-cold diethyl ether was added and the process was repeated to yield solid peptide. The resulting solid peptide was dissolved in dH_2O and lyophilized to yield crude product peptide as a white powder.

4.2.4 *GEL ELECTROPHORESIS*

SDS-PAGE was used to visualise protein following nitration, a similar method to that described in section 3.2.4.5.

1mm thick gels were cast using a Hoefer dual gel caster (Amersham Bioscience). A 10% running gel contained: 3.95ml dH_2O, 3.35ml 30% acrylamide, 2.5ml 1.5M Tris pH6.8, 100µl 10% SDS in dH_2O, 100µl 10% ammonium persulphate (APS) in dH_2O, 4µl TEMED. 5% stacking gel contained: 1.7ml dH_2O, 415µl 30% acrylamide, 315µl 1M Tris pH8.8, 25µl 10% SDS, 25µl 10% APS, 2.5µl TEMED. The running gel was poured and left to set with a layer of butanol. Alcohol was then washed off removed and stacking gel with a 10 well comb added.

Samples were boiled for 5min in loading buffer (10x loading buffer - 0.625M Tris, 10% SDS, 50% glycerol, 0.005% Bromophenol Blue, pH6.8) before loading. Gels were run at 70V in running buffer (25mM Tris base, 250mM glycine, 0.1% SDS, pH8.3).

Protein bands were then visualised using SimplyBlue SafeStain (Invitrogen), using the microwave protocol in the manufacturer's instructions.

4.2.5 *CITRULLINATION*

Citrullination work was carried out in the Laboratory of Molecular Immunology, Rega Institute, Katholieke Universiteit Leuven, Belgium with the help of Prof. Paul Proost and Dr Eva Moelants.

4.2.5.1 Basic principles

Citrullination is difficult to detect as it only gives a 1Da mass increase and the loss of one positive charge. The modification is therefore difficult, if not impossible, to detect by mass spectrometry. A 1Da shift will fall within the isotope peak range of the uncitrullinated protein if the sample is mixed. Citrullination increases the hydrophobicity of the protein, and therefore its retention time on a HPLC column, which may allow detection if the mass spectrometer is sufficiently sensitive. In this study citrullinated chemokine was further modified to allow detection.

The citrulline side chain has a distinctive ureido group (-NC(=O)N-) which is the target of reactions for specific identification of citrulline. At low pH, modification with antipyrine and 2,3-butanedione leads to a mass gain of 238Da per citrulline residue, as described in (Holm *et al.*, 2006; Stensland *et al.*, 2009). Reaction of 2,3-butanedione with the ureido group of citrulline forms a reactive imidazolone derivate (M+50), followed by the nucleophilic addition of antipyrine (M+188) to this imidazolone ring, as shown in Figure 4-2. Citrulline residues modified in this way are sufficiently different to arginine to be detected by mass spectrometry and antibodies can be raised against the modified residue.

4.2.5.2 CCL5 citrullination and modification

Citrullination was performed as described in Moelants *et al.*, 2011. CCL5 was incubated with PAD (from rabbit skeletal muscle; Sigma, P1584) for 90mins at 37°C at an enzyme–substrate molar ratio of 1:10. Incubations with PAD were carried out in 40mM Tris with 2mM $CaCl_2$ (pH7.4). Citrullinated CCL5 was then chemically modified using 50mM antipyrine (Fluka), 16% TFA and 12.5mM 2,3-

butanedione (Fluka) and incubated for 2hr at 37°C in the dark. The reaction mixture is highly acidic and so needed to be neutralised before use in further experiments. This was achieved by dialysis using Slide-A-Lyzer MINI Dialyse Units, 2000 MWCO (Thermo Scientific) overnight in 0.05% Tween/PBS (pH7.4) at RT in the dark.

Before modification was performed ELISA were carried out to ensure that the highly acidic conditions for modification did not destroy the epitope of the anti-CCL5 antibodies used in the subsequent sandwich ELISAs to allow detection of the modified chemokine.

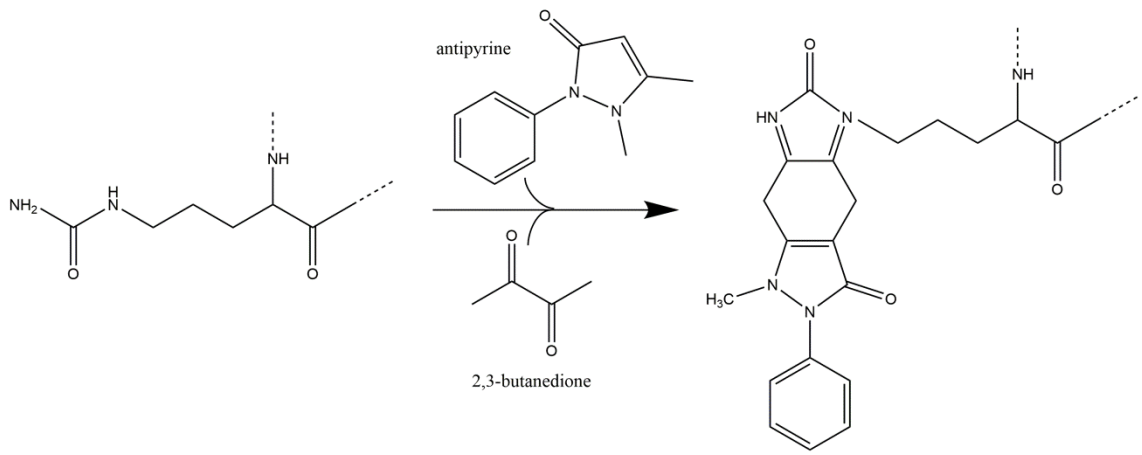


Figure 4-2 Chemical modification of citrulline by antipyrine and 2,3-butanedione.
Reaction of peptidylcitrulline with 50mM antipyrine and 12.5mM 2,3-butanedione at low pH results in a total mass gain of +238.

4.2.5.3 Citrulline-specific ELISA

Citrullination was quantified using a specific sandwich ELISA based on that developed for CXCL8 (Moelants *et al.*, 2011). The coating antibody used was a mouse monoclonal anti-CCL5 antibody (1µg/ml in PBS; from Duoset, R&D Systems). Chemically modified citrullinated CCL5 was detected using specific polyclonal antibodies against chemically modified citrulline residues, generated in rabbit by Moelants *et al.*, 2011, and a secondary anti-rabbit IgG-HRP antibody (1/2500; Jackson ImmunoResearch Laboratories). The ELISA was developed using TMB.

A normal CCL5 Duoset (R&D Systems), as described in the chemokine purification section, was also performed to show CCL5 is not lost during modification and dialysis (see Table 6).

4.3 RESULTS

4.3.1 *CHEMOKINE NITRATION*

Chemokine expression has been shown in chapter 3 to be increased by oxidative stress. To see if this oxidative environment can also modify chemokines directly CCL2, CCL5 and CXCL8 were incubated with peroxynitrite and nitration measured. Direct ELISAs using an antibody against 3-nitrotyrosine show peroxynitrite can nitrate tyrosine residues in all three chemokines (Figure 4-3 and Figure 4-4)

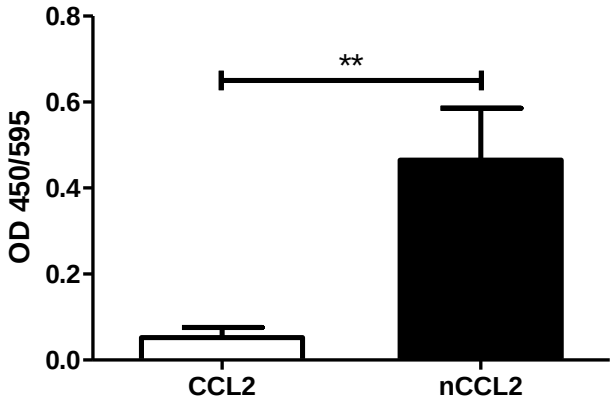


Figure 4-3 Nitration of CCL2 by peroxynitrite
Direct ELISA using a polyclonal antibody against 3-nitrotyrosine shows following peroxynitrite incubation tyrosine residues in CCL2 are nitrated. CCL2 – wild type chemokine, nCCL2 – chemokine following modification. t-test, p = 0.0033, representative of three experiments.

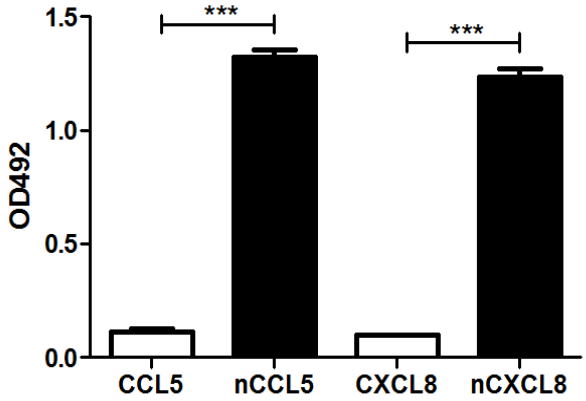


Figure 4-4 Nitration of CCL5 and CXCL8 by peroxynitrite
Direct ELISA using a polyclonal antibody against 3-nitrotyrosine shows following peroxynitrite incubation tyrosine residues in CCL5 and CXCL8 are nitrated. t-test, p = 0.009, representative of three experiments.

CCL2 was focused on to establish the exact residues modified by peroxynitrite; this was determined using trypsin digest and LC-MS/MS. Both tyrosines (Tyr13 and Tyr28; Figure 4-5 and Figure 4-6) and the tryptophan (Trp59; Figure 4-7) in CCL2 were all targets of nitration by peroxynitrite. Methionine oxidation was also found but, as this is a common artefact during mass spectrometry, cannot be attributed to modification by peroxynitrite (Morand *et al.*, 1993).

To further confirm the targets of nitration in CCL2, peptides were synthesised of the five residues surrounding the second tyrosine and the tryptophan in the CCL2 sequence. Peptides have an acetyl capped N-terminus and C-terminal amide to mimic the sequence being within a protein as closely as possible. Synthesis was by SPSS and purity of the synthesis determined by mass spectrometry (Figure 4-8 and Figure 4-9). These peptides were then subject to modification by peroxynitrite, and subsequent nitration detected by mass spectrometry. Figure 4-8 shows tyrosine nitration, peroxynitrite incubation increased the half mass peak by 22.5Da (369.7 - 347.2 = 22.5), an overal increase in 45Da. Tryptophan nitration was also detected (Figure 4-9) confirming that both these residues are targets of modification.

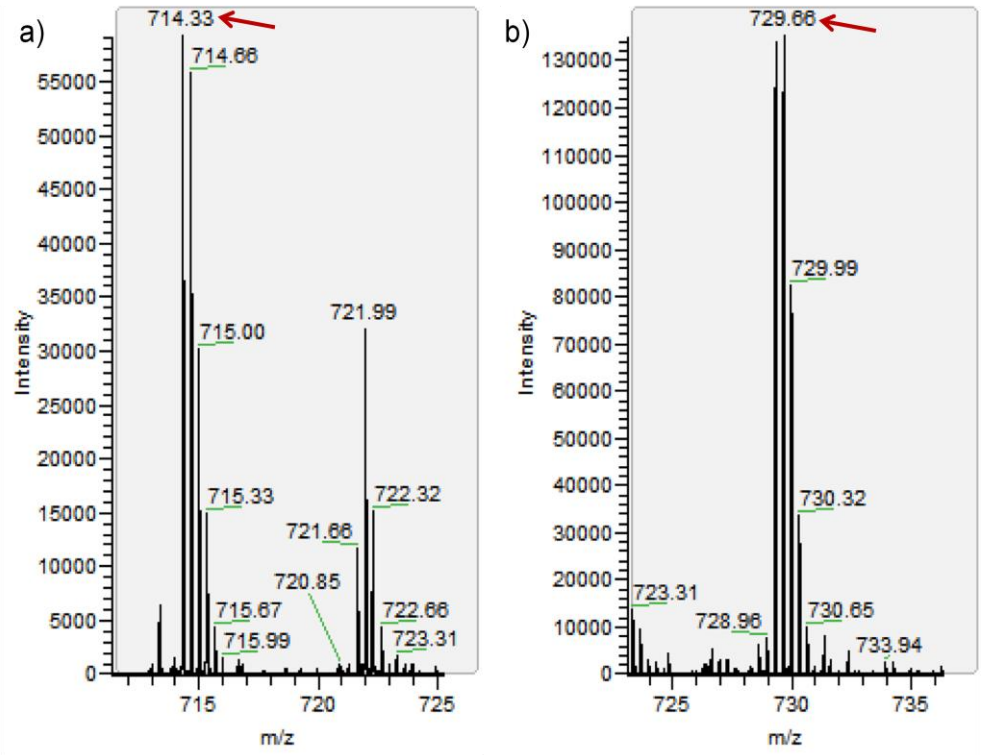


Figure 4-5 MS/MS spectra of CCL2 peptide including Tyr13
Peroxynitrite modified CCL2 was analysed by LC-MS/MS following trypsin digest to establish which residues were modified. Shown is the peptide containing Tyr13 (-.QPDAINAPVTCCYNFTNR.K). a) unmodified (714.3) b) tyrosine nitration (729.6). n=2

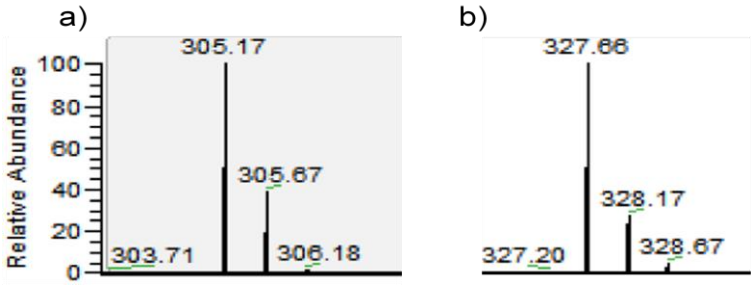


Figure 4-6 MS/MS spectra of CCL2 peptide including Tyr28
Peroxynitrite modified CCL2 was analysed by LC-MS/MS following trypsin digest to establish which residues were modified. Shown is the peptide containing Tyr28 (R.LASYR.R). a) unmodified (305.17), b)tyrosine nitration (327.66). n=2

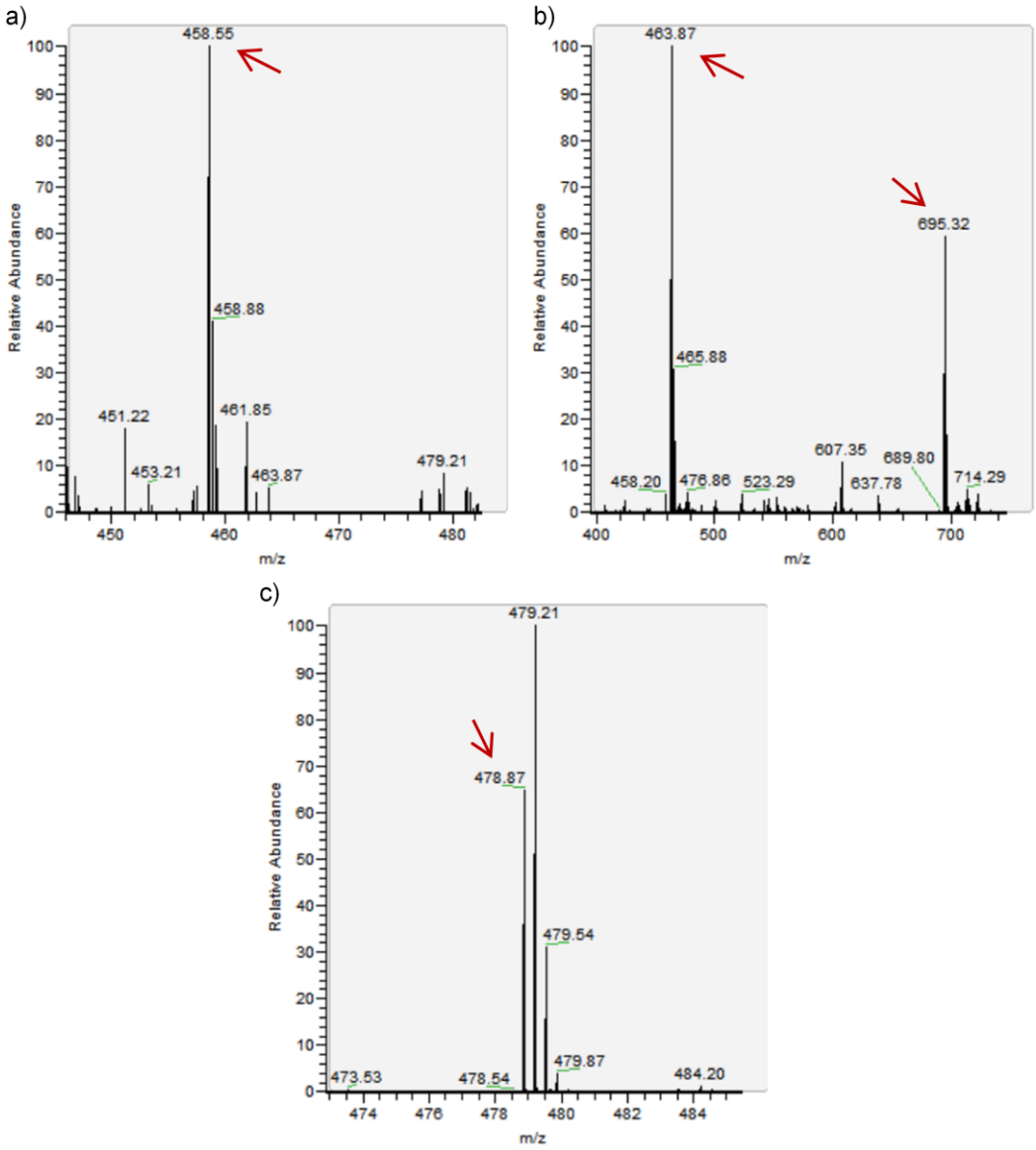
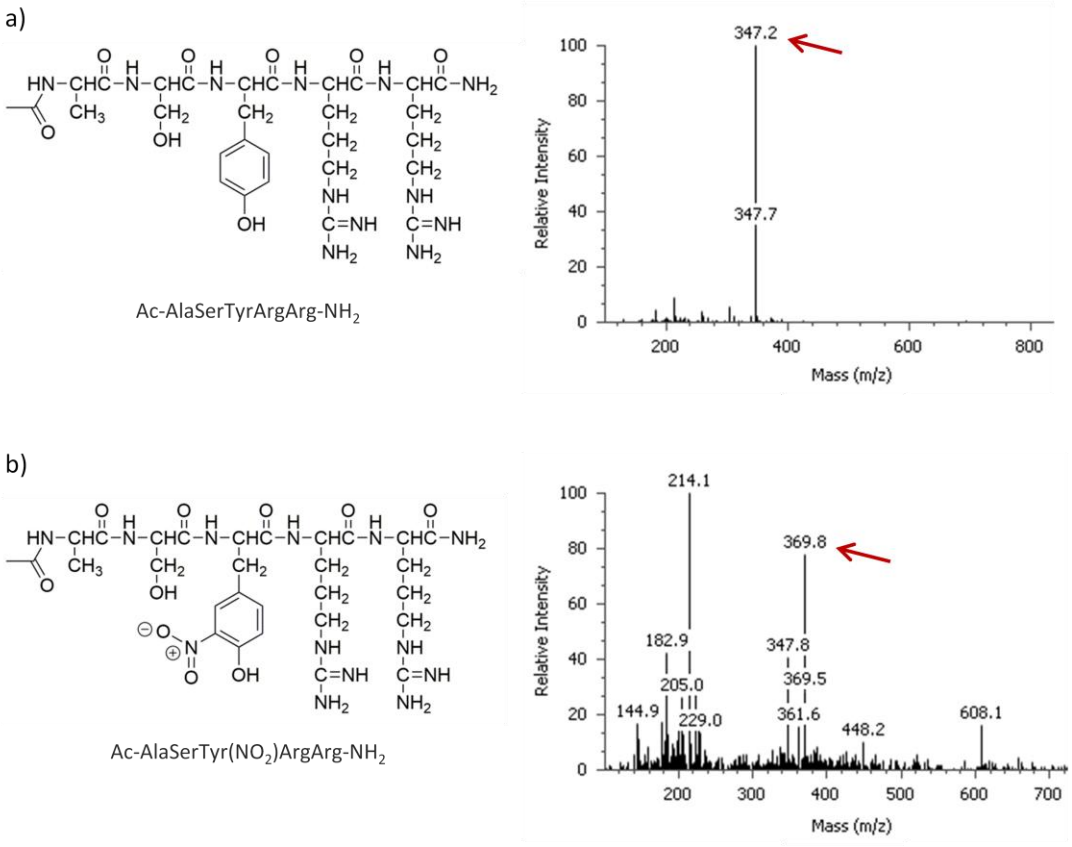


Figure 4-7 MS/MS spectra of CCL2 peptide including Trp59
Peroxynitrite modified CCL2 was analysed by LC-MS/MS following trypsin digest to establish which residues were modified. Shown is the peptide containing Trp59 (K.WVQDSMDHLDK.Q). a) unmodified (458.5), b) methionine oxidation (463.87 and 695.3; can occur during MS), c) methionine oxidation with tryptophan nitration (478.87). n=2

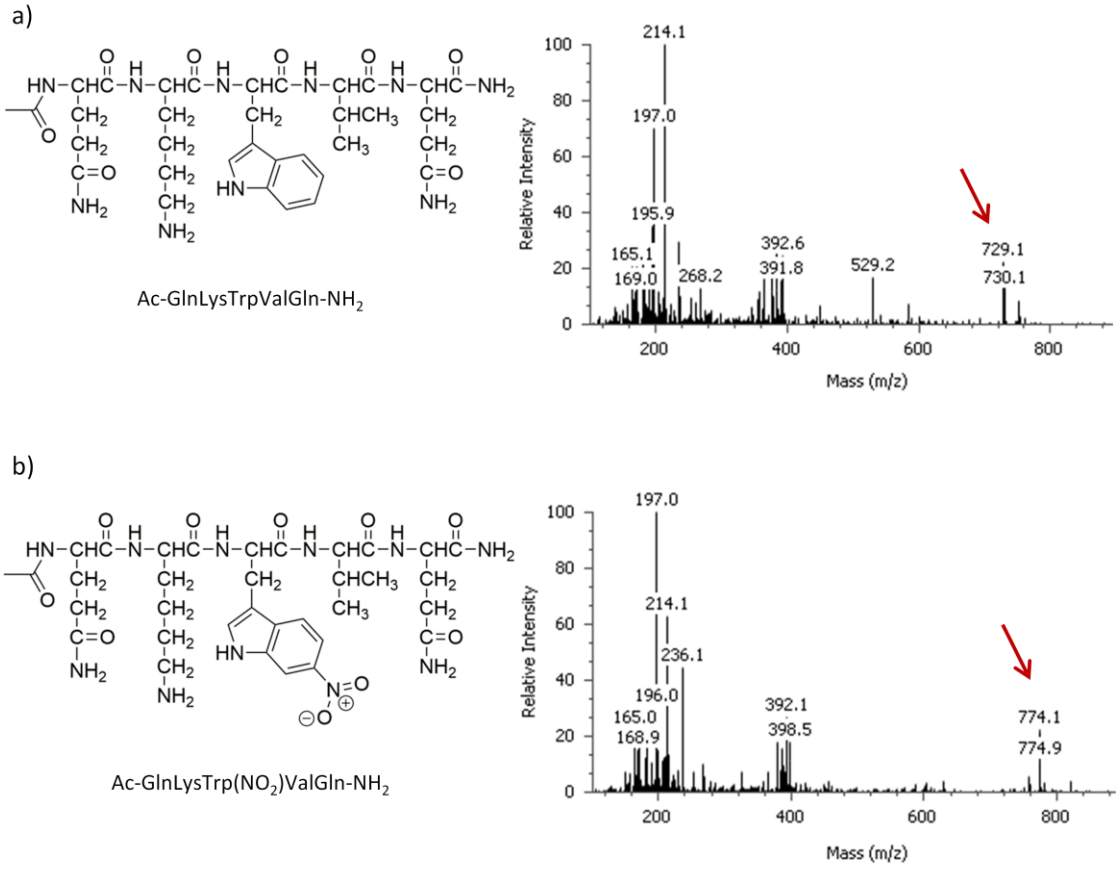


Predicted: Ac-ASYRR-NH_2 peptide MW of 692 and equivalent nitration MW of 737

Ac-ASYRR-NH_2: **MS *m/z* (ESI+) $[M+H]^+$ unobserved, 347.2 ($[M+2H]^{2+}$. $C_{29}H_{50}N_{12}O_8$ requires 347.2)**

Ac-$ASY_{(NO2)}$RR-NH_2: **MS *m/z* (ESI+) $[M+H]^+$ unobserved, 369.8 ($[M+2H]^{2+}$. $C_{29}H_{49}N_{13}O_{10}$ requires 369.7)**

Figure 4-8 Synthesis and nitration of the peptide ASYRR
a) Peptide structure (exact mass 692.37) and electrospray ionisation mass spectrometry (ESI-MS) plot showing correct peptide synthesis.
b) Peptide structure (exact mass 737.36) and ESI-MS plot showing nitration of the peptide following incubation with peroxynitrite.



Predicted: Ac-QKWVQ-NH_2 peptide MW of 728 and equivalent nitration MW of 773

Ac-QKWVQ-NH_2: **MS *m/z* (ESI+) 729.2 ($[M+H]^+$.$C_{34}H_{53}N_{10}O_8$ requires 729.4), 365.2 ($[M+2H]^{2+}$. $C_{34}H_{54}N_{10}O_8$ requires 365.2)**

Ac-QKW$_{(NO2)}$VQ-NH_2: **MS *m/z* (ESI+) 774.1 ($[M+H]^+$. $C_{34}H_{52}N_{11}O_{10}$ requires 774.4) 387.2 ($[M+2H]^{2+}$. $C_{34}H_{52}N_{11}O_{10}$ requires 387.7)**

Figure 4-9 Synthesis and nitration of the peptide QKWVQ
a) Peptide structure (exact mass 728.40) and electrospray ionisation mass spectrometry (ESI-MS) plot showing correct peptide synthesis
b) Peptide structure (exact mass 773.38) and ESI-MS plot showing nitration of the peptide following incubation with peroxynitrite.

Chemokine nitration was found to alter protein recognition by some antibodies. For CCL2, both the monoclonal and polyclonal antibodies tested could not detect the nitrated form of the protein, as shown in Figure 4-10. Similar findings were found for CXCL8 (Figure 4-11) as neither a sandwich ELISA using both monoclonal and polyclonal antibodies against CXCL8, nor a direct ELISA using the same polyclonal, could detect the nitrated form of the chemokine. For CCL5 however the epitope damage was not as severe as although the monoclonal antibody tested could not detect the nitrated form, the polyclonal could. Figure 4-12a shows a sandwich ELISA for CCL5 is unable to efficiently detect the nitrated form of the chemokine. A second sandwich ELISA was developed using an anti-3-nitrotyrosine antibody as the capture antibody and the same anti-CCL5 polyclonal detection antibody. Figure 4-12b shows this ELISA can specifically detect nCCL5 and so the anti-CCL5 polyclonal used in this instance is able to detect the nitrated form of the chemokine.

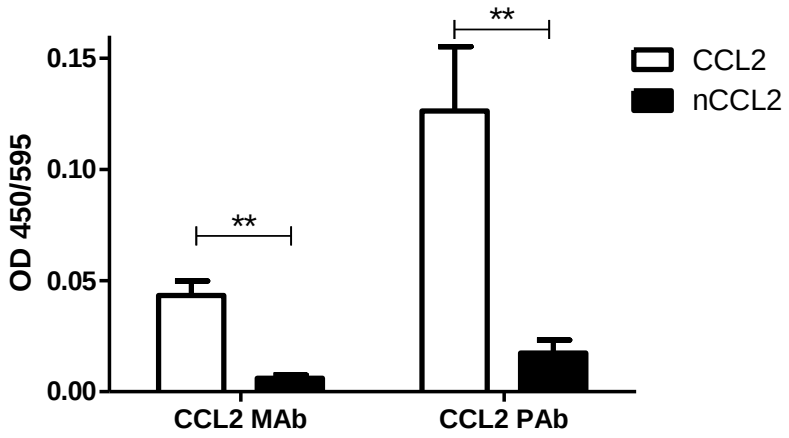


Figure 4-10 CCL2 nitration by peroxynitrite prevents detection by ELISA
By direct ELISA both the R&D monoclonal (MAb; MAB2791) and polyclonal (PAb; AB-279-NA) antibody can detect wild type CCL2 but cannot detect the nitrated form (nCCL2). Statistics - t-test, MAb - p =0.0014, PAb - p =0.0062. Representative of three experiments.

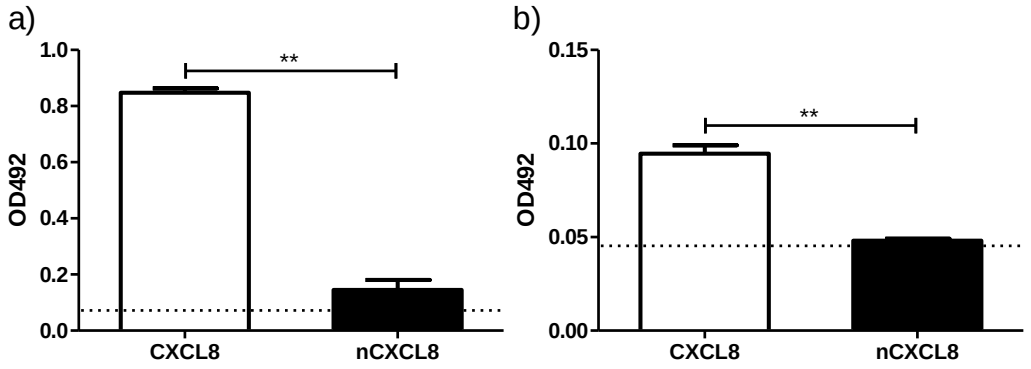


Figure 4-11 CXCL8 nitration by peroxynitrite prevents detection by ELISA
By a) sandwich ELISA (Duoset) and b) direct ELISA (using polyclonal from Duoset), wild type CXCL8 can be detected but the nitrated form cannot. Negative control (diluent only) displayed by dotted line. Representative of three experiments.

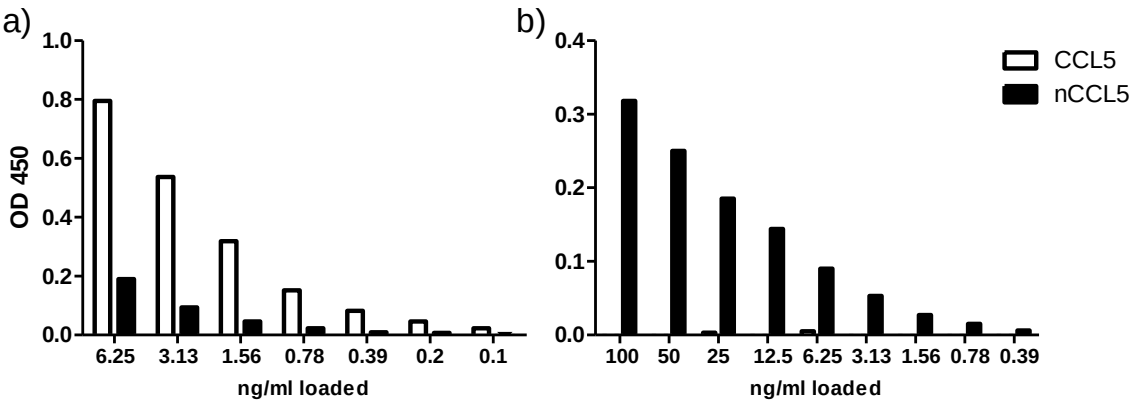


Figure 4-12 Detection of nitrated CCL5 by ELISA
CCL5 incubated with peroxynitrite was analysed on two sandwich ELISAs. a) CCL5 Duoset (R&D Systems), b) polyclonal anti-3-nitrotyrosine capture antibody with biotinylated polyclonal anti-CCL5 detection antibody. Representative of three experiments.

To ensure loss of epitope was not due to protein loss two methods were used, and for these experiments BSA was used instead of chemokine. Firstly, total protein level was assessed using a BCA assay (Figure 4-13). This showed that total protein levels were the same with or without peroxynitrite. To assess protein degradation, SDS-PAGE was performed. As seen in Figure 4-14, protein is not degraded using this method of nitration as equal levels of protein are present in the corresponding lanes.

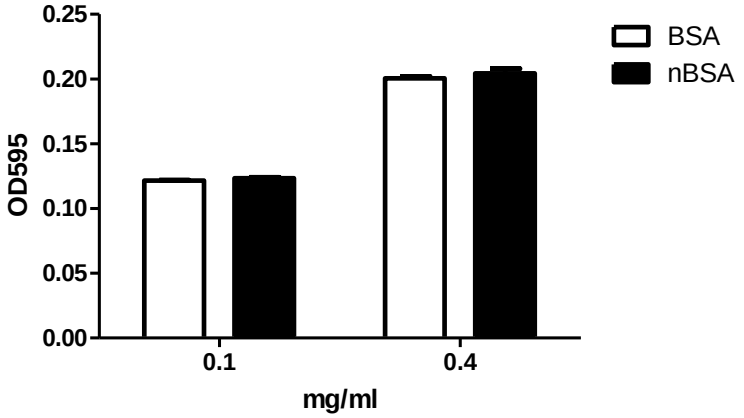


Figure 4-13 Protein levels following nitration
BCA assay kit was used to confirm no loss of protein following nitration. BSA was incubated with peroxynitrite for 10min, dialysed, then protein levels measured by BCA assay. n=2

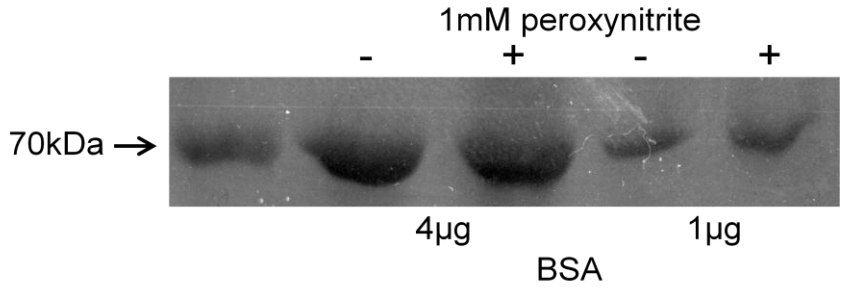


Figure 4-14 SDS-PAGE of BSA following nitration
SDS-PAGE was used to confirm no degradation of protein following nitration. BSA was incubated with 1mM peroxynitrite for 10min at 37°C, run on a 10% SDS-PAGE and stained with SimplyBlue SafeStain (Invitrogen). Representative image of three experiments.

To determine whether nitrated chemokine could be detected in an *in vivo* setting, the ELISAs used in Figure 4-12 were used to test chemokine previously isolated from platelet samples in Prof Paul Proost's lab. The platelets were taken from healthy volunteers between 1988-1995 but no further information was available. Figure 4-15 shows that CCL5 was present in all batches tested but in no samples were detectable levels of nitrated tyrosine found.

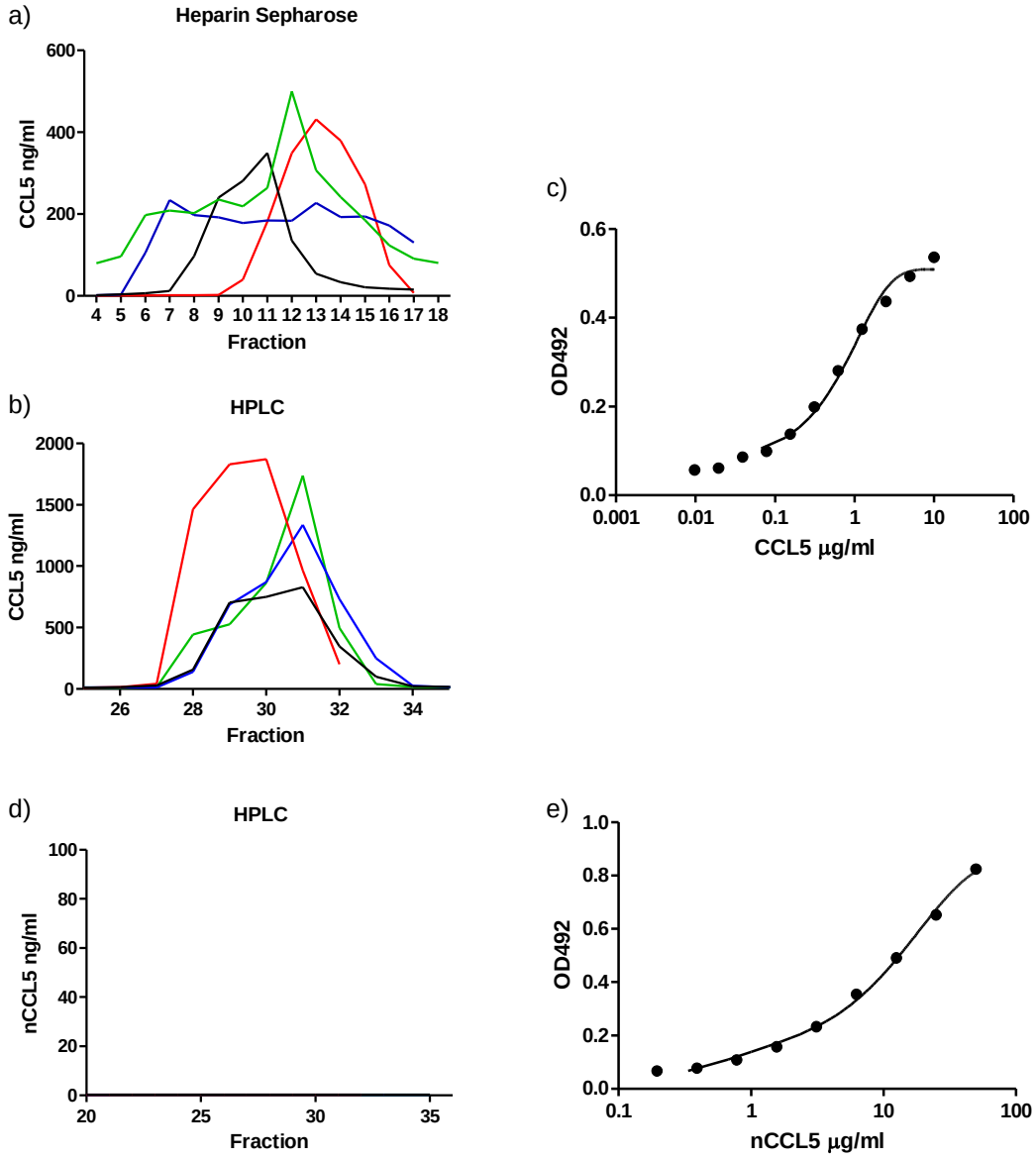


Figure 4-15 CCL5 isolated from platelets does not have detectable levels of nitration
a & b show levels of CCL5 present in several batches of platelet samples purified by heparin-sepharose and then HPLC columns. c) example standard curve used for CCL5 concentration calculations. CCL5 Duoset (R&D Systems) was used for CCL5 detection.
d & e used a specific nCCL5 ELISA to determine levels of nitration in the samples. d) nCCL5 concentrations in HPLC fractions, e) standard curve.

4.3.2 *CHEMOKINE CITRULLINATION*

Previously, CXC chemokines have been shown to be targets of citrullination by PAD. This preliminary study shows that CCL5 is also a target of these enzymes. As citrullination is only a 1Da change in mass this cannot easily be detected but further specific chemical modification of the citrulline creates a detectable difference. The modification process requires strongly acidic conditions; Figure 4-16 shows these conditions do not alter epitope and so sandwich ELISAs can be used for the detection of the modified chemokine. Antibodies have been made against modified citrulline and can be used in ELISAs, allowing the detection of citrullinated chemokine. Figure 4-17a shows an enzyme:substrate ratio of 1:10 is sufficient for citrullination of CCL5 but 1:100 is too low for detectable modification.

The modification citrullination is the result of enzymatic modification of arginine residues by PAD enzymes. To establish the potential for chemokine citrullination in the models used in this study, the presence of such enzymes was determined in liver and kidney epithelial cell lines (H69 and HKC8 respectively) and primary neutrophils and PBMC. There are five forms of human PAD and PAD2 and PAD4 were chosen for analysis using qPCR as they have previously been detected in liver and kidney (Moelants *et al.*, 2012). These results show that PAD4 was present at low levels in both epithelial cell lines but PAD2 was absent. RNA of both enzymes was present in both neutrophils and PBMCs (Figure 4-18).

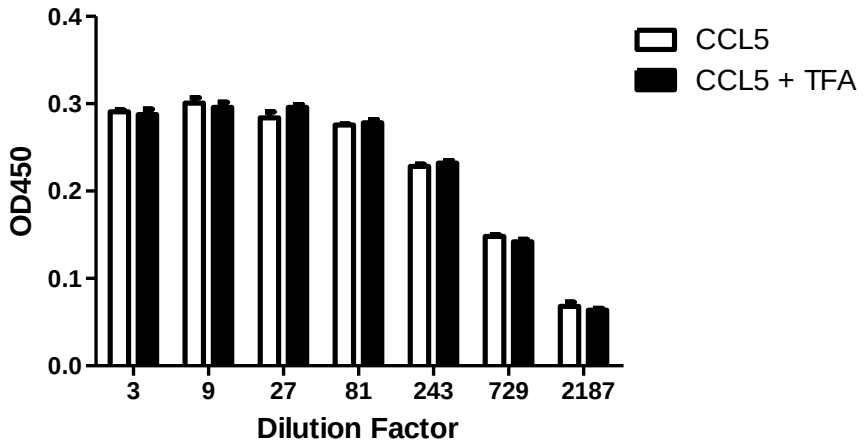


Figure 4-16 Incubation of CCL5 with TFA does not impair detection
Detection of CCL5 following incubation with TFA using a CCL5 Duoset (R&D Systems). Detection was using TMB and OD from blank samples subtracted. Performed in duplicate.

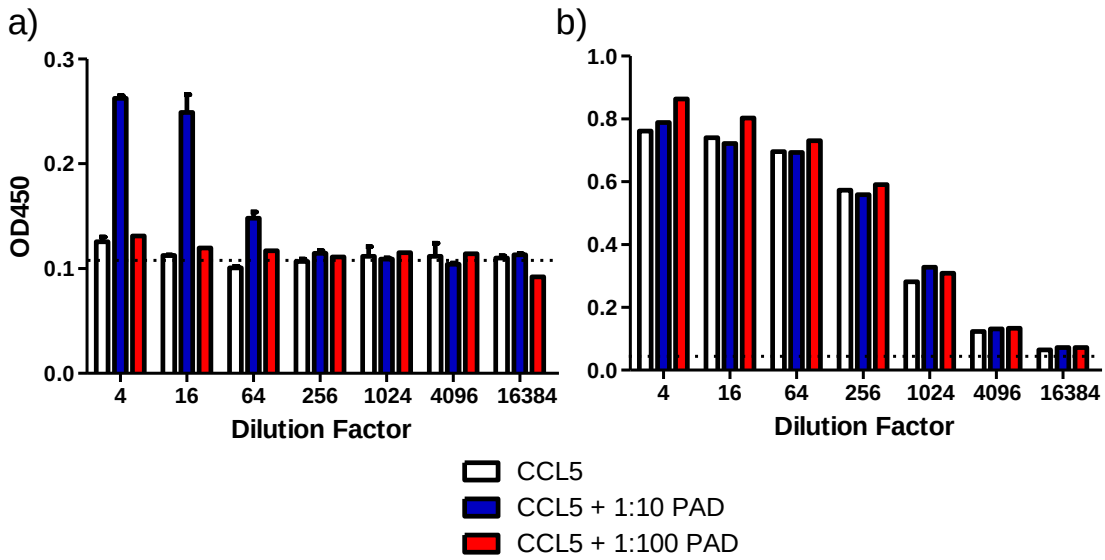


Figure 4-17 CCL5 citrullination
CCL5 incubated with 1:10 or 1:100 enzyme:substrate molar ratio of PAD for 90mins then the citrulline modified using antipyrine and 2,3-butanedione. a) ELISA using anti-CCL5 capture antibody and detected using an antibody specific for modified citrulline. b) CCL5 sandwich ELISA. Negative control (diluent only) displayed by dotted line. E/S 1:10 representative of four experiments.

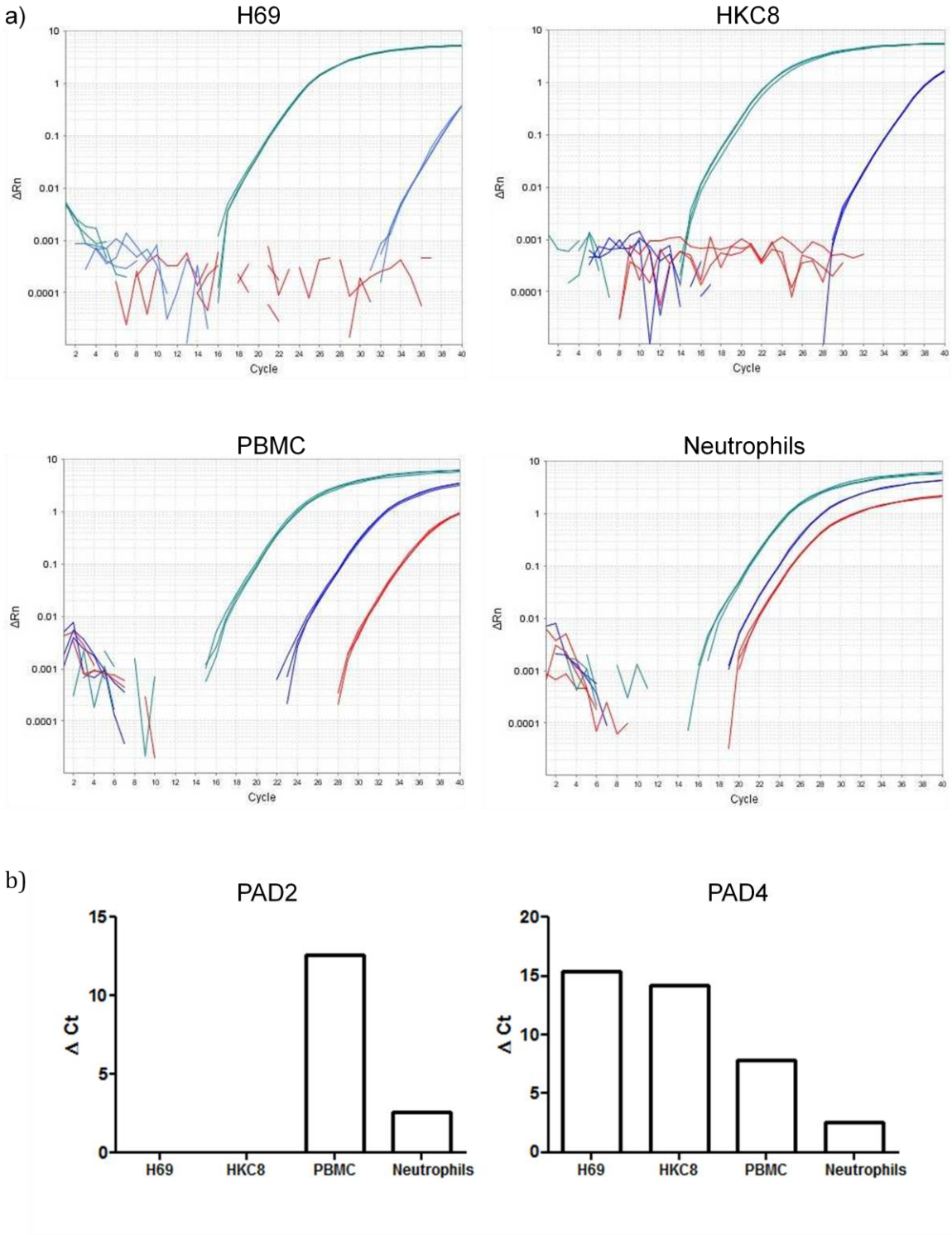


Figure 4-18 Presence of PAD in epithelium
a) Taqman qPCR amplification plots showing PAD2 (red), PAD4 (blue) and GAPDH (green) gene expression in H69 and HKC8 cell lines and primary PBMC and neutrophils. b) delta Ct values relative to GAPDH. n=2

4.4 DISCUSSION

The regulation of chemokines is a complex process, especially within the inflammatory environment, and one which is not fully understood. The findings in this chapter of new targets of post-translational modification, namely the less well studied nitration and citrullination, further add to the complexities.

All three prototypical inflammatory chemokines assayed were found to be nitrated following incubation with peroxynitrite. For CCL5 and CXCL8, the current lack of mass spectrometry data means that the exact residues modified are unknown, but 3-nitrotyrosine formation occurs, as shown by ELISA. In the case of CCL2, more detailed analysis was performed and Tyr13, Tyr28 and Trp59 were all found to be nitrated. This is in contrast to a previous study which only identified nitrated tryptophan in human CCL2 (Molon *et al.*, 2011). Neither study found oxidised histidine residues or other modifications which peroxynitrite could be predicted to cause (Pacher *et al.*, 2007).

As discussed in the introduction to this chapter, assessing mechanisms for the detection of both modified and wild type chemokines is important. Nitrated CCL2 was unable to be detected by either antibody tested, although the loss of epitope of the monoclonal antibody used can be easily explained, the complete loss of detection by a polyclonal is harder to comprehend. Protein degradation is one explanation for this loss of antibody recognition and for this reason additional experiments were carried to establish if peroxynitrite incubation causes such degradation. The results show that it is loss of antibody sensitivity and not protein degradation which prevents the detection of nCCL2. This again is consistent with findings reported by (Molon *et al.*, 2011), but due to a lack of details in the methods section of this paper, the antibody used is unknown and therefore it is unsure whether these are the same findings or further adding to the number of commercially available antibodies which cannot detect nCCL2. Similar findings for CXCL8 and CCL5 show that the lack of detection of nitrated chemokines with some currently produced antibodies is not a finding exclusive to CCL2. The observation that the polyclonal antibody tested for nCCL5 detection was able to detect the nitrated form of the chemokine further supports evidence that it is epitope loss and not protein degradation and highlights the need for a thorough screen of

available antibodies. It is crucial to determine which forms of chemokines are being detected with which antibody based detection techniques and if large quantities of chemokine are potentially being missed. The difference in detection difficulties between CCL2 and CXCL8, and CCL5 highlight that this is not a 'one fits all' problem.

Although attempts to identify nitrated chemokine from biological samples were unsuccessful this is not entirely surprising and does not prove that chemokine nitration does not occur *in vivo*. This study was carried out as a test for the newly developed nCCL5 ELISA, for which chemokine in a soluble form is needed. Previously isolated CCL5 was the only chemokine source readily available at the time. However this had been purified from the platelets of healthy individuals and therefore there it is unlikely there were high concentrations of peroxynitrite and other reactive species present to cause nitration. Some of the samples were 20 years old and the stability of nitration at -80 for this length of time is unknown. It is therefore possible that samples were originally nitrated although detection is no longer possible. To fully test this ELISA as a method for nCCL5 detection, chemokine from an inflammatory environment in a soluble form needs to be used, for example synovial fluid from a patient with rheumatoid arthritis or urine from a patient with an inflammatory kidney disease.

Chemokine nitration has previously been identified *in vivo* for CCL2 by IHC and ELISA (Molon *et al.*, 2011), although the antibody used in this study did not give positive results by ELISA or western blot in our hands (data not shown). One explanation for this could potentially be that Molon *et al.,* 2011 identified Trp59 as the sole site of nitration, and three sites have been found in this study. The antibody may be specific for nitration only at one position and so unable to detect more heavily nitrated CCL2. *In vivo* detection of nitrated protein in sites rich in chemokines has been shown in chapter 3 and also in numerous other studies of transplantation and inflammatory environments (Macmillan-Crow *et al.*, 1996; Moon *et al.*, 2008) and so chemokine modification is likely to occur.

Citrullination is the other modification studied in this chapter and so far work has focused on CXC chemokines and other cytokines including TNF-α (Loos *et al.*,

2008; Proost *et al.*, 2008; Loos *et al.*, 2009; Struyf *et al.*, 2009; Moelants *et al.*, 2013b). The findings in this study show that CC chemokines can also be targets of modification by PAD. Although no functional work has yet been carried out on this modified chemokine, previous work on citrullination would suggest that modification of arginine residues in this manner would lead to a decrease in *in vivo* function due to a decrease in GAG binding ability and potentially receptor binding as well. Further work is needed to determine if citrullination is also a downregulator of proinflammatory CC chemokines. The results of the PCR data support previous findings of PAD expression in liver and kidney cells and in leukocytes (Moelants *et al.*, 2012). Although PAD2 has previously been found in liver and kidney it may not be specifically expressed in the epithelium and so not present in the cell lines tested.

Data in this chapter demonstrates that nitration, a post-translational modification which occurs during inflammation and IRI, occurs to chemokines. Not only does this modification occur to the chemokines evaluated, but also impairs their detection. CCL5 has been shown to be subject to citrullination, another inflammation associated post-translational modification. Overall, this adds to the complexity of chemokine regulation and highlights the need for determining which forms of chemokine are being detected when establishing chemokine presence, or absence, especially in an inflammatory environment.

5 FUNCTIONAL EFFECTS OF CHEMOKINE NITRATION

5.1 INTRODUCTION

The biological behaviour of chemokines within the inflammatory environment is often assumed to be that of wild type chemokines, as assessed *in vitro*. However due to the complexities of both inflammation and chemokine regulation, this is not necessarily the case. Chemokine levels in a disease do not always correlate to chemokine function within that environment.

Chapter 3 demonstrated that chemokines can be modified during stress (section 3.3.3), and within the highly oxidative environments of inflammation and IRI, protein nitration can occur (section 3.3.4). Section 4.3.1 went on to show chemokines can be targets of such nitration by reactive nitrogen species but the function of these modified chemokines is yet to be assessed. This is the aim of this chapter. Not only do the mechanisms for chemokine modification need to be established, but the function of these modified chemokines also needs to be characterised. *In vitro* observation of the modified chemokines however is not sufficient. Confirming that these modifications occur *in vivo* and are not altered by GAG binding, or other post-translational modifications is also important (Sadir *et al.*, 2004; Ellyard *et al.*, 2007; Proost *et al.*, 2008).

Chemokines with altered function have been detected in inflammatory disease. For example CXCL10 truncation abrogates function (Proost *et al.*, 2001; Van den Steen *et al.*, 2003a). Truncated CXCL10 has been found in hepatitis C patients (Casrouge *et al.*, 2011; Ragab *et al.*, 2013; Riva *et al.*, 2014). These highlight the need to determine the function of chemokines present in disease, as truncated CXCL10 was identified in a study which aimed to resolve the paradox that high CXCL10 levels correlated with low response to therapy in hepatitis C patients; the CXCL10 present is an antagonist. Other chemokine modifications during disease have also been suggested, for example, citrullination of CXCL12 during Crohn's disease, a modification which abrogates function (Struyf *et al.*, 2009), and chemokine truncation in multiple cancers as reviewed by Van Damme *et al.*, 2004. Not all modifications decrease chemokine function. N-terminal cleavage of ELR^+ CXC chemokines predominantly enhances neutrophil recruitment. For example, CXCL7

cleavage is necessary for function (Walz and Baggiolini, 1990) and CXCL8 (6-77) is more active than the full length form (Van Damme *et al.*, 1988). Pathogens can also modify chemokines to evade the immune system and so evaluating the function of these chemokines and processes for modification may also help with infection control and prevention (Mikolajczyk-Pawlinska *et al.*, 1998; Edwards *et al.*, 2005; Kurupati *et al.*, 2010; Moelants *et al.*, 2014).

The proportion of chemokines which undergo modification is unknown, and there are multiple post-translational modifications identified which are yet to be examined for their effect on chemokines, including acetylation and carbamylation. Few studies have examined the level of modification which occurs but one showed that truncated CCL5 makes up less than 10% of the CCL5 produced by platelets, but in whole blood this percent rises to 60 (Struyf *et al.*, 1998a). Other cell types are also able to produce large amounts of the modified chemokine with 95% and 100% of CCL5 produced by diploid fibroblasts and sarcoma cells respectively being truncated (Struyf *et al.*, 1998a).

Even if the proportion of chemokines modified is relatively low, the effects can still be significant. Chemokines which are still able to bind the receptor but not signal can be antagonistic to the other 'normal' chemokines present (Proost *et al.*, 2001), as can chemokines which are able to bind and signal normally but are unable bind GAGs. In such cases chemokine gradients cannot be formed but receptor desentisiation can occur resulting in an anti-inflammatory effect (O'Boyle *et al.*, 2009; Ali *et al.*, 2010). Modified chemokines may also be able to form homo- or hetero-oligomers with wild type chemokines, potentially further altering function.

All this evidence highlights the need to establish the function of chemokines within the inflammatory environment and not merely the simple presence of the proteins. For example, any biopsy stained for chemokines is only seeing part of the story; *in vivo* function cannot be established by antibody staining. This is especially important in inflammatory conditions where many modifications are possible.

5.1.1 *SPECIFIC AIMS*

This chapter examined the alteration in chemokine biology following nitration by peroxynitrite, focusing on CCL2. Specifically these aspects were examined:

- Chemotaxis - bare membrane, transendothelial and *in vivo*
- Receptor binding and intracellular signalling
- GAG binding

5.2 SPECIFIC MATERIALS AND METHODS

5.2.1 *RADIOLIGAND BINDING ASSAY*

Radioligand competition assays were performed to assess differences in CCR2 binding between CCL2 and nCCL2. These are competition assays in which radiolabelled CCL2, in this case labelled with ^{125}I, is used as a tracer to compete with unlabelled chemokine to determine ligand affinity for the receptor. For these experiments the affinities of CCL2 and nCCL2 for CCR2b were determined. The higher the radiation level at the end of the experiment, the less unlabelled protein is bound and so the lower the affinity of the chemokine for its receptor.

200,000 HEK-CCR2b per tube were resuspended in 30µl binding buffer (1mM $CaCl_2$, 5mM $MgCl_2$, 50mM HEPES, 0.5% BSA, pH7.2). 100µl CCL2 or nCCL2 in binding buffer was then added at a final concentration of 0.1-650nM. Finally a tracer amount of [^{125}I]-CCL2 (Perkin Elmer) in 20µl binding buffer was added and tubes incubated on ice with agitation for 2hr. Cells were then washed twice using wash buffer (binding buffer with 0.5M NaCl) before resuspending cells in 1ml dH_2O. A negative control with no un-labelled chemokine added was also performed. Counts per minute (CPM) were measured using a gamma counter (Perkin Elmer; Medical Physics department, Royal Victoria Infirmary, Newcastle, UK) with the help of J Lloyd and E Howell and background radiation readings subtracted from results. All radiation work was performed in the designated radiation laboratory. Experiments were performed three times in triplicate and IC50 values calculated using 'one site competition analysis' in GraphPad Prism 5.0.

5.2.2 *PROTEOME PROFILER ANTIBODY ARRAYS*

To see if CCL2 and nCCL2 trigger different intracellular signalling pathways in THP-1 Proteome Profiler™ antibody arrays (R&D Systems) were used. The kits used in this study were the human phospho-MAPK array and the human phospho-kinase array and were carried out according to the manufacturer's instructions. Briefly, membranes which are pre-spotted with capture antibodies were blocked and then incubated with a mix of cell lysate and biotinylated-detection antibody overnight. After washing, membranes were incubated with streptavidin-HRP followed by chemi-luminescent reagent and manually developed. Densitometry

analysis was performed using Photoshop, and the density value normalised to reference spots on each membrane, minus the negative control value was plotted.

5.2.2.1 Cell Stimulation and Lysis

THP-1 were allowed to settle out of suspension overnight as previous work found centrifugation caused high levels of background MAPK activation (Chaffey, 2004). Cells were then gently resuspended at approximately $2.5x10^{-6}$ cells per ml in serum free RPMI for 2hr at 37°C prior to a 5min incubation (O'Boyle *et al.*, 2007) with or without 10nM chemokine. Cell lysate was then prepared as per kit instructions. Briefly, cells were washed with PBS and resuspended at 1 x 10^7 cells/ml in lysis buffer. For lysis to occur samples were incubated at 4°C for 30min with agitation before centrifuging for 5min at 14 000xg. To ensure equal sample loading, protein levels were then determined using a BCA assay and compared to a standard curve (Figure 5-1), as described in section 2.7, and 250µg protein added to each membrane.

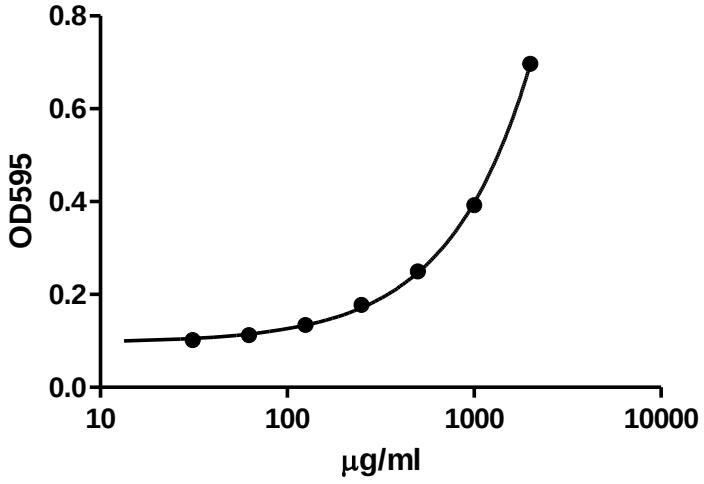


Figure 5-1 BCA protein assay standard curve
Example standard curve from a BCA assay used to determine the protein concentration in cell lysates.

5.2.3 *HEPARIN BINDING ASSAYS*

5.2.3.1 Development of solid-phase heparin binding assay

Solid phase heparin assays were performed to establish alteration in heparin binding between wild type and nitrated CCL2. These assays are ELISA based assays, similar to the basic protocol described in section 2.5.1, but instead of a capture antibody, plates are coated with heparin. Due to technical difficulties there were many stages in the development of these assays.

First, Immulon 4HBX microtitre plates were coated overnight with 250µg/ml poly-L-lysine (Sigma) at 4°C to create a positively charged surface for heparin to bind to (all volumes are 100µl). Heparin (25µg/ml, Sigma) was then incubated for 2hr at RT before blocking with 1% BSA/PBS for 1hr. 10-1000ng/ml CCL2 was added for 1hr and then detected using a polyclonal anti-CCL2 antibody (1/1500, 2hr, RT; AB-279-NA, R&D Systems) and anti-goat-HRP (1/5000, 2hr, RT; A5420, Sigma). The assay was developed using OPD but there was no detectable signal.

In order to determine if the failure to detect bound CCL2 was due to a lack of heparin binding, 1,9-Dimethyl-methylene blue (DMMB) solution (35mM NaCl, 40mM glycine, 40µM DMMB, pH3.0) was used. This should change from blue to purple in the presence of GAGs. Poly-L-lysine however was found to prevent this colour change occurring, presumably by interfering with the charge which is important for the colour change, so this method could not be used to check that heparin was bound to the plate.

To establish if the lack of detection was a problem with the antibodies used a direct ELISA was performed, using the basic protocol described in section 2.5.1.1. Reagents from a CXCL12 Duoset kit (R&D Systems) were used as a positive control. CCL2 detection was performed as above. The assay was developed using both OPD and TMB, stopped using 1M H_2SO_4 and read at 492nm and 450/595nm respectively. The results showed that the detection system used does detect both CCL2 and CXCL12 in a direct ELISA and that TMB (R&D Systems – from Duoset) is a more sensitive development method (Figure 5-2).

To further investigate if heparin immobilisation was the problem a 4HBX plate coated with poly-L-lysine and a Heparin/GAG binding plate (Iduron) were compared. Both plates were incubated with heparin overnight prior to performing a similar binding assay as first performed. Protocol alterations were made as follows, as recommended by Iduron for use with plates. Plates were coated with 100µl heparin per well – 25µg/ml in standard assay buffer (SAB; 100nM NaCl, 50mM sodium acetate, 0.2% v/v Tween 20, pH7.2) and incubated overnight at RT. Plates were then washed three times in SAB prior to blocking with 150µl/well, 1% BSA/PBS for 1hr at 37°C. Plates were washed before 100µl/well chemokine in 1%

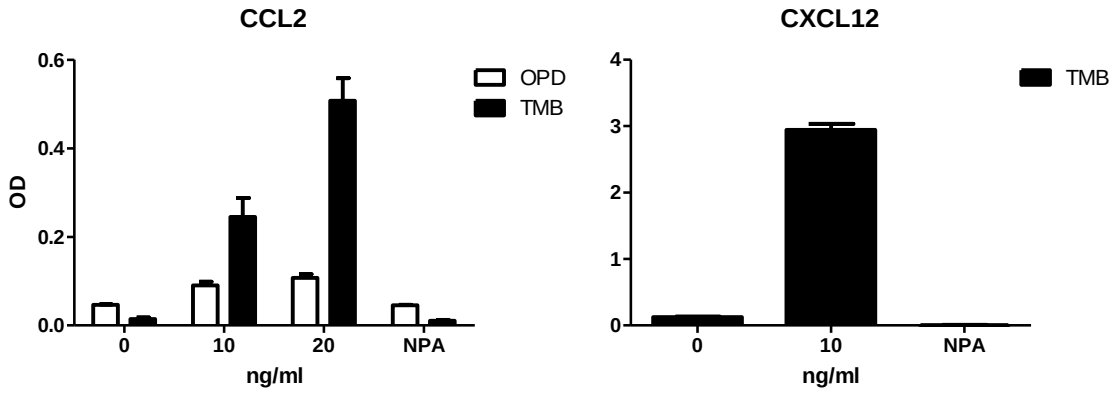


Figure 5-2 Direct ELISA for CCL2 and CXCL12
By direct ELISA both CCL2 and CXCL12 could be detected using OPD and TMB showing the antibodies and detection methods are suitable for the detection of chemokine. No primary antibody (NPA) controls are shown.

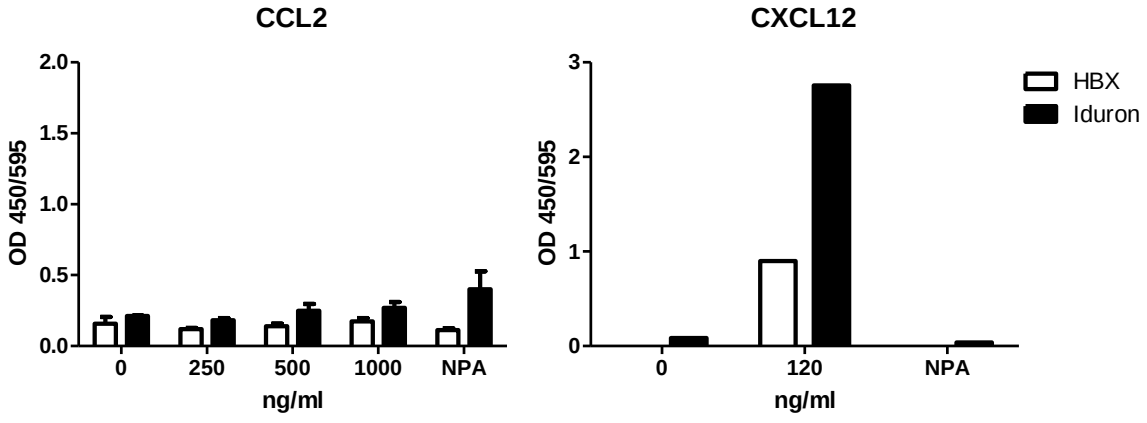


Figure 5-3 Comparison of heparin binding strategies
HBX plates coated with poly-L-lysine and GAG binding plates (Iduron) were compared for their ability to bind heparin, and subsequently chemokine. No primary antibody (NPA) controls are shown.

BSA/PBS added for 2hr at 37°C. Suitable detection antibodies were used and developed using TMB. CXCL12 was detectable on both plates within minutes. After 1hr there was no visible detection of CCL2 above background (Figure 5-3).

If the anti-CCL2 antibody used detected an epitope which overlapped with the heparin binding site the antibody would either be unable to bind, or would compete off the heparin and both CCL2 and antibody would wash off, either way would result in no detection. For this reason a second CCL2 antibody was tested (MAB2791, R&D Systems). Using this antibody heparin bound CCL2 was detected (Figure 5-4).

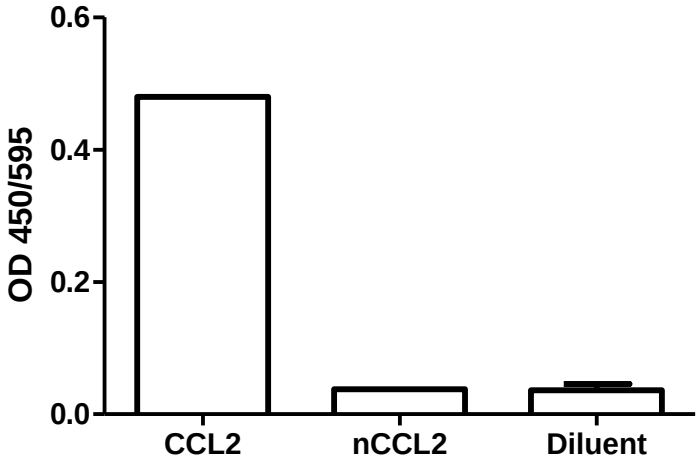


Figure 5-4 Detection of heparin bound CCL2
CCL2 bound to immobilised heparin can be detected using the monoclonal antibody (MAB2791, R&D Systems) with appropriate secondary antibody and TMB.

5.2.3.2 Optimised method

Heparin/GAG binding plate (Iduron) was coated with 100µl heparin per well – 25µg/ml in SAB and incubated overnight at RT. Plates were then washed three times in SAB prior to blocking with 150µl/well, 1% BSA/PBS for 1hr at 37°C. Plates were washed before 100µl/well chemokine in 1% BSA/PBS was added for 2hr at 37°C. Plates were then washed prior to incubation with detection antibody. For CCL2 this was MAB2791, R&D Systems, 0.5µg/ml in block buffer for 2hr at RT. After washing, detection antibody was added for 2hr at RT (anti-mouse IgG-HRP, Sigma; 1/5000). For nCCL2 the detection antibodies were anti-3-nitrotyrosine

(1/1000; Millipore) and anti-rabbit IgG-HRP (Sigma; 1/5000). The assay was then developed using TMB, 100µl/well, and stopped with 50µl 1M H_2SO_4. Plates were then read at 450nm with 595nm correction.

5.2.4 *SURFACE PLASMON RESONANCE*

5.2.4.1 General principles

Surface plasmon resonance (SPR) is a powerful and reproducible technique used to study the interaction between molecules without the need for labelling and is the phenomenon used by Biacore systems. The technique involves immobilising one molecule (ligand) onto the surface of a sensor chip, and then flowing the other molecule of interest (analyte) over the surface. Binding of the molecules to the sensor surface generates a response which is proportional to the bound mass, and can be sensitive to changes of a few picograms per mm^2. As binding events are monitored in real time a range of interaction characteristics can be determined.

Biacore chips consist of a glass slide coated with a 50nm layer of gold, acting as an electrically conducting surface; SPR occurs when polarised light hits this surface. Electron charge density waves called plasmons are generated, reducing the light reflected at a specific angle (the resonance angle) in proportion to the mass bound to the chip surface (see Figure 5-5). The interface must be in conditions of total internal reflection for SPR to occur. Alterations in binding can therefore be read out in resonance units (RU).

All SPR work was performed in the Institut de Biologie Structurale, Grenoble, France with the help of Dr Hugues Lortat-Jacob using a Biacore 3000. The running buffer used was HBS-P (10mM HEPES pH7.4, 150mM NaCl, 0.005% P20). Unless stated all reagents are from GE Healthcare.

5.2.4.1 Streptavidin Immobilisation

The surface of Biacore chips are coated with a matrix of covalently attached carboxymethylated dextran, an unbranched carbohydrate polymer which is flexible, allowing movement of the attached ligand. For the experiments in this study a CM4 chip was used. This has a lower level of carboxymethylation than

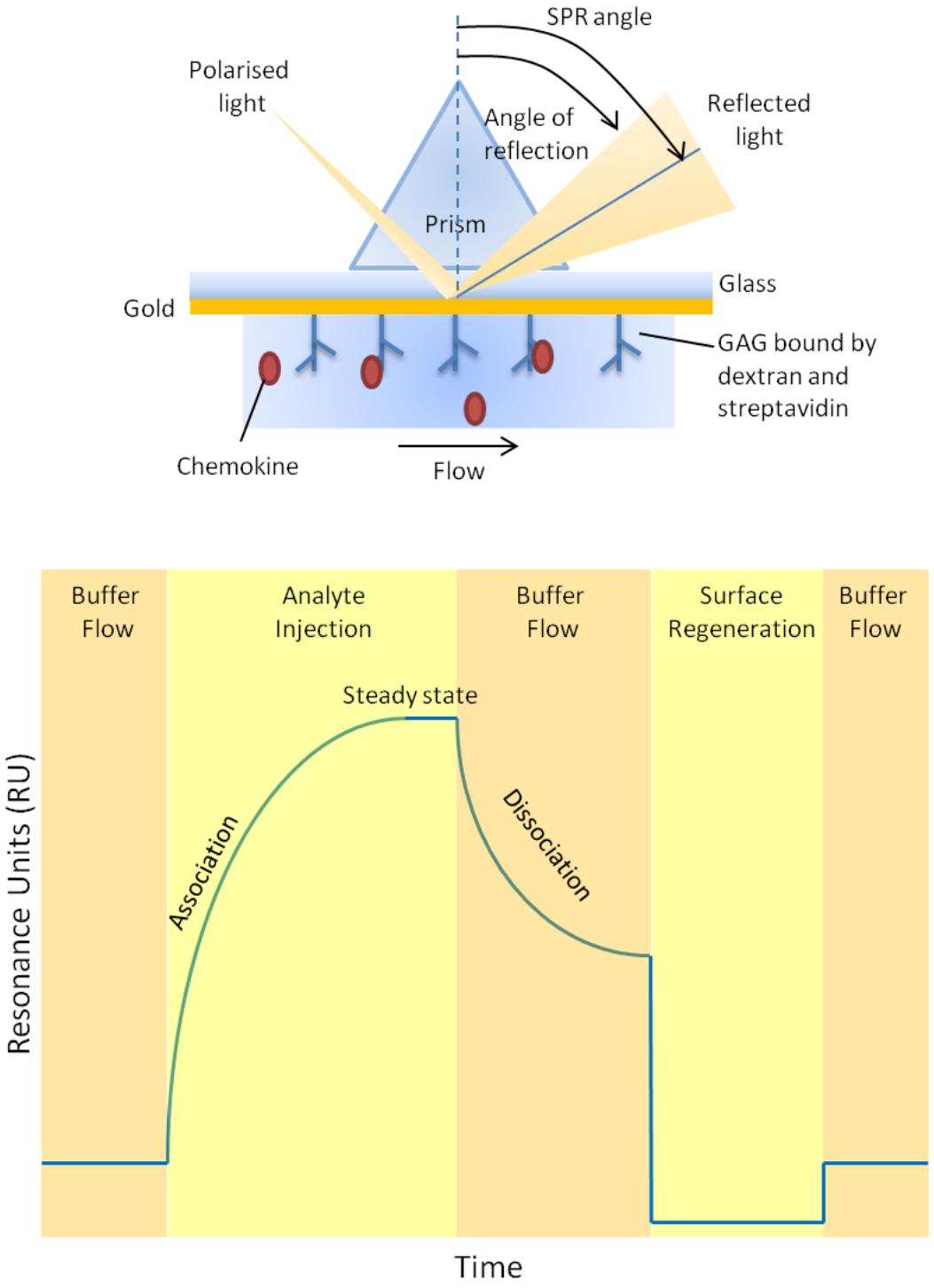


Figure 5-5 Surface plasmon resonance
Upper panel - schematic of surface plasmon resonance. GAG is immobilised to the gold plated sensor surface using dextran and streptavidin. As the chemokine (analyte) binds to the GAG, the refractive index shifts and the SPR angle alters. The movement is the signal monitored, and the change over time forms the sensorgram.
Lower panel - sensogram cartoon showing alteration in RU with association and dissociation of the (chemokine) analyte. The more analyte binds to the chip surface, the larger the increase in RU.

other chips, resulting in reduced ligand immobilisation and a lower surface charge density. This helps to reduce the non-specific binding of positively charged molecules, for example chemokines, and is beneficial when investigating kinetics as low levels of ligand immobilisation levels are optimal. There are several methods for covalent attachment to the chip surface, for streptavidin amine coupling is used. This is stable, allowing the chip to be reused multiple times. For coupling the surface must first be activated with 50µl 0.2M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and 50µl 0.05M N-hydroxysuccinimide (NHS). This activates the carboxyl groups on the chip surface, forming reactive succinimide esters which can spontaneously react with nucleophilic groups such as amines, in this case covalently attaching streptavidin to the chip surface.

The immobilisation process is shown in Figure 5-6. 50µl streptavidin (0.2 mg/ml, Sigma, in 10mM acetate buffer, pH 4.2) was injected over the activated surface. The buffer must be below the isoelectic point of the ligand, but needs to be above the pKa of the surface (pH3.5), in this case pH4.5 was used as the pI of streptavidin is ~pH5. Once streptavidin is immobilised, 1M ethanolamine HCl, pH8.5, is injected as it deactivates any of the remaining active groups and removes any unbound ligand which would interfere with subsequent heparin immobilisation.

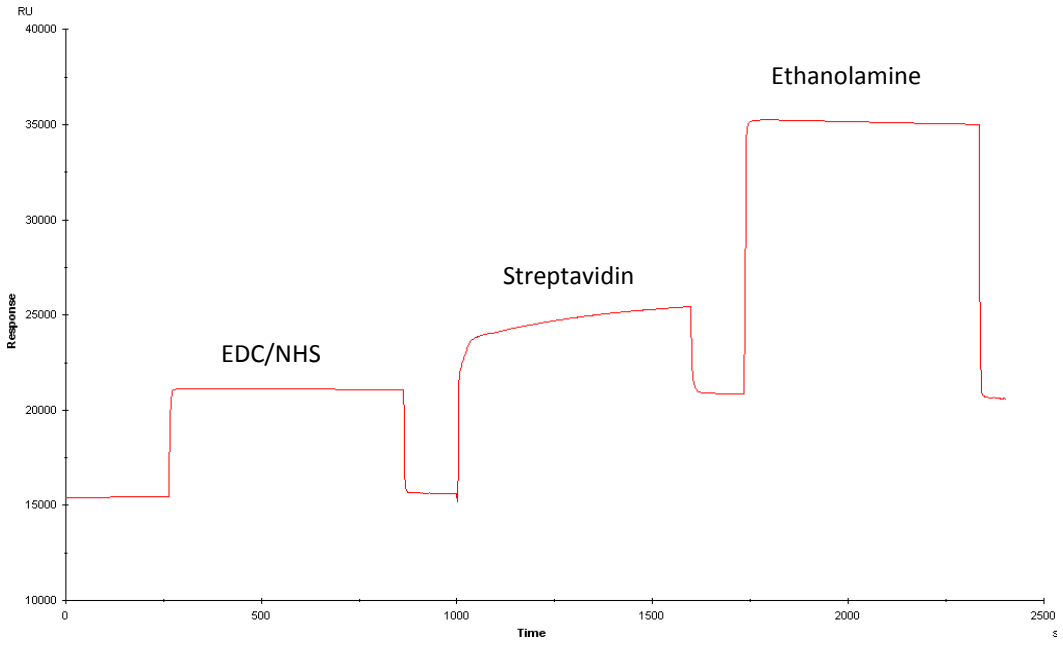


Figure 5-6 Streptavidin immobilisation
Trace showing the activation of a CM4 chip using EDC and NHS, followed by streptavidin immobilisation and deactivation by ethanolamine.

5.2.4.2 GAG Immobilisation

a) GAG Biotinylation

Heparin and heparan sulphate were biotinylated to allow immobilisation on to the chip by streptavadin. Mono-biotinylation at the reducing end of the GAG is important for correct presentation when immobilised.

Biotinylation was performed by H. Lortat-Jacob as described previously (Sadir *et al.*, 2001; Saesen *et al.*, 2013). Briefly, 1mM heparin (9kDa; Sigma) or heparan sulphate (12kDa; Sigma) in PBS was reacted with 10mM biotin-LC-hydrazide for 24hr at RT. This was then dialysed against water to remove any unreacted biotin, and freeze dried for storage.

b) Immobilisation

Biotinylated heparin and heparan sulphate were immobilised as follows. 5-20µg/ml biotinylated heparin in 300mM NaCl, was injected at 10µl/min for 30sec followed by a 2M NaCl wash to remove unbound heparin. Injections were repeated until a total RU of 50-150 was achieved (see Figure 5-7). For heparan sulphate immobilisation, a 5µl injection of 50µg/ml biotinylated heparan sulphate was injected at a flow rate of 5µl/min. The surface was again washed with 2M NaCl and injections repeated until an RU increase of 200 reached.

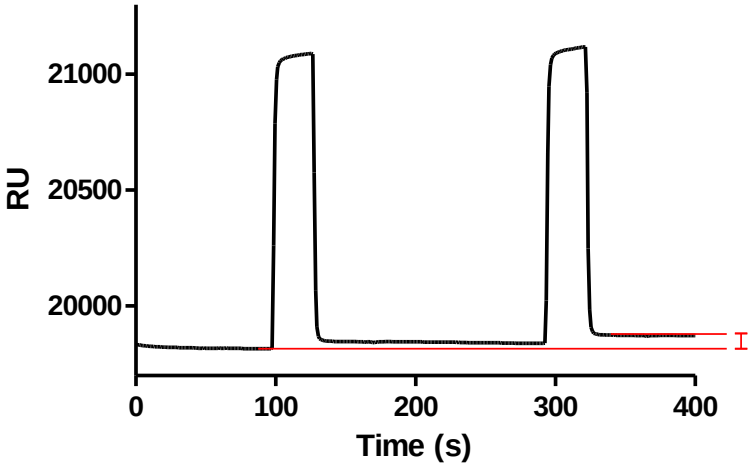


Figure 5-7 Heparin immobilisation
Plot showing immobilisation of heparin to the streptavidin coated chip. Two injections were needed to reach the RU increase required. The increase in RU from baseline is shown by the red lines.

5.2.4.3 Chemokine SPR

Following preparation of the chip surface, SPR was performed to compare the GAG binding properties of CCL2 and nCCL2.

A range of chemokine concentrations (66-500nM) were flowed across the chip at 25µl/min for 5mins followed by a 500sec dissociation phase.

RU from a flow cell coated with streptavidin only was subtracted from the results from GAG coated flow cells and analysis was performed using BIAevaluation 4.1.

5.2.5 *IN VIVO CHEMOTAXIS*

In vivo chemotaxis assays were performed using the air pouch model outlined in section 2.8.2. Eight-week old female BALB/c mice (Charles River, UK) were used for generation of air pouches and animal work was performed by Prof. Simi Ali and technicians in the Comparative Biology Centre, Newcastle University. On day 6, each pouch was injected with 1ml PBS containing either 10µg CCL2, nCCL2 or PBS alone. After 18hr recruited cells were recovered by lavaging the pouch. The exudates were centrifuged (500xg, 5min) and cell pellets resuspended in 1% FBS/PBS. Resuspended cells were then split into tubes to allow cells to be both counted and identified. Cells were counted by haemocytometer, or stained for mCD3 and mCD45 and analysed by flow cytometry. Figure 5-8 shows the gating strategy used to identify cell populations in the pouch and was performed with the help of Dr Chris Lamb. The percentage of each population was established and multiplied by the total number of cells in each pouch to calculate the total number of, for example, $CD45^+$ cells which have migrated into the pouch.

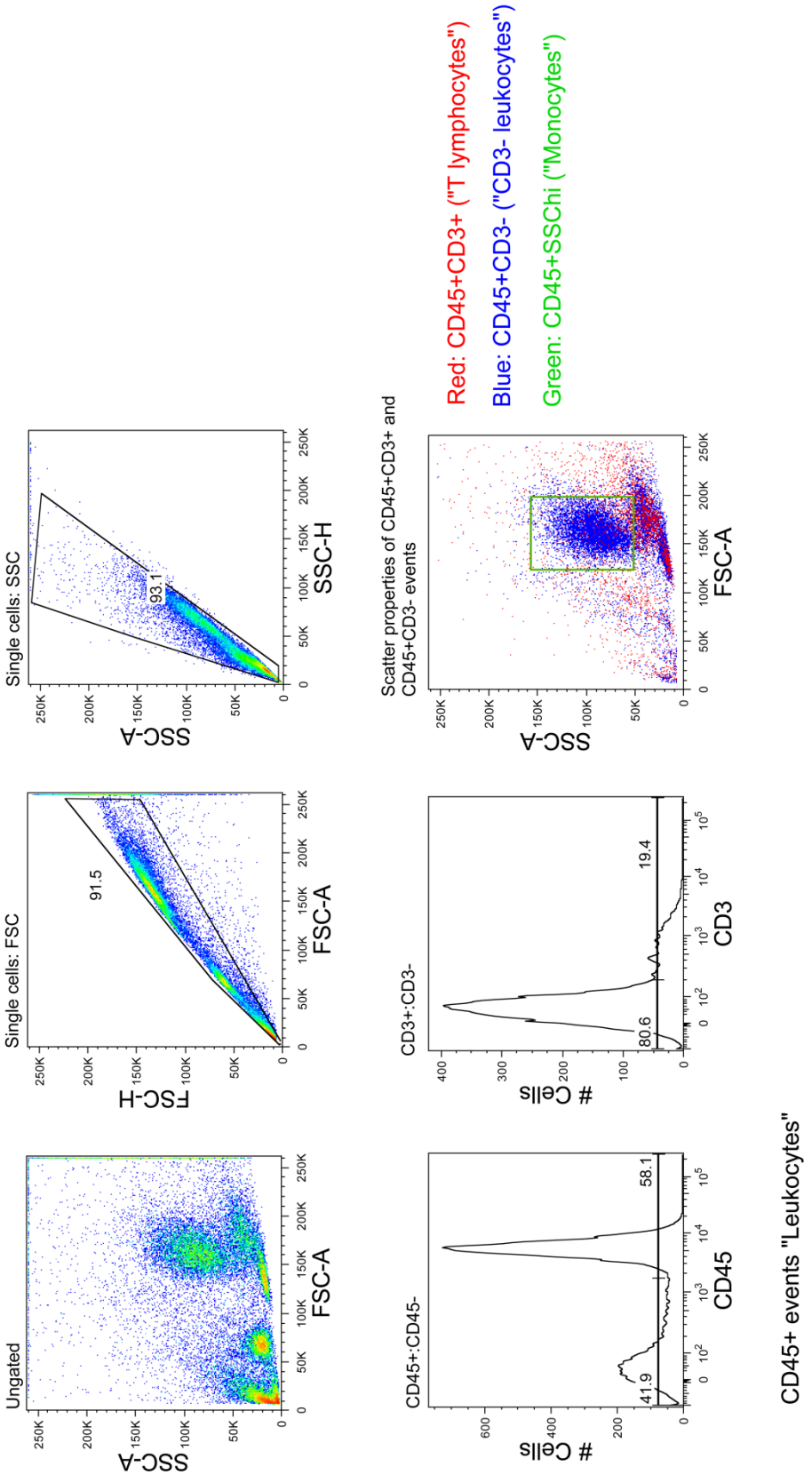


Figure 5-8 Flow cytometry gating strategy for the air pouch chemotaxis assay

5.3 RESULTS

5.3.1 *EFFECT OF NITRATION ON BARE MEMBRANE CHEMOTAXIS*

The previous chapter demonstrated that incubation with peroxynitrite leads to the nitration of chemokines. This chapter shows how nitration alters the function of these chemokines. Figure 5-9 and Figure 5-10 show CCL2 nitration decreases its ability to recruit both monocytes and T cells in bare membrane chemotaxis assays. Two sources of chemokine were used, Almac (synthetic) and Peprotech (recombinant), to confirm these findings. For monocyte chemotaxis although minor differences were seen between the two CCL2 sources these were only statistically significant at 1nM nCCL2 ($p=0.0019$). For T cell recruitment there was no overall differences between Almac and Peprotech although in some experiments slight differences were seen. For example in the one shown in Figure 5-10 Almac CCL2 and nCCL2 has significantly higher increase in recruitment at 1nM. Of note is that nitration of CCL2 in these experiments does not completely abrogate function even though it is decreased in comparison to wild type.

An increasing concentration of peroxynitrite used for CCL2 modification increases the reduction of monocyte recruitment (Figure 5-11). At high concentrations however there is a risk that some protein degradation could occur and so this decrease may not be specifically due to an increase in nitration.

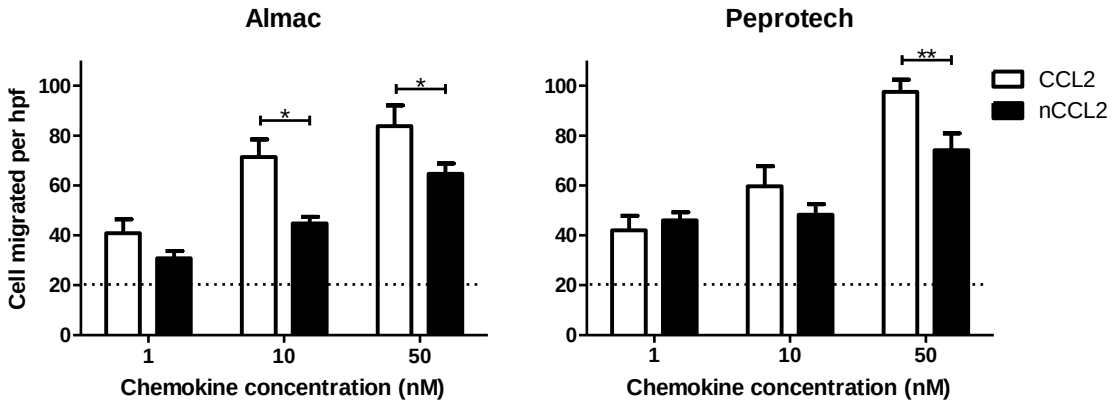


Figure 5-9 The functional effect of CCL2 nitration on monocyte recruitment
Chemotaxis using PBMC with both wt and peroxynitrite modified CCL2 (nCCL2) from both Almac and Peprotech was performed. Cells were placed in a 3μm filter above a chemokine containing well, incubated at 37°C for 90min, then migrated cells counted. Graph shows migrated cells adhered to the filter per high power field (mean of 5 fields per filter shown). No chemokine control displayed by dotted line. Representative data of three experiments, performed in triplicate.

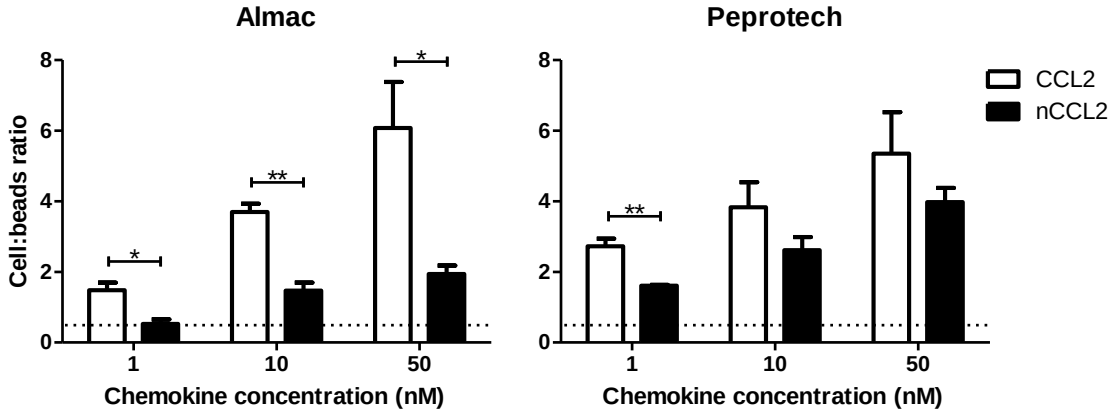


Figure 5-10 The functional effect of CCL2 nitration on T cell recruitment
Chemotaxis using PBMC with both wt and peroxynitrite modified CCL2 (nCCL2) from both Almac and Peprotech was performed. Cells were placed in a 3μm filter above a chemokine containing well, incubated at 37°C for 90min, then migrated cells counted. Graph shows cells migrated into the lower well as a ratio to beads by flow cytometry. No chemokine control displayed by dotted line. Representative data of three experiments, performed in triplicate.

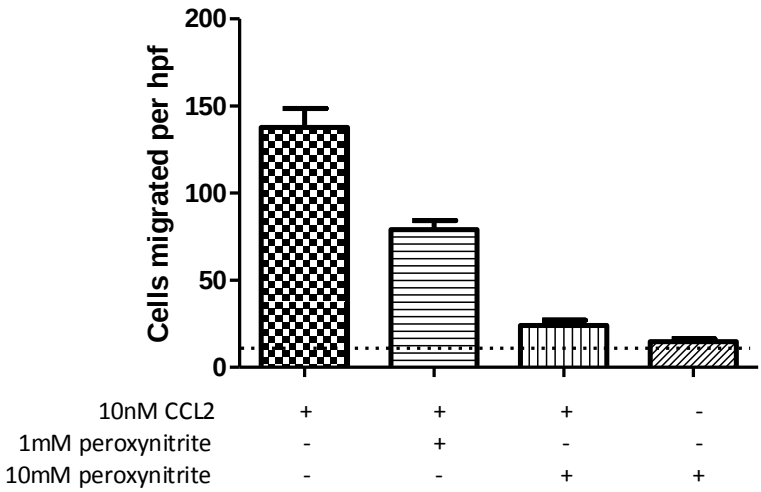


Figure 5-11 The effect of peroxynitrite concentration on CCL2 nitration and the functional effect on monocyte recruitment
CCL2 was incubated with 1 or 10mM peroxynitrite for 10min at 37°C prior to chemotaxis using PBMC. Cells were placed in a 3μm filter above a chemokine containing well, incubated at 37°C for 90min, then migrated cells counted. Graph shows migrated cells adhered to the filter per high power field (mean of 5 fields per filter shown). No chemokine control displayed by dotted line. Representative data of three experiments, performed in triplicate.

To determine if this decrease in function following nitration was a finding specific to CCL2 or a more wide-ranging effect, the other chemokines examined in Chapter 4 were also tested. Like CCL2, nitration of CCL5 significantly decreased its ability to recruit both monocytes and T cells at a range of chemokine concentrations (Figure 5-12). Unlike CCL2, function was completely lost following nitration, a much more

severe effect on function than was found with CCL2. Figure 5-13 shows CXCL8 function is also decreased following nitration. At 10nM there is no recruitment of neutrophils in response to nCXCL8.

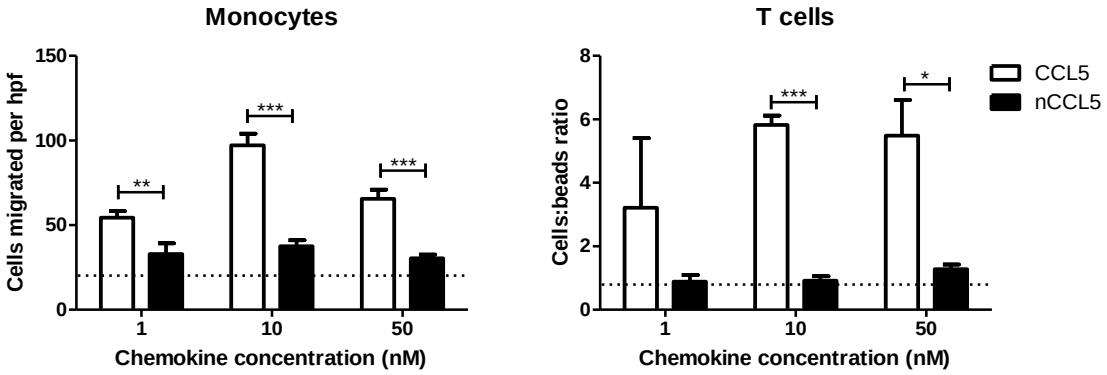


Figure 5-12 The functional effect of CCL5 nitration on monocyte and T cell recruitment
Chemotaxis using PBMC with both wt and peroxynitrite modified CCL5 (nCCL5) was performed. Cells were placed in a 3μm filter above a chemokine containing well, incubated at 37°C for 90min, then migrated cells counted. Left – Graph shows migrated monocytes adhered to the filter per high power field (mean of 5 fields per filter shown). Right - Graph shows T cells migrated into the lower well as a ratio to beads by flow cytometry. No chemokine control displayed by dotted line. Representative data of three experiments, performed in triplicate.

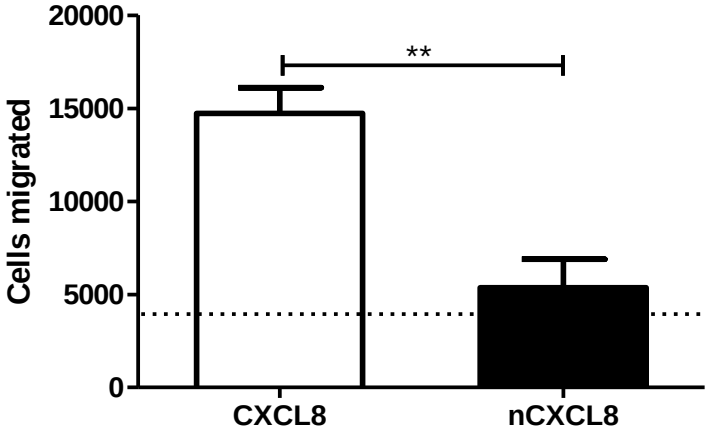


Figure 5-13 The functional effect of CXCL8 nitration on neutrophil recruitment
Chemotaxis using neutrophils with both wt and peroxynitrite modified CXCL8 (nCXCL8) was performed. Cells were placed in a 3μm filter above a well containing 10nM chemokine, incubated at 37°C for 90min, then migrated cells counted. Graph shows cells migrated into the lower chamber and counted by haemocytometer. No chemokine control displayed by dotted line. Combined data of three expreiments, performed in triplicate.

5.3.2 *Effects of Nitration on Single Receptors*

To establish if the effect of nitration on cell recruitment was mediated through chemokine receptors, chemotaxis assays were performed using transfected cells expressing only the chemokine receptor of interest. Figure 5-14 confirms the expression of CCR2b, the receptor for CCL2, on HEK transfectants. Chemotaxis assays showed that at 10nM, HEK-CCR2b recruitment was significantly impaired following CCL2 nitration (Figure 5-15). This suggests that the decrease in T cell and monocyte recruitment is mediated through this receptor.

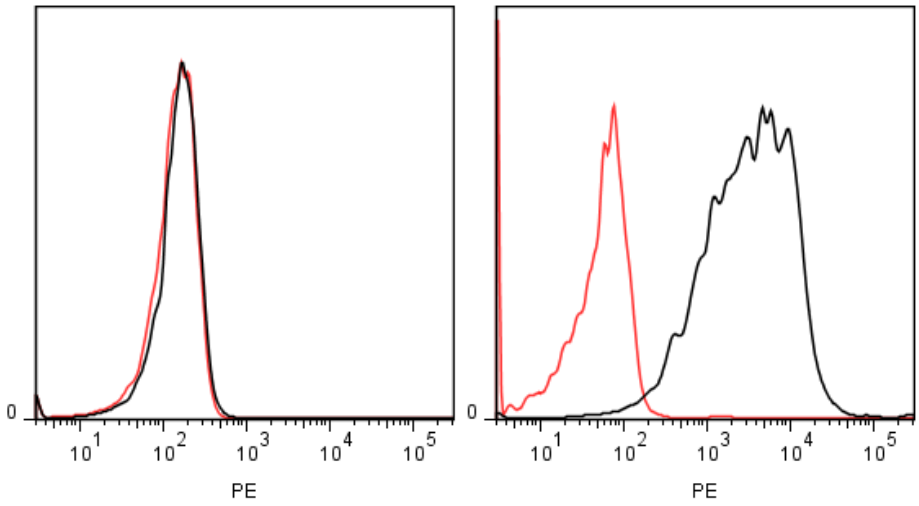


Figure 5-14 CCR2 expression on HEK-CCR2b transfectants
Representative flow cytometry staining of wild type HEK (left) and HEK-CCR2b transfectants (right) for CCR2 (black) and isotype control (red).

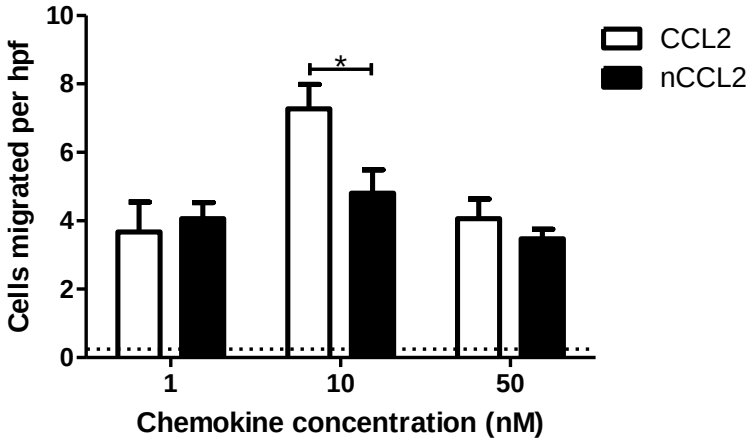


Figure 5-15 The functional effect of CCL2 nitration on HEK-CCR2b recruitment
Chemotaxis using HEK-CCR2b with both wt and peroxynitrite modified CCL2 (nCCL2) was performed. Cells were placed in a fibronectin coated 8µm filter above a chemokine containing well, incubated at 37°C for 6hr, then migrated cells counted. Graph shows migrated cells adhered to the filter per high power field (mean of 5 fields per filter shown). No chemokine control displayed by dotted line. Representative data of three experiments, performed in triplicate. * p = 0.0198

There is precedent for post-translational modification changing the receptor usage of the chemokine. For this reason chemotaxis assays with CHO-CCR5 were also performed with CCL2 and nCCL2. Receptor expression on the transfectants was confirmed in Figure 5-16 and chemotaxis in Figure 5-17 shows neither CCL2 nor its nitrated form can recruit cells through CCR5. This assay also demonstrated decreased recruitment of cells in response to nCCL5 compared to CCL5 (Figure 5-17) showing that, as with CCL2/CCR2, at least some of the decrease in PBMC recruitment is mediated through this receptor.

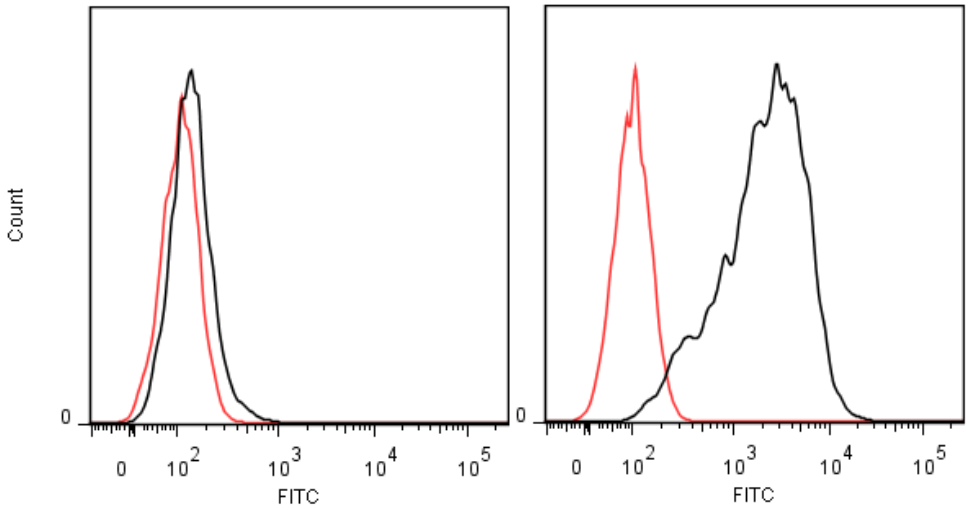


Figure 5-16 CCR5 expression on CHO-CCR5 transfectants
Representative flow cytometry staining of wild type CHO (left) and CHO -CCR5 transfectants (right) for CCR5 (black) and isotype control (red).

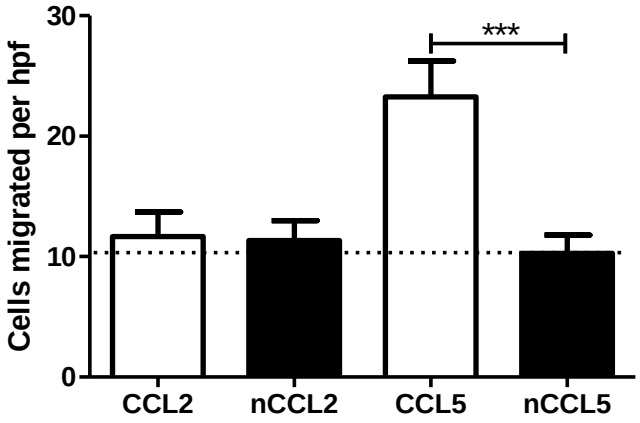
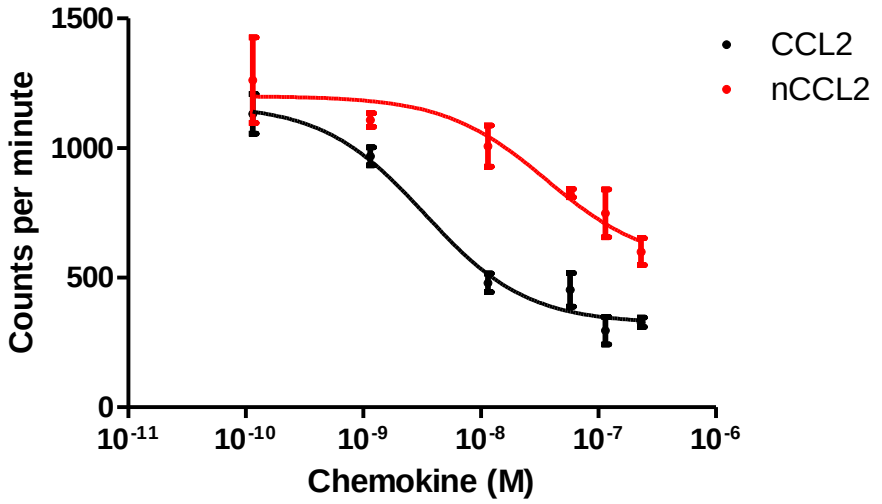


Figure 5-17 The functional effect of nitration on CHO-CCR5 recruitment
Chemotaxis using CHO-CCR5 with both wt and peroxynitrite modified CCL2 and CCL5 was performed. Cells were placed in a fibronectin coated 8µm filter above a well containing 10nM chemokine, incubated at 37°C for 5hr, then migrated cells counted. Graph shows migrated cells adhered to the filter per high power field (mean of 5 fields per filter shown). No chemokine control displayed by dotted line. Representative data of three experiments, performed in triplicate. t-test, *** p=0.0005.

To determine if the decrease in recruitment of HEK-CCR2b in response to nCCL2 compared to CCL2 was due to a decrease in CCR2b binding, radioligand binding assays were performed. Figure 5-18 shows nCCL2 binds the receptor with a ten-fold lower affinity than the wild type form of the chemokine. The assay was performed three times in triplicate and the details of each repeat are shown in Figure 5-18. Although there is variation between the repeats an approximate ten-fold decrease was found each time. In two of the three repeats the measurements at 10nM are significantly different by t-test. Overall however, the IC50 values of CCL2 and nCCL2 are not statistically different.



	CCL2			nCCL2		
Repeat	1	2	3	1	2	3
IC50	1.56×10^{-9}	6.30×10^{-10}	3.33×10^{-9}	1.90×10^{-8}	8.18×10^{-9}	3.65×10^{-8}
95% CI						
Upper	2.43×10^{-11}	4.43×10^{-11}	1.50×10^{-9}	5.72×10^{-12}	5.77×10^{-10}	1.12×10^{-8}
Lower	1.00×10^{-7}	8.97×10^{-9}	7.39×10^{-9}	6.30×10^{-5}	1.16×10^{-7}	1.19×10^{-7}

Figure 5-18 The effect of CCL2 nitration on CCR2 binding
Radioligand binding assays comparing the ability of CCL2 and nCCL2 to bind CCR2. Assays were carried out using HEK-CCR2b, and ^{125}I-CCL2 used as a tracer. Graph shows nitration decreases receptor binding ten-fold (IC50 CCL2=3nM, nCCL2=36nM). Representative of three experiments. Table gives details of the individual repeats with the 95% confidence intervals (CI).

5.3.3 *Effects of Nitration on Intracellular Signalling*

To establish if there were differences in downstream signalling following THP-1 stimulation with either CCL2 or nCCL2, Proteome Profiler arrays were performed. THP-1 express the appropriate receptor, CCR2, as demonstrated in Figure 5-19. An example from the phospho-kinase array is shown in Figure 5-20. No significant alterations were found in intracellular signalling between CCL2 and nCCL2 although for most proteins examined phosphorylation is above that of PBS, the negative control. Although there do appear to be some differences, for example to AMPKα1, the difference is small and unlikely to be biologically significant.

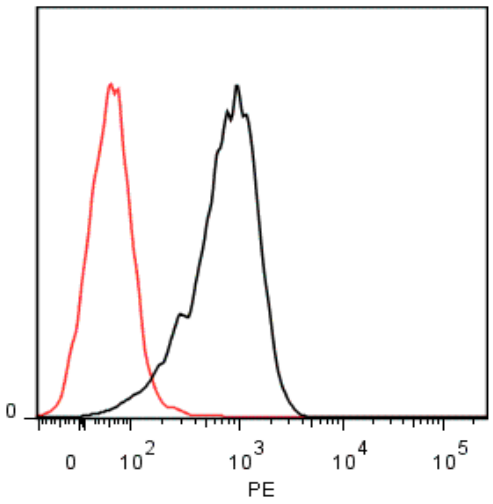


Figure 5-19 CCR2 expression on THP-1
Representative flow cytometry staining of THP-1 for CCR2 (black) and isotype control (red).

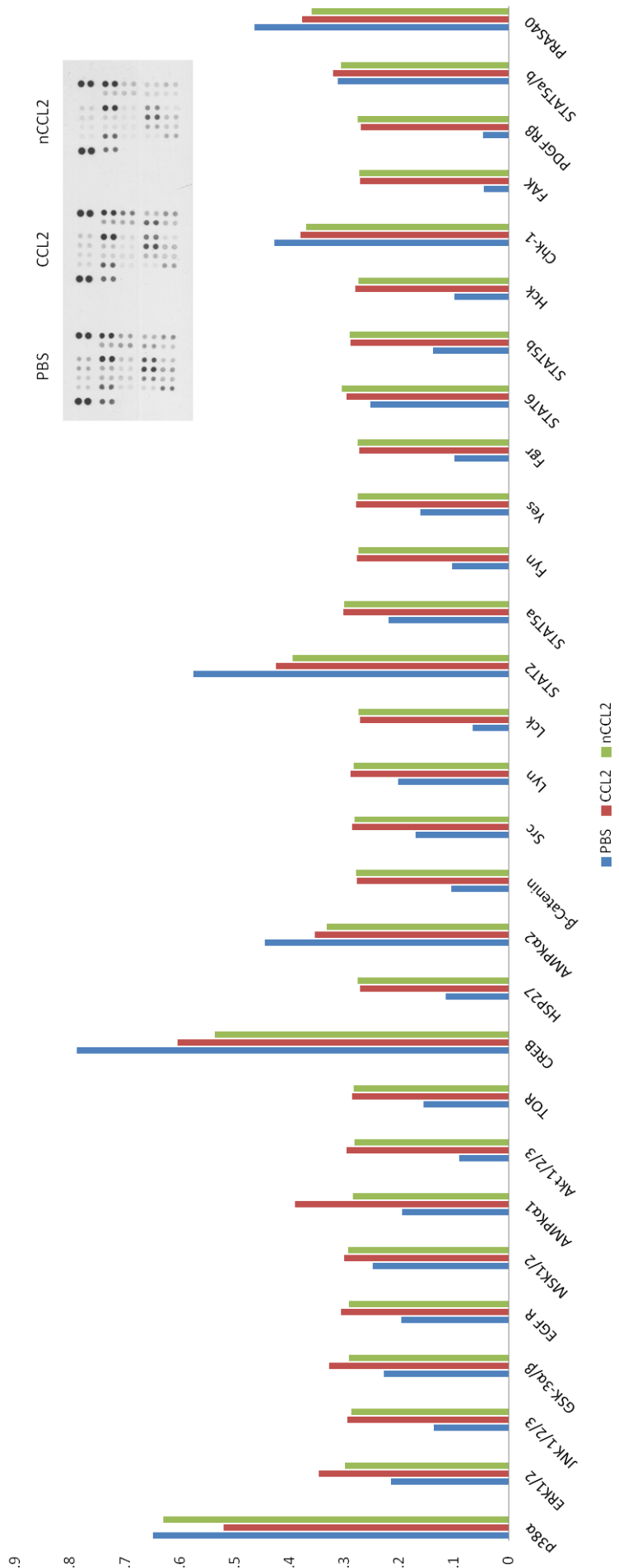


Figure 5-20 The effect of CCL2 nitration on downstream signalling
A human phospho-kinase Proteome Profiler antibody array (R&D Systems) was used to asses alteration in intracellular signalling following 5min stimulation of serum-starved THP-1 cells with 10nM CCL2 or nCCL2, or PBS as a control. n=1

5.3.4 *EFFECTS OF NITRATION ON GAG INTERACTIONS*

GAG binding is an important property of chemokines for *in vivo* function. If nitration altered the ability of CCL2 to interact with GAGs was assessed by two methods. Solid-phase heparin binding assays (Figure 5-21) indicated that nCCL2 did not bind to immobilised heparin. Due to the complications of detecting nCCL2 using antibodies, as discussed in sections 5.2.3 and shown in Figure 4-10, the same method of detection could not be used for both CCL2 and nCCL2. This means direct comparison could not be made between the two results. To eliminate this problem, Biacore, an antibody free technique, was used. Biacore confirmed that nCCL2 does not bind to heparin unlike the wild type form (Figure 5-22). A decreased alteration in RU was seen with a decrease in heparin density on the chip surface for both forms (Figure 5-23). The same lack of binding of nitrated CCL2 is also seen with heparan sulphate (Figure 5-24), a more physiologically important GAG.

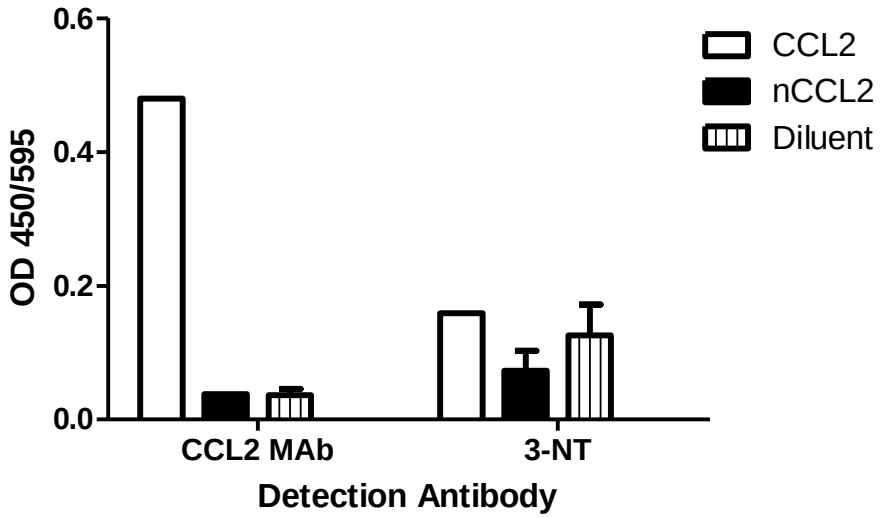


Figure 5-21 Solid-phase heparin binding assays with CCL2 and nCCL2
Solid-phase heparin assays were performed with CCL2 and nCCL2, and bound chemokine detected using anti-CCL2 or anti-3-nitrotyrosine (3-NT) antibodies. Representative of two experiments.

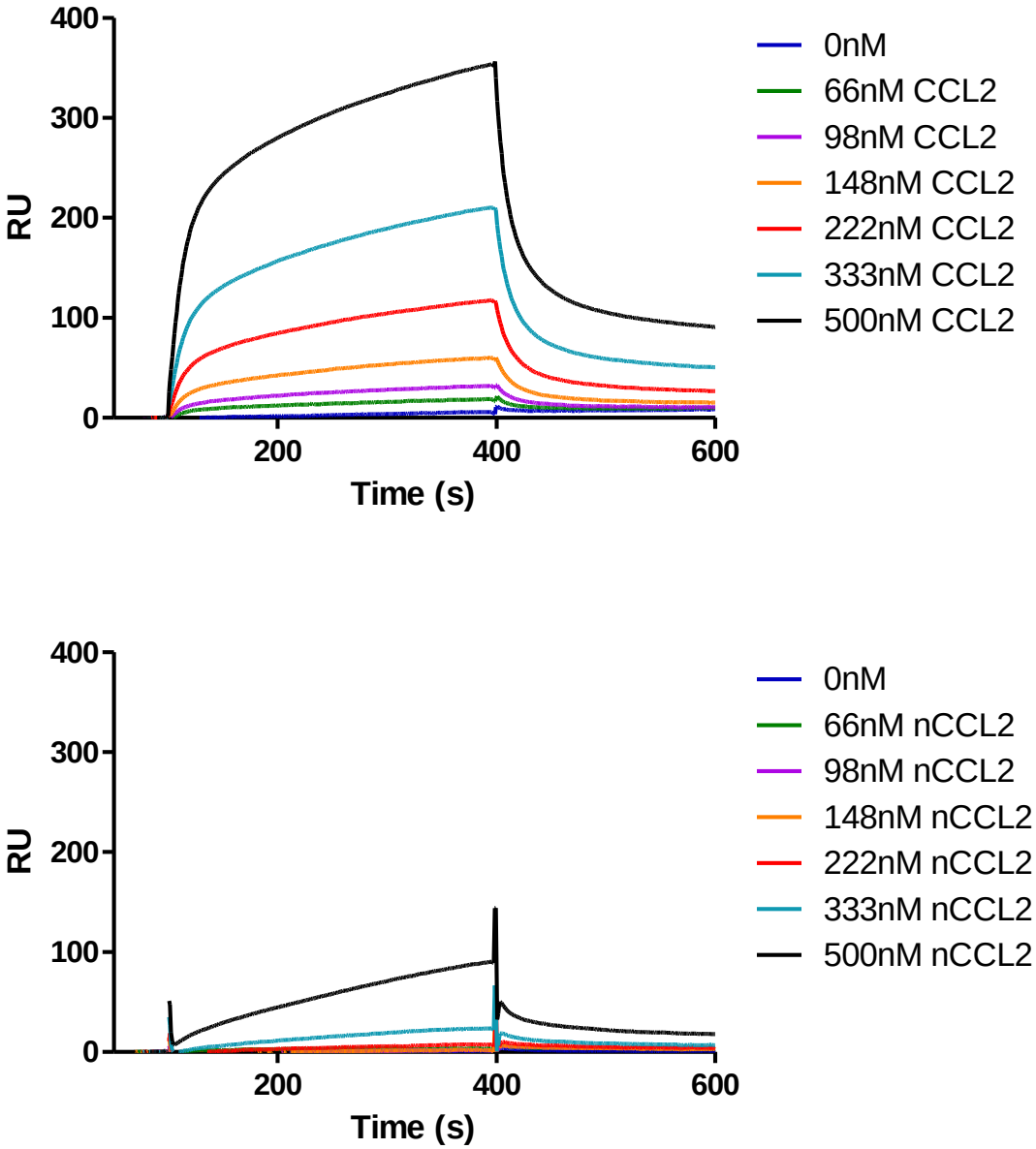


Figure 5-22 Alteration in higher density heparin interaction following CCL2 nitration
Biacore was used to assess the alteration in heparin interaction following CCL2 nitration. 66-500nM chemokine was flowed over immobilised heparin (142RU bound) and the alteration in resonance units (RU) shown. Upper – CCL2, Lower – nCCL2. Representative data of two experiments

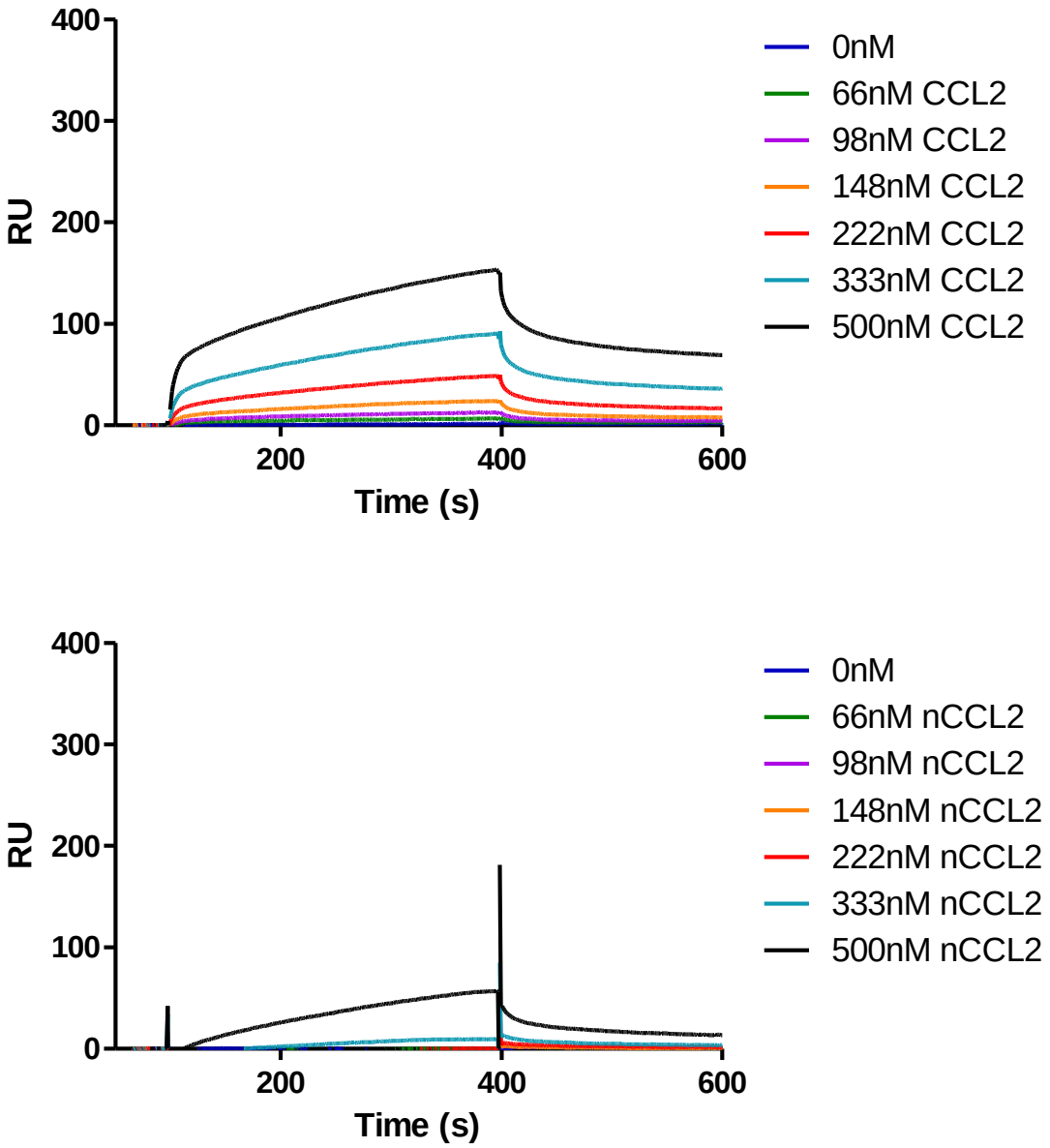


Figure 5-23 Alteration in lower density heparin interaction following CCL2 nitration
Biacore was used to assess the alteration in heparin interaction following CCL2 nitration. 66-500nM chemokine was flowed over immobilised heparin (50RU bound) and the alteration in resonance units (RU) shown. Upper – CCL2, Lower – nCCL2. Representative data of two experiments

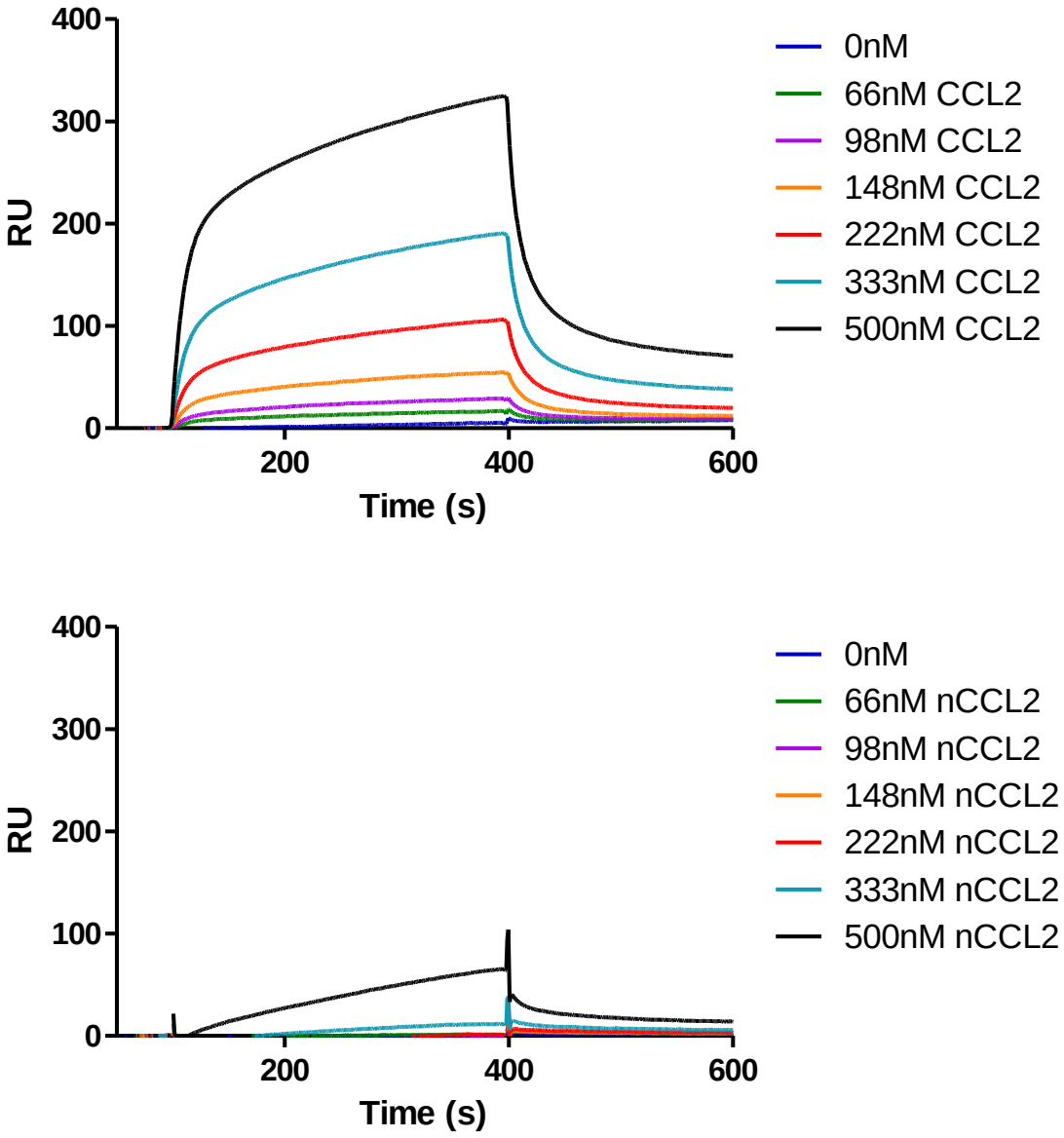


Figure 5-24 Alteration in heparan sulphate interaction following CCL2 nitration
Biacore was used to assess the alteration in heparan sulphate interaction following CCL2 nitration. 66-500nM chemokine was flowed over immobilised heparan sulphate (237RU bound) and the alteration in resonance units (RU) shown. Upper – CCL2, Lower – nCCL2. Representative data of two experiments

5.3.5 *TRANSENDOTHELIAL CHEMOTAXIS*

The chemotaxis assays performed so far in this study have been using a bare membrane system. Transendothelial chemotaxis assays are a more physiological approach to studying chemokine function as cells need to cross the endothelium *in vivo* for extravasation. In this system chemokines need to bind GAGs as well as activate appropriate receptors for cell migration to occur. Figure 5-25 shows that in such assays CCL2 nitration abolishes the recruitment of monocytes, a more substantial loss in activity than was found in bare membrane assays which is consistent with the loss of GAG binding.

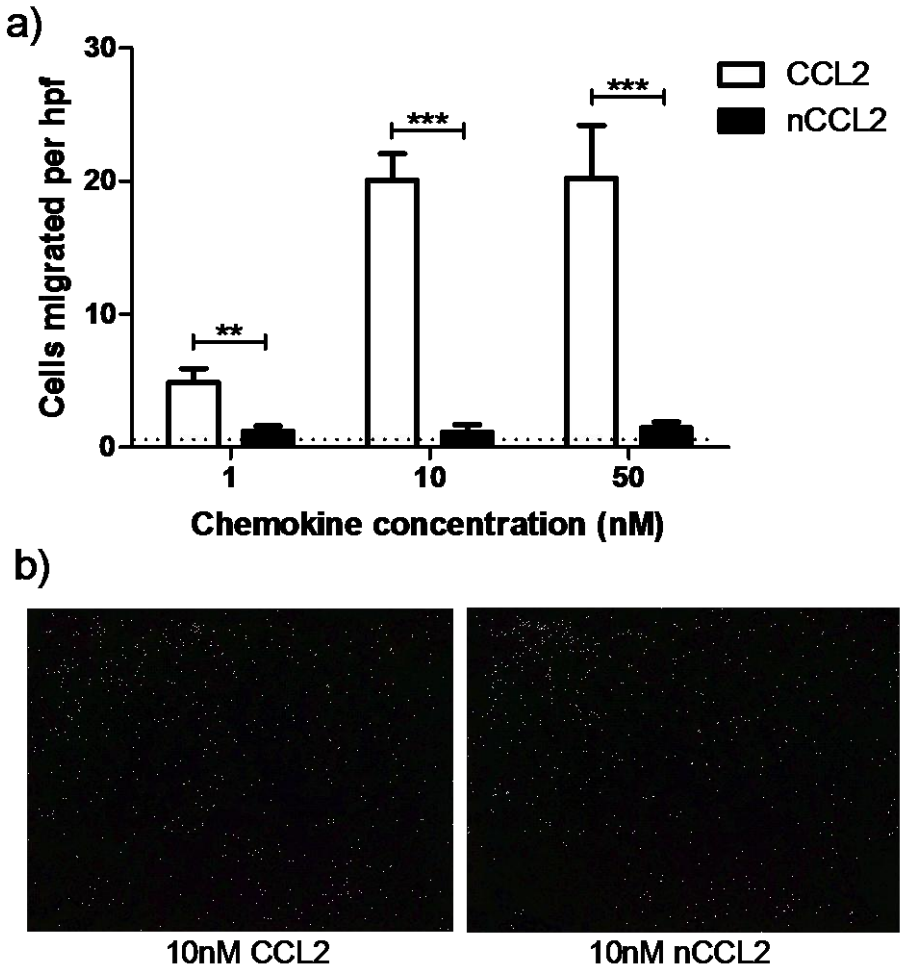


Figure 5-25 The functional effect of CCL2 nitration on monocyte recruitment across an endothelial layer

Transendothelial chemotaxis using PBMC with both wt and peroxynitrite modified CCL2 (nCCL2) was performed. Cells were placed in a 3µm filter with an endothelial monolayer above a chemokine containing well, incubated at 37°C for 90min, then migrated cells counted. a) Graph shows migrated cells adhered to the filter per high power field (mean of 5 fields per filter shown). No chemokine control displayed by dotted line. Representative data of three experiments, performed in triplicate. ** p=0.0029, **** p<0.0001 b) representative images of migrated cells at 10nM.

5.3.6 *IN VIVO CHEMOTAXIS*

CCL2 nitration has been shown to impair function in both bare membrane and transendothelial assays. To see if nitration also decreases cell recruitment compared to wildtype CCL2 *in vivo* an air pouch chemotaxis model was performed. An initial pilot study was performed which indicated a decrease in the function of nCCL2 compared to CCL2. Cell counts are shown in Figure 5-26. Due to low animal numbers and animal death, along with anomalies in the PBS control, this did not reach significance and so robust conclusions could not be drawn.

The experiment was therefore repeated with larger groups of eight mice and data agrees with the previous assay that nitration inhibits CCL2 function. Total cell counts from each pouch are shown in Figure 5-27. Data from lavages contaminated with blood have been excluded. Recruitment in response to CCL2 is significantly higher than to both PBS and nCCL2, with nCCL2 and PBS not being statistically significant. Using flow cytometry analysis the number of migrated $CD3^+$ and $CD45^+$ cells was calculated. Figure 5-28a shows $CD45^+$ cells are the majority of cells migrated into the pouch, consisting of both T cells (Figure 5-28b) and monocytes (Figure 5-28c). All three cell populations follow the same pattern, with nitration preventing recruitment. Another observation from these experiments is that the spread of data is always less for nCCL2 than the other two groups. Although not statistically different to PBS, the negative control, the mean recruitment in the nCCL2 group is lower than that of the PBS treated group in all cases.

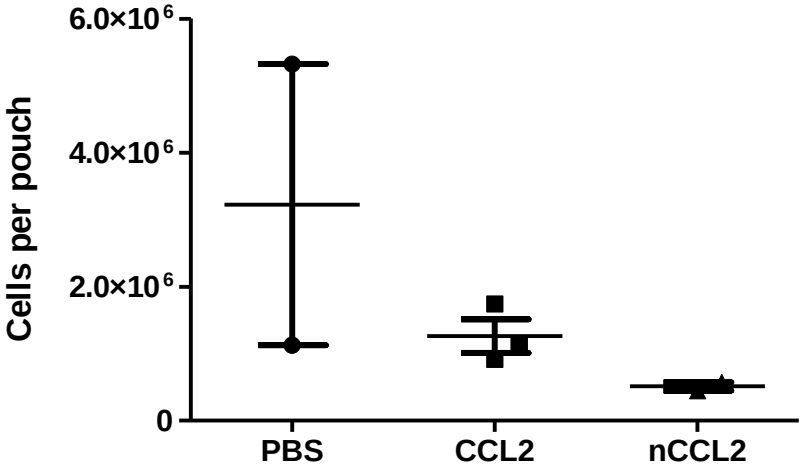


Figure 5-26 Cell counts from initial air pouch experiment
Total cell migration into air pouches 18hr after intrapouch administration of 10μg CCL2, nCCL2 or PBS only control. Cells were counted by haemocytometer. Each symbol represents an animal.

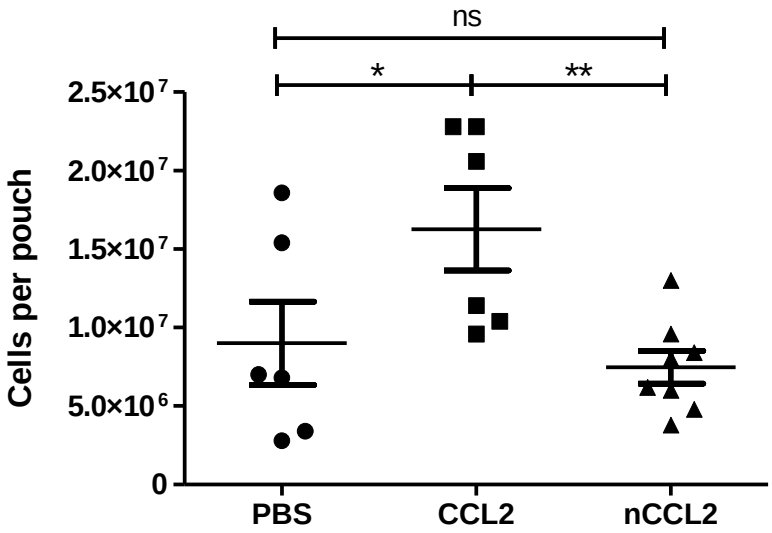


Figure 5-27 The functional effect of CCL2 nitration on cell recruitment *in vivo*
Total cell migration into air pouches 18hr after intrapouch administration of 10μg CCL2, nCCL2 or PBS only control. Cells were counted by haemocytometer. Statistics - one-tailed t-test - * p=0.04, ** p=0.0024, ns p=0.28. Each symbol represents an animal.

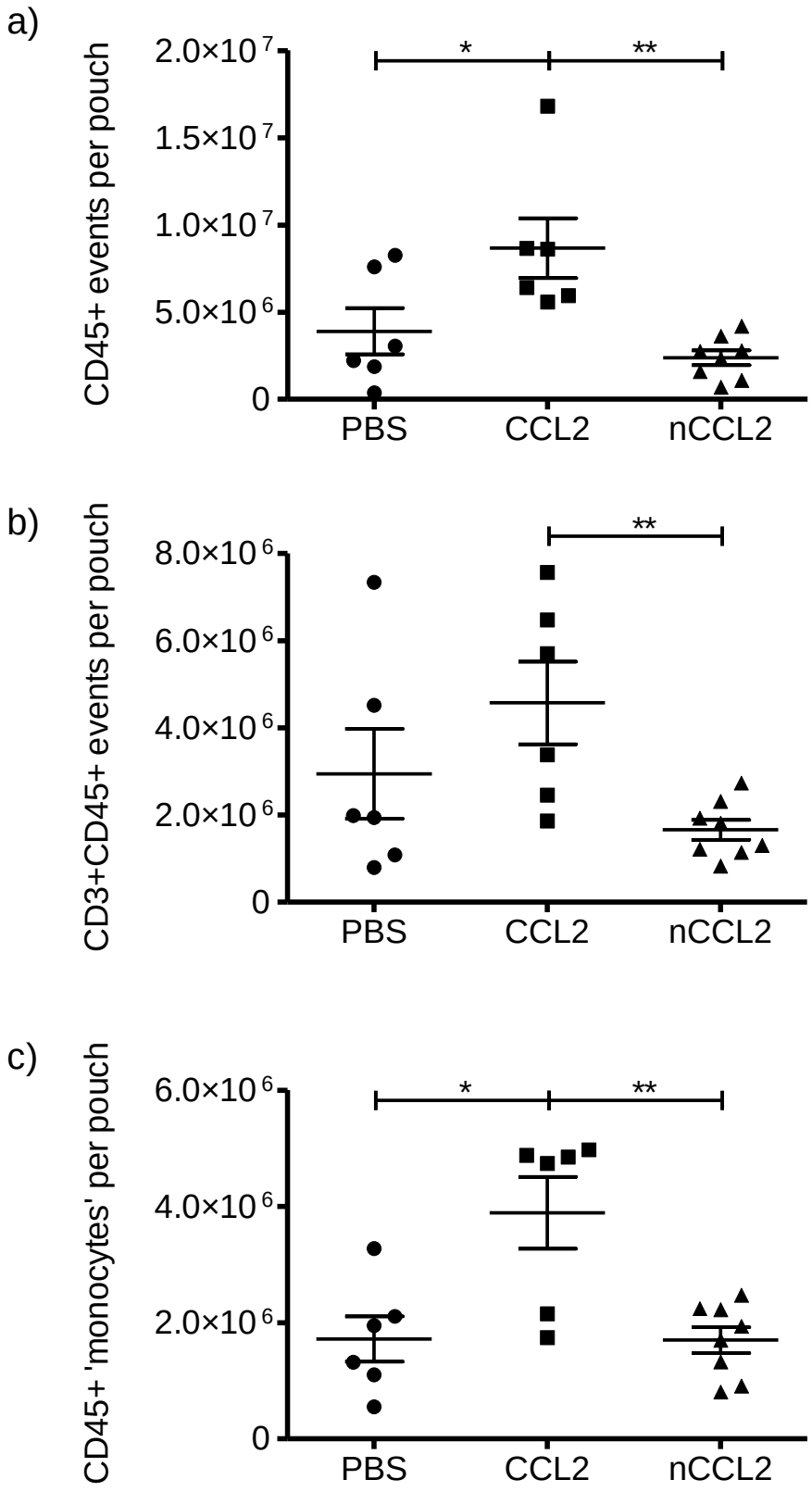


Figure 5-28 Effects of CCL2 nitration on the migration of different cell types *in vivo*
Cells migrated into air pouches 18hr after intrapouch administration of 10μg CCL2, nCCL2 or PBS only control. Cell populations were identified by flow cytometry and absolute cell numbers calculated using data from haemocytometer counts. a) $CD45^+$ migrated cells, b) $CD3^+CD45^+$ cells, c) monocytes identified by both CD45 positivity and FSC/SSC characteristics. Each symbol represents an animal.

5.4 DISCUSSION

The function of chemokines is tightly regulated, especially during times of stress. This chapter has shown the profound effects of chemokine nitration on chemokine function, a modification identified in chapter 4 which could potentially occur during oxidative stress.

All three prototypical inflammatory chemokines found to be nitrated following incubation with peroxynitrite in chapter 4, namely CCL2, CCL5 and CXCL8, have decreased cell recruiting ability compared to the wild type proteins. For CCL2, in bare membrane transwell assays, the chemotactic potential for both T cells and monocytes following nitration is greatly reduced but the chemokine still has some function. The soluble diffusion gradient necessary for cell migration in this assay has still been formed but the chemokine has lost some function. The largest decrease being seen for T cell recruitment at 50nM where nitration decreases recruitment by 74%, however nCCL2 still causes recruitment significantly above the negative control ($p=0.0182$). For CCL5 and CXCL8 however the loss of function is more severe. Nitrated CCL5 and CXCL8 do not cause migration significantly different to the no chemokine control. This suggests that though no selectivity has been shown for which chemokines are able to be modified by peroxynitrite, the magnitude of the effects are varied. The reasons for this have not been elucidated but CCL5 contains more potential modification sites than CCL2 which may be a factor. Although both CCL2 and CCL5 have one tryptophan, CCL2 only has two tyrosines whereas CCL5 has five. In all cases assayed so far, nitration can be described as an anti-inflammatory modification.

These findings for CCL2 are in some aspects inconsistent with findings by Molon *et al.* 2011 which found that, like this study, T cells do not migrate in response to nCCL2 but states that $CD14^+$ cells do respond. However closer examination of the data in that paper shows that although there is no alteration in $CD14^+$ cell response at 10nM chemokine, there is a significant decrease in response to nCCL2 compared to wild type at lower concentrations. This is the opposite of my findings which only gave a significant decrease in monocyte recruitment at 10nM or higher, a contrast to T cells in which significant differences could be seen at all chemokine concentrations.

The nitrated chemokines used in this study are potentially a mix of proteins with varied levels of nitration. Although this does not allow the exact determination of which residues are crucial for function this heterogeneous mix is arguably more physiological. It is very unlikely *in vivo* that all chemokine would be subject to the same level of modification. The half-life of radicals is generally short and the diffusion distance small, so chemokines not immobilised to, or present near, the site of RNS production may not be modified. Also, an area which has not been examined in this study is which other situations and modifications can modulate nitration. For example, if CCL2 is bound to heparin is it still subject to nitration? GAG binding has previously been shown to prevent enzymatic modifications such as the CD26 cleavage of CXCL12 (Sadir *et al.*, 2004) which, although a different type of modification to the chemical reaction achieved with RNS, highlights how other aspects of *in vivo* biology can alter post-translational modification.

To see if the decrease in migration in response to nCCL2 was mediated through the receptor CCR2b, migration assays using HEK transfectants were performed. These showed decreased recruitment at 10nM showing that at least some of the lack of function is mediated through the receptor. CCL2 can also bind the isoform CCR2a but this receptor has a lower level of surface expression due to cytoplasmic retention (Wong *et al.*, 1997). For this reason CCR2b was chosen as the isoform to use in this study. Similar chemotaxis experiments were performed with nCCL5 and cells transfected with CCR5, one of the three CCL5 receptors. These experiments again showed decreased migration was mediated through the appropriate receptor and, fitting with the PBMC chemotaxis data, the effect of nitration is more severe for CCL5 than CCL2. Other CCL5 receptors have yet to be examined.

Other post-translational modifications of chemokines have been shown to not only alter function through the appropriate receptors for wild type chemokine but to also have the ability to alter receptor usage. For example CD26 cleaved CCL4 can still bind CCR5 but unlike the full form can also bind CCR1 and CCR2 (Guan *et al.*, 2002). For this reason chemotaxis assays with CCR5 transfectants were also performed with CCL2 and nCCL2. CCL2 nitration does not change its ability to bind CCR5 but this does not rule out the possibility that binding to other receptors is altered. However, the PBMC and HEK-CCR2b chemotaxis assays gave similar

results suggesting the effects seen are mainly CCR2b dependent. It is therefore unlikely that binding to other receptors has been affected by nitration.

To determine if the decrease in function was due to a lack of receptor binding, radioligand binding assays were performed. These showed that CCL2 nitration causes a 10 fold decrease in CCR2b binding but due to variation between repeats, overall the two forms were not statistically different. Previous studies suggest that although this level of decrease in receptor binding will effect chemotaxis, it is not sufficient to abrogate function and for antagonism (Paavola *et al.*, 1998) and so the biological importance of such a decrease even if significance had been reached would be limited. Two crucial receptors which are yet to be examined are ACKR1 and ACKR2. If nCCL2 also has decreased binding to these atypical receptors it could be predicted that there will be higher levels of the chemokine present as it cannot be sequestered. This may have profound effects on the *in vivo* biology.

Previous work from our group and others demonstrates that chemokine receptors display biased agonism (O'Boyle *et al.*, 2007; Zidar *et al.*, 2009). For example CCR2, the receptor of most interest for this study, has four ligands. CCL8 activates different signalling pathways to the other three ligands, CCL2, CCL7 and CCL13. CCL8 cannot inhibit adenylate cyclase, does not induce nuclear translocation of ERK1 and has a delayed response in Akt activation compared to the other three ligands (O'Boyle *et al.*, 2007). To determine if CCL2 nitration could also alter the downstream signalling pathways activated through CCR2, signalling arrays were performed. These assays show a general increase in phosphorylation following stimulation but there were no differences between CCL2 and nCCL2. It is however of note that phosphorylation is not uniformly an 'on' switch and can be used to downregulate signalling. Although the nitrated form of the chemokine does not bind the receptor with such high affinity as the wild type, it may be that at 10nM there is sufficient chemokine present to activate the 'threshold' number of receptors needed for signalling; a binary response and therefore not so sensitive to a small decrease in binding. This data is only n=1, due to experimental cost, and the assays performed are not fully quantitative so further work is needed to determine if there are differences in signalling and the biological implications of this data.

GAG interaction is an important aspect of chemokine biology and how nitration alters this was assessed by both solid-phase heparin binding assays and Biacore. Both these techniques show that nCCL2 is unable to bind heparin. It is of note that Biacore also shows that nCCL2 is unable to bind the less sulphated GAG heparan sulphate, which is more biologically relevant than heparin. This data suggests that even though nCCL2 is able to bind CCR2b, albeit with less affinity than the wild type protein, it will not function *in vivo* due to an inability to bind GAGs.

Biacore is a very complex and powerful technique which can in some circumstances be used to determine the Kd of interactions. In this instance however it was not possible. Nitration caused a complete loss of binding so no kinetic data could be determined. For CCL2, binding was achieved but no kinetic models could successfully be fitted to the data. This is most likely due to the complexities of CCL2-GAG interactions. CCL2 can be present as a monomer, dimer and in larger oligomers and these multiple forms means that establishing kinetics is difficult. There are two distinct 'on' phases seen in Figure 5-22 - Figure 5-24, an initial rapid increase in RU, followed by a more gradual phase, suggestive that more than one form is present. These multiple phases could be rapid binding of pre-formed dimers or higher structures followed by slower binding of monomers. With further optimisation kinetics may be found for CCL2, and have previously been found by other groups (Piccinini *et al.*, 2010; Salanga *et al.*, 2014). This was not optimised during this study as the purpose of the experiment was to establish differences between the wild type and nitrated forms and not necessarily to determine exact kinetics.

GAG density has been shown in some cases to affect chemokine binding so levels of immobilised heparin were assessed. However as discussed, affinities could not be determined and so differences between the heparin densities could not be shown. Wild type CCL2 affinity would not be expected to alter greatly between GAG densities however if the decrease in binding following nitration was due to a decrease in oligomerisation potential, the affinity would be expected to decrease on low density surfaces (Salanga *et al.*, 2014). In this study very little binding was found following nitration even on higher density surfaces so this phenomenon is unlikely to be seen.

Difficulties in setting up the solid-phase heparin assay as described in section 5.2.3.1 are interesting from the technical aspect. The polyclonal anti-CCL2 antibody (AB-279-NA, R&D Systems) originally tested was unable to detect heparin bound chemokine. Monoclonal antibodies have been specifically designed to investigate chemokine-GAG interactions (Burns *et al.*, 1998) but polyclonal antibodies interfering with GAG binding have not been reported. As GAG bound chemokine is a vital context physiologically it is an important observation. This is similar to the concepts in the results Figure 4-10 - Figure 4-12 showing that due to chemokine post-translational modification, not all chemokine can be detected by antibodies and so physiologically relevant chemokine is being missed. These findings highlight, potentially more importantly that chemokine, with most likely 'normal' function also cannot be detected if it is GAG bound and functional.

The residues nitrated in CCL2 were established in chapter 4, but exactly which modified residues are responsible for the alteration in function is unknown. Tyr13 is one of the residues modified and has previously been shown to be crucial for CCL2 function. It is involved in receptor binding and signalling (Beall *et al.*, 1996; Paavola *et al.*, 1998; Jarnagin *et al.*, 1999) and so modification of this residue could explain the decrease in receptor binding and bare membrane chemotaxis. As discussed earlier however the nitrated CCL2 used in this study is likely to be a heterogeneous mix of levels of nitration and so the exact involvement of this and other residues cannot be confirmed.

The residues directly involved in GAG binding are positively charged amino acids such as arginine and lysine, neither of which is subject to nitration. Studies modifying such residues in CCL2 cause a decrease in GAG binding and therefore *in vivo* function (Chakravarty *et al.*, 1998; Lau *et al.*, 2004). Post-translational modifications such as citrullination which alter the charge of arginine residues also have profound effects on GAG binding (Proost *et al.*, 2008). One potential explanation for nitration altering GAG binding is by disrupting dimerisation. As discussed in section 1.3.4 dimerisation is crucial for GAG binding. P8A and Y13A are obligate monomers with decreased GAG binding potential (Lau *et al.*, 2004; Handel *et al.*, 2008; Salanga *et al.*, 2014), however the loss of GAG binding in these studies was not as severe as found following nitration. Dimerisation therefore is

unlikely to be the sole explanation for decreased function. Figure 5-29 highlights the position of Tyr 13 along the dimer interface and as Tyr 13 is subject to nitration this residue could again be the explanation for decreased function. If nitration affects the dimerisation ability of Tyr13, either by altering hydrogen bonding potential or altering the conformation of the protein then GAG binding will be affected.

Another explanation for the substantial loss of GAG interaction could potentially be that nitration unfolds the protein. The chemokine however cannot be completely unfolded as there is at least some retention of receptor binding. Modification of Trp59, a residue found to be nitrated, could be predicted to cause protein unfolding and structure distortion (Personal communication with Prof Tracy Handel at the Gordon Research Conference, 2014). If this occurred but left the N-terminal region structurally intact, the N-terminus would be predicted to still bind the receptor, with lower efficacy, but loss of general structure would prevent dimerisation and therefore GAG interaction. Detailed structural and biochemical analysis needs to be performed to determine exactly how nitration affects CCL2 function, and that of other chemokines.

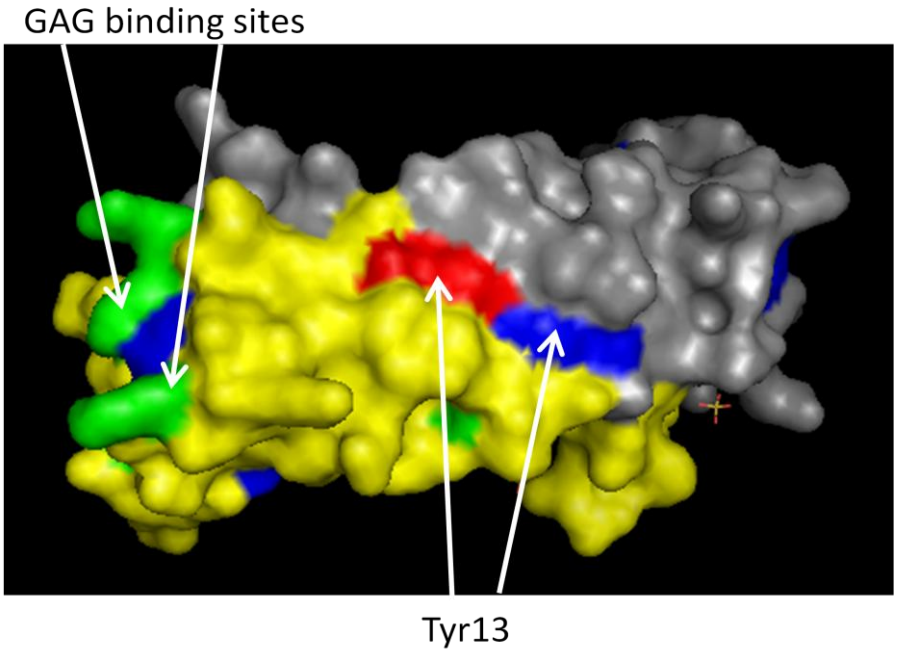


Figure 5-29 CCL2 dimer structure
Cartoon of a CCL2 dimer with potential nitration sites highlighted. Tyr13 from both monomers can be seen along the dimer interface. Monomers - yellow and grey, GAG binding sites – green, nitration sites - blue and red.

To confirm that loss of GAG binding following nitration causes the predicted loss of function, transendothelial chemotaxis assays, a more physiologically relevant variation of the technique requiring apical presentation of the chemokine for cell migration were performed. These showed complete abrogation of CCL2 function following nitration, a much more severe effect than was seen in the bare membrane assays. This confirms the GAG interaction data which suggests nitration prevents chemotaxis across the endothelium, a requirement for *in vivo* chemotaxis, due to lack of GAG binding.

Establishing the *in vivo* implications of CCL2 nitration were the final experiments in this study. These assays confirmed all the data discussed so far that nitration abrogates the chemotactic potential of CCL2. Other interesting observations from this assay are the high level of basal recruitment into pouches in the absence of chemokine, and the differences seen between PBS and nCCL2 groups. The creation of a murine air pouch is in itself a proinflammatory event. It is therefore unsurprising that relatively large numbers of cells were recovered from the PBS air pouches. Numbers are comparable to previous similar studies from our group (Ali *et al.*, 2005b; O'Boyle *et al.*, 2009). The large spread of data seen in the PBS and CCL2 group is potentially because of variation during air pouch creation. As mentioned, creating an air pouch is inflammatory and the positioning of the needle or other experimental variations may alter the level of background inflammation induced, and the natural production of murine chemokines and associated cell recruitment. For nCCL2 treated mice however the spread of data is always less and also the mean value is lower than that of the PBS control. This decrease compared to control could be explained if nCCL2 causes heterologous receptor desensitisation, which may decrease both the spread of data and the level of background recruitment. It is actively anti-inflammatory, not just lacking proinflammatory properties. Further experiments need to be performed to determine if this phenomenon does occur in response to nCCL2.

Overall the data in this chapter meets the three aims. If protein nitration, a modification which can occur during inflammation, occurs to chemokines, the function of the chemokines affected is reduced. Function decreased in all three forms of chemotaxis assay performed: bare membrane, transendothelial and *in*

vivo. This functional abrogation in the latter two is predominantly due to a decrease in GAG interaction and not mediated through the receptor CCR2b. Chemokine nitration is potentially a mechanism for self-regulation, preventing excessive cell recruitment during inflammation.

6 FINAL DISCUSSION

6.1 SUMMARY OF AIMS AND OUTCOMES

- To determine how tissue stress effects the production of the chemokines CCL2, CCL5, CXCL8 and CXCL10

The tissue and stress specific nature of chemokine responses is known. Chapter 3 established the range of responses of liver and kidney epithelium, the cell lines H69 and HKC8 respectively, to a number of tissue stresses. These stresses are either present during inflammation and transplantation, for example ROS, or non-physiological models of stress, such as PMA. Results from qPCR experiments showed CXCL8 had the greatest capacity for induction both in terms of fold increase and the range of stimuli which could upregulate it; CCL5 demonstrated little increase at the time points assayed.

The chemokines produced during stress were functional, as verified by both *in vitro* and *in vivo* chemotaxis assays. The effects of such a mix of chemokines can be blocked using the CXCR3 agonist PS372424, but not a CXCR3 blocking antibody, fitting with a previous study showing PS372424 can cause heterologous receptor desensitisation and therefore block chemotactic responses to chemokines, beyond those which are ligands to CXCR3. The chemokines within this mix may have undergone post-translational modification and therefore do not have 'normal' function. This was demonstrated by the purification of truncated CCL5 from HKC8 cells which had been subject to oxidative stress.

The concurrent presence of chemokines and nitro-oxidative stress was also demonstrated by immunohistochemical detection of CCL2 and 3-NT. These were found in liver, both in inflammatory conditions and ischaemia. Importantly, dramatically increased levels of 3-NT were found in a murine model of kidney IRI, a cause of injury known to considerably increase chemokine production.

- To investigate which chemokines are subject to post-translational modification. Specifically if CCL2, CCL5 and CXCL8 undergo the oxidative stress associated modification, nitration, and if CCL5 citrullination occurs

Chemokines are induced and present during periods of oxidative stress. The first section of chapter 4 aimed to assess if chemokines are subject to nitration by incubation with the RNS peroxynitrite. Results show that CCL2, CCL5 and CXCL8 can all undergo nitration, and that in CCL2 the residues modified are the two tyrosines and the tryptophan. Nitration alters our ability to detect all three chemokines by disrupting the epitope recognised by several commercially available antibodies.

Studies of citrullination, another inflammation associated post-translational modification, have so far focussed on CXC chemokines. Here CCL5 was also found to be a target of PAD enzymes. Such enzymes are expressed by the liver and kidney epithelial cell lines used in other areas of this study and so citrullination could be predicted to occur in these cells, dependant on calcium levels.

- Characterise the functional effects of nitration, focussing on CCL2

Chemokine modification by peroxynitrite has been shown and chapter 5 established the functional implications of the modification. In bare membrane chemotaxis assays all three chemokines tested showed decreased chemotactic potential following nitration. More detailed further studies were then performed using CCL2. These showed that although the decrease in chemotaxis seen in bare membrane assays could be attributed to CCR2b, as shown by chemotaxis with appropriate transfectants, a biologically significant decrease in receptor binding was not seen. No difference in intracellular signalling was detected but robust conclusions cannot be drawn from the signalling assays performed in this study due to lack of repetition.

Nitration also disrupted the GAG binding potential of CCL2, detected by solid-phase binding assays and Biacore. This lack of GAG interaction lead to a complete loss of function in transendothelial chemotaxis assays. Crucially, this was also seen in an *in vivo* air pouch model of chemotaxis where migration of both T cells and monocytes was lost.

6.2 IMPLICATIONS

Chemokines are the key regulators of inflammation, controlling both its initiation and resolution. Fine tuning of the chemokine response is therefore crucial for the prevention of unnecessary and prolonged inflammation. As many diseases have an inflammatory aspect, it is not surprising that the chemokine system is a major focus for drug development. Unfortunately due the complexities of the chemokine system few of these drugs have made it to market (Horuk, 2009; Roy *et al.*, 2014). Although many drugs appear to work *in vitro* and in animal models, few make it through phase II clinical trials. This is for a multitude of reasons, including lack of efficacy and unpredicted off-target effects. This highlights the need for greater knowledge of the basic biology of human chemokines. This study attempted to unravel more of the complexities of chemokine regulation that may occur in the inflammatory environment.

The results of this study are summarised in Figure 6-1. Oxidative, as well as other forms of stress stimulate epithelial cells to produce increased levels of chemokine. This is not however an on/off response, as both the cell type and stimulus affect the chemokine upregulated. The chemokines produced in such situations however do not necessarily have an easily predicted function. As well as altering chemokine levels, stress can also modify both the levels and function of multiple enzymes and molecules which are capable of post-translational chemokine modification. PAD and CD26 are the enzymes responsible for such modifications that were assessed in this study. Although no evidence has been provided here that either of the enzymes is increased in epithelium during inflammation, PAD, the enzyme responsible for citrullination, is regulated by calcium levels which are known to increase during oxidative stress. CD26, a protease which can cleave chemokines, for example trimming the two N-terminal amino acids from CCL5, is expressed on T cells and other cells in inflammatory infiltrate (Morimoto and Schlossman, 1998). Therefore even if it is not directly transcriptionally regulated by stress, the levels of the enzyme at sites during inflammation are increased.

The effects of the co-incubation of chemokines with the RNS peroxynitrite is the main focus of this study. Levels of nitration by this reactive species have been shown to increase dramatically in the transplant and inflammation associated

situations examined. This study has shown nitration, caused by peroxynitrite, to be an anti-inflammatory modification, downregulating chemokine function. It could therefore be proposed that even though chemokine production is upregulated by oxidative stress, the increase in overall chemokine function would not be as great as predicted. This suggests a feedback loop wherein the same stimulus which increases chemokine production, also decreases chemokine function. This is a similar auto-inhibitory concept to a study of CCL8 which showed the same stimulus, in this case IL-1β and IFNγ, which increases CCL8 production, also increases proteolytic cleavage of the chemokine. This cleavage results in an antagonist form of CCL8 (Struyf *et al.*, 1998b). Overall, despite there being an increase in protein, the levels of functional chemokine do not increase to the same extent.

The majority of studies carried out so far which assess the involvement of chemokines in disease assume the function of the chemokines detected to be 'normal'. Not only is this an over simplification of the situation, especially *in vivo*, but may be a confounding factor for the failure of chemokine based drug trials. The functional ability and therefore the potential biological role of each chemokine during disease needs to be fully established. Due to the chemokine specific effects of each modification, no general paradigm can be drawn; systematic analysis is needed. This analysis is needed not just in terms of alteration of function following modification but also alteration of detection. The majority of current detection methods do not differentiate between different forms of chemokine.

Findings in this study show that nitration can, in some instances, prevent chemokine detection and highlight the need to establish which methods are able to identify which chemokines. Both commercial CCL2 antibodies tested were unable to bind nCCL2, however the situation is not universal as one CCL5 antibody could detect the nitrated form of this chemokine. Generalisations therefore should not be made. Studies using such antibodies to detect chemokine levels in inflammatory environments, for example Robertson *et al.*, 2000, are therefore not 'seeing' the whole picture. This is arguably not important for the nitration modifications discovered in this study as although the nitrated chemokines generally cannot be detected, they also have decreased function. Other modifications however may

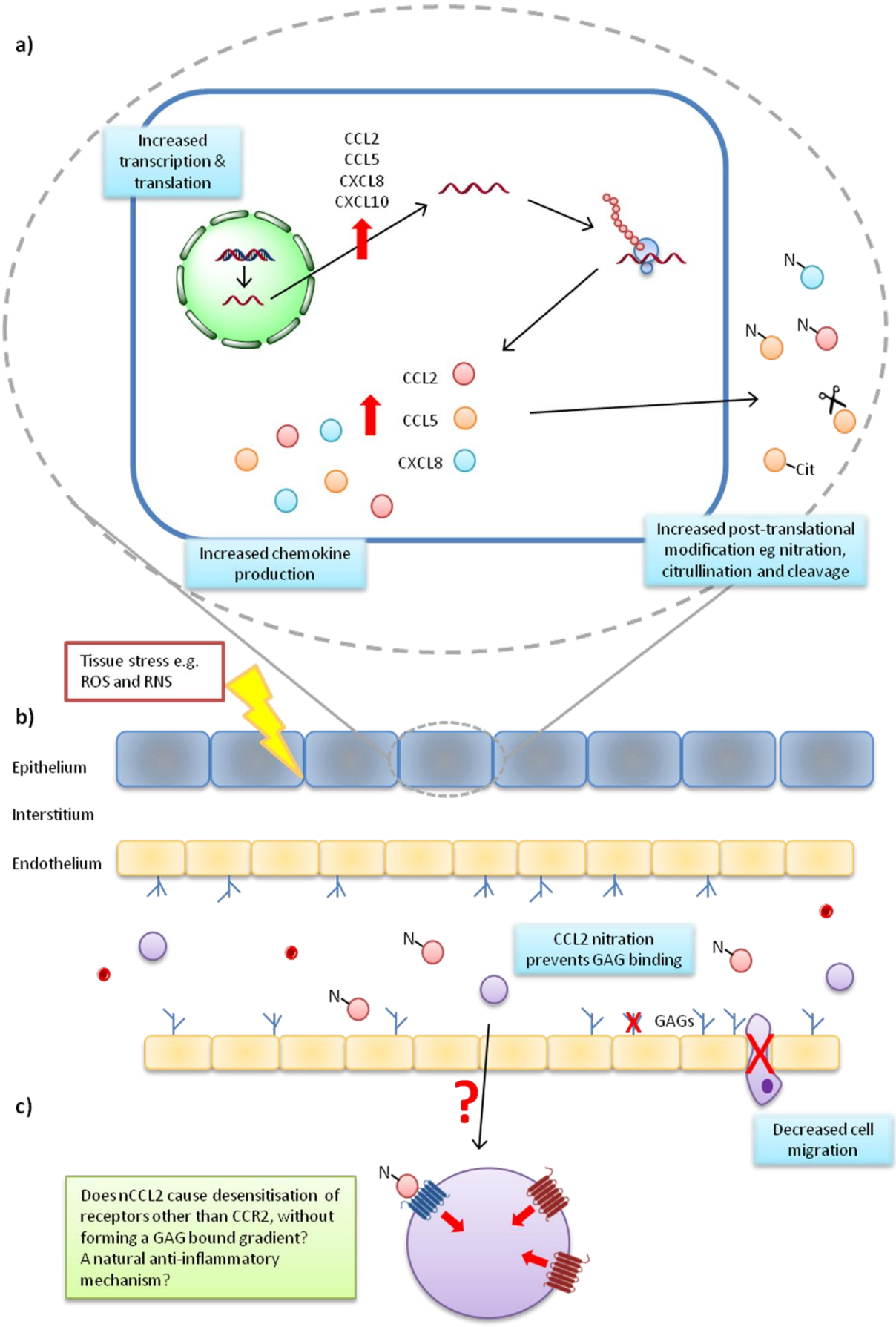


Figure 6-1 Chemokine regulation during inflammation

Chemokine biology is complex especially during times of stress, this figure summaries the findings of this study and some ideas for future work.
a) Following tissue stress the transcription and production of numerous chemokines increases, as does the potential for chemokine post-translational modification. Data presented in this thesis shows nitration of CCL2, CCL5 and CXCL8 can occur although its presence is yet to be determined *in vivo*. CCL5 is also subject to citrullination, and N-terminal processing by CD26.
b) CCL2 nitration abrogates function *in vivo*. The effects are predominantly due to an inability to interact with GAGs and therefore transendothelial migration cannot occur.
c) Other non-GAG binding chemokines are able to not only desensitise the appropriate chemokine receptor but also others. If nCCL2 can desensitise receptors other than CCR2, and therefore have more extensive anti-inflammatory effects, this will have profound implications for *in vivo* chemokine regulation during times of stress.

retain function but are unable to be detected and vice versa. Ensuring antibodies used can detect GAG bound chemokine is also important. It is imperative to determine the function and true levels of chemokines present otherwise roles in disease will be missed.

Further analysis of the role of chemokines in disease is necessary and may help to explain certain paradoxes found between clinical outcome and chemokine levels. For example the finding that increased CXCL10 truncation is found in hepatitis C patients, and explains why increased levels of the chemokine correlate with decreased response to treatment, the opposite would be predicted (Riva *et al.*, 2014).

In many studies transcription does not fully correlate with protein levels (Li *et al.*, 2014). There are numerous stages of regulation throughout protein production for example during translation, but lack of detection of modified proteins may also have an effect. The ability of nitration to prevent detection by antibody could explain a discrepancy between PCR and detectable protein levels in inflammatory environments. This however was not directly evaluated in this study. Although cells were treated by peroxynitrite and the subsequent alteration in chemokine levels assessed by both qPCR and ELISA, samples were taken 24 or 48hr after treatment for protein measurement. Due to the short half-life of peroxynitrite it will no longer be active once increased protein production occurs. Chemokine nitration is unlikely to occur in such situations without a repeat dose of peroxynitrite or co-incubation with cells capable of producing oxidative burst. No nCCL5 was found in peroxynitrite treated HKC8 samples when assessed by mass spectrometry, however for the reasons described above this finding was not

unexpected. Data in this study emphasise the importance of looking at chemokine function and not merely protein transcription or translation, especially if the protein levels are determined by antibody based techniques which do not differentiate between the forms present. There are reports in the literature of antibodies specific for the nitrated form of individual chemokines being developed.

Work in this study suggests chemokine nitration could be an *in vivo* method of chemokine downregulation, preventing chronic inflammation and associated damage. Recent studies also show that chemokine nitration, and subsequent non-function, may be a mechanism by which cancers can evade the immune system (Molon *et al.*, 2011). The only situations evaluated for levels of protein nitration in this study are those of increased oxidative stress such as IRI and inflammatory liver conditions. However diseases can arise due to a lack of reactive species production, for example primary immunodeficiencies such as chronic granulomatous disease (CGD). These patients have problems with recurrent infection due to defects in the NADPH oxidase in phagocytes. This inhibits pathogen killing as they are unable to perform oxidative burst. As well as the anticipated problems with infection, there is increasing interest in observations that these patients also suffer from increased inflammation (Magnani *et al.*, 2014). If nitration is a mechanism for the resolution of inflammation, the inability of these patients to produce RNS and therefore allow nitration to occur may go some way to explaining this inflammation dysregulation.

6.3 Future Directions

There are three main areas of future work regarding the biology of nCCL2 to come from this study: finding the structure of nCCL2 and so determining exactly how the modification disrupts function, establishing if nCCL2 is capable of heterologous desensitisation and therefore affecting the function of chemokines other than CCL2, and finally finding *in vivo* presence of nitrated chemokine in a transplant or other human inflammatory setting.

The first area of future work builds on observations relating to the regulation of chemokine production by stress. This analysis was predominantly performed using qPCR which only gives relative results. Therefore even though the fold

increase of chemokine production in response to different stresses has been established, the overall importance of the chemokines induced in the epithelial cells studied is unknown. It would be interesting to compare changes in chemokine levels between rich sources present in inflamed tissues, for example macrophages and epithelium. Is a ten-fold increase in chemokine production from epithelium an overwhelming increase in protein production? Or is the basal level significantly lower than of the infiltrating immune cells that even relatively large upregulation by epithelial cells will not be significant pathologically?

Nitration of the residue Tyr13 is likely to be the modification predominantly responsible for the loss of CCL2 function following peroxynitrite incubation due to its crucial role in both dimerisation and signalling. This study has only used a heterogenous mix of nitration levels and so the exact residues responsible for the functional change seen are unknown. For this reason more detailed structural and biochemical studies need to be performed. Evaluation of the structural consequences of nitration especially on dimerisation and protein unfolding is necessary, for example by NMR. Establishing the role of each nitrated residue may also help to explain differences found with the study by Molon *et al.*, 2011 which only found nitrated tryptophan.

In vivo chemotaxis assays showed that nCCL2 is unable to induce cell recruitment into a murine air pouch. This work should be extended to determine whether nitration not only downregulates the function of the chemokine modified, but also can have wider anti-inflammatory effects. This is depicted in Figure 6-1c. Previous experiments carried out by our group, similar to the air pouch model involving PS372424 (section 3.3.2), show that giving a non-GAG binding agonist, downregulates recruitment in response to a functional chemokine. To test this, the effect of nCCL2 i.v. on recruitment of immune cells to a CCL5 containing air-pouch could be assessed. Determining the wider reaching effects of chemokine nitration is crucial to understanding its potential role in the regulation in vivo chemokine biology.

Not only does the basic biology of chemokine nitration need to be determined but the presence of nitrated chemokine needs to be established in transplantation and

other human inflammatory settings discussed in this study. Molon *et al.*, 2011 found nitrated CCL2 in cancer sections, and this study has shown increased 3-NT in transplant situations with increased chemokine production. It is therefore almost certain that the modification will occur. Developing a suitable ELISA or refining mass spectrometry analysis would help with the finding of such modifications, of both CCL2 and other chemokines. An nCCL2 specific antibody has been developed and used by Molon *et al.*, 2011. However as mentioned previously the targets of nitration found in that study were different to this, and so the suitability of the antibody to detect all forms of nCCL2 is uncertain.

It would also be of interest to determine if this modification is prevented if the chemokine is already GAG bound. If so, this would have profound implications for the global effects of nitration as a downregulator of chemokine function as significant amounts of chemokine could no longer be subject to modification if protected by GAG binding.

6.4 CONCLUSIONS

Chemokines are crucial for the induction and resolution of inflammation; it is therefore not surprising that the methods of their regulation alter during times of stress. This study shows that inducers of tissue stress can increase chemokine production in a chemokine, tissue and stress specific manner. The chemokines produced in such situations are able, unsurprisingly, to induce chemotaxis but the chemokine present may not have the predicted functions due to post-translational modifications.

Nitration, a modification caused by reaction with RNS, increases in times of oxidative stress for example IRI and inflammation. This is a known protein modification but previously little work has established its effects on chemokine biology. CCL2, CCL5 and CXCL8 are all targets of nitration and in all three cases is an anti-inflammatory modification. Nitration also affects the detection of these chemokines in antibody based techniques. For CCL2, the focus of this work, the recruitment of both T cells and monocytes is decreased. This is in some part due to interaction with the receptor CCR2b but predominantly due to an inability to bind GAGs. This interaction is crucial to the *in vivo* function of chemokines as shown by

the lack of cell migration into an air pouch model in response to nCCL2. Overall this work demonstrates the complexities of chemokine regulation during inflammation and highlights how much more there is to discover.

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PUBLICATIONS ARISING DURING THIS STUDY

Transplantation and inflammation: implications for the modification of chemokine function. Catriona E Barker, Simi Ali, Graeme O'Boyle, John A Kirby. Immunology, October 2014; 143(2):138-45. PMID:24912917

Figure selected for cover image

Autoantibodies to CD59, CD55, CD46 and CD35 are not associated with atypical haemolytic uraemic syndrome (aHUS). Rachael Watson, Emma Wearmouth, Amy-Claire McLoughlin, Arthur Jackson, Sophie Ward, Paula Bertram, Karim Bennaceur, Catriona E. Barker, Isabel Y. Pappworth, David Kavanagh, Susan M. Lea, John P. Atkinson, Timothy H.J. Goodship, Kevin J. Marchbank. Mol Immunol, August 2014. pii: S0161-5890(14)00187-4. PMID:25150608

Development of a robust protocol for gene expression analysis using formalin-fixed, paraffin-embedded liver transplant biopsy specimens. Emily Thompson, Alastair D Burt, Catriona E Barker, John A Kirby, John G Brain. J Clin Pathol, September 2013; 66(9):815-8. PMID:23757038

Inhibition of CXCR4-CXCL12 chemotaxis in melanoma by AMD11070. G O'Boyle, I Swidenbank, H Marshall[1], CE Barker, J Armstrong, S A White, S P Fricker, R Plummer, M Wright, PE Lovat. Br J Cancer, 30 April 2013;108(8):1634-40 PMID:23538388

A CXCR3 agonist prevents human T cell migration in a humanized model of arthritic inflammation. Graeme O'Boyle, Christopher Fox, Hannah R Walden, Joseph DP Willet, Emily R Mavin, Dominic W Hine, Jeremy M Palmer, Catriona E Barker, Christopher A Lamb, Simi Ali, John A Kirby. PNAS, 20 March 2012;109(12):4598-603 PMID:22392992

ORAL PRESENTATIONS

Catriona Barker, Graeme O'Boyle, Simi Ali and John Kirby. CCL2: the immunoregulatory potential of oxidative stress, Chemotactic Cytokine Gordon Research Seminar, Vermont, USA, 27th July 2014.
- Abstract selected for oral presentation by GRS and funds were awarded to cover registration and accommodation costs.

G O'Boyle, C Barker, C Fox, H Walden, D Hine, S Douglass, J Willet, E Mavin, C Lamb, X Wang, P Newton, S Ali, J Kirby. A small molecule agonist of the chemokine receptor CXCR3 prevents experimental graft-versus-host disease, Chemotactic Cytokine Gordon Research Seminar, Vermont, USA, 27th July 2014.

G O'Boyle, C Barker, C Fox, H Walden, D Hine, S Douglass, J Willet, E Mavin, C Lamb, X Wang, P Newton, S Ali, J Kirby. A small molecule agonist of the chemokine receptor CXCR3 prevents experimental graft-versus-host disease. Immunology North East Symposium, Northumbria University, 26th June 2014.

Catriona Barker, Graeme O'Boyle, Simi Ali and John Kirby. Chemokine modification in inflammation, Student Seminar Series, Institute for Cellular Medicine, Newcastle University, 21st May 2014

Catriona Barker, Graeme O'Boyle, Simi Ali and John Kirby. How does the inflammatory environment affect inflammation? Meeting the MRC Mission, 18th October 2013

Catriona Barker, Graeme O'Boyle, Simi Ali and John Kirby. Chemokines and Oxidative Stress, Rega Institute, KU Leuven, Belgium, 4th June 2013

Catriona Barker. A BRC funded PhD studentship – Progress and Opportunities, NIHR Training School Welcome Event, 13th February 2013

Catriona Barker, Graeme O'Boyle, Simi Ali and John Kirby. Chemokine Post-Translational Modification in Inflammation, Student Seminar Series, Institute for Cellular Medicine, Newcastle University, 15th January 2013

G O'Boyle, C Fox, H Walden, J Palmer, C Barker, D Hine, S Douglass, J Willet, E Mavin, C Lamb, X Wang, S Ali, J Kirby. A CXCR3 agonist prevents human T cell migration in a humanized model of arthritic inflammation. Chemotactic Cytokine Gordon Research Seminar, Italy, 26th May 2012.

POSTER PRESENTATIONS

Catriona Barker, Graeme O'Boyle, Simi Ali and John Kirby. CCL2: the immunoregulatory potential of oxidative stress, Chemotactic Cytokine Gordon Research Seminar and Conference, Vermont, USA, July 2014.

G O'Boyle, C Barker, C Fox, H Walden, D Hine, S Douglass, J Willet, E Mavin, C Lamb, X Wang, P Newton, S Ali, J Kirby. A small molecule agonist of the chemokine receptor CXCR3 prevents experimental graft-versus-host disease Chemotactic Cytokine Gordon Research Seminar and Conference, Vermont, USA, July 2014.

Catriona Barker, Graeme O'Boyle, Simi Ali and John Kirby. Chemokine post-translational modification in inflammation, BSI Summer School - 21st Century Immunology: From the Basics to the Bedside, Newcastle, July 2013.

Catriona Barker, Graeme O'Boyle, Simi Ali and John Kirby. The role of oxidative stress in chemokine biology, 3rd NIHR Experimental Medicine Research Training Camp, Ashridge Business School, June 2012.

Catriona Barker, Graeme O'Boyle, Simi Ali and John Kirby. The role of oxidative stress in chemokine biology, Chemotactic Cytokine Gordon Research Seminar and Conference, Italy, May 2012.

G O'Boyle, C Fox, H Walden, J Palmer, C Barker, D Hine, S Douglass, J Willet, E Mavin, C Lamb, X Wang, S Ali, J Kirby. A CXCR3 agonist prevents human T cell migration in a humanized model of arthritic inflammation. Chemotactic Cytokine Gordon Research Seminar and Conference, Italy, May 2012.

Catriona Barker, Graeme O'Boyle, Simi Ali and John Kirby. The role of reactive nitrogen species in tissue damage, 2nd NIHR Experimental Medicine Research Training Camp, Ashridge Business School, June 2011.

AWARDS

British Society for Immunology Travel Award - £1000 - allowed attendance of the Chemotactic Cytokine Gordon Research Seminar and Conference, July 2014.

Newcastle University Graduate School Travel Grant - £500 - allowed attendance of the Chemotactic Cytokine Gordon Research Seminar and Conference, July 2014.

MRC Centenary Early Career Award – allowed collaboration with Prof. Paul Proost, Molecular Immunology Group, Rega Institute, KU Leuven, Belgium on chemokine citrullination and purification (3 month lab visit, £7014).