REGULATION OF CXCL8 FUNCTION DURING INFLAMMATION

Beatriz Martínez Burgo

A thesis submitted in accordance with the requirements for the degree of Doctor of Philosophy

Institute of Cellular Medicine,
Newcastle University, UK
2nd May 2018

Ischaemia-Reperfusion Injury (IRI) is a major contributor to acute organ dysfunction. Chemokines play a key role in leukocyte recruitment to the injured tissue. CXCL8 is a critical inflammatory mediator of neutrophil migration in a range of transplant surgeries. In addition to binding to their G protein-coupled receptor (GPCR) on the leukocyte surface, chemokines also interact with endothelial surface glycosaminoglycan (GAG). Chemokine activity can be regulated by several means, including binding to GPCRs, GAGs, and through post-translational modifications (PTMs).

GAGs bind and immobilise chemokines at high concentrations at the site of injury. This directs the migration and activation of leukocytes, contributing to local inflammation. We aimed to interfere with this binding in order to modulate neutrophil recruitment through the use of chemokine peptides. A truncated version of wild type CXCL8 corresponding to the GAG-binding region of CXCL8, located towards the C-terminal α-helix, the E70K peptide and a scrambled peptide were chemically synthesised (90% purity). Their GAG binding was shown by Surface Plasmon Resonance. Flow-based neutrophil adhesion was significantly decreased by WT peptide, E70K peptide or scrambled peptide ($p<0.01$), with no significant difference among them, suggesting a competitive role of peptides at displacing CXCL8 from GAG. *In vitro* neutrophil transendothelial chemotaxis mediated by CXCL8 gradient was decreased by E70K peptide ($p<0.001$) as opposed to WT or scrambled peptides. Thus, E70K peptide higher positive charge might contribute to higher specificity to bind polyanionic GAG.

Post-translational nitration appears to render CXCL8 non-functional, reducing migration completely ($p<0.001$). The presence of Reactive Nitrogen Species and CXCL8 in kidney biopsies was assessed by immunofluorescence. There was an association between grade of injury and nitration. CXCL8 expression did not significantly change.

Our study has generated a better understanding of CXCL8 function, particularly the process involved in targeted competitive modulation and the role of chemokine nitration as a negative regulator. This could offer therapeutic opportunities to protect the organ from neutrophil-derived damage during IRI.

Contents

List of Figures

List of Tables

List of Abbreviations

AA	Amino Acid
AKI	Acute Kidney Injury
Arg	Arginine (Arg or R)
APN	Acute Pyelonephritis
APS	Ammonium Persulphate
ATCC	American Type Culture Collection
ATN	Acute Tubular Necrosis
BSA	Bovine Serum Albumin
c-AMP	cyclic Adenosine Monophosphate
CKD	Chronic Kidney Disease
cDNA	Complementary DNA
CD	Circular Dichroism
CD11b	Macrophage-1 antigen or integrin αM –β2
CD45	Cluster of Differentiation 45
CF	Cystic Fibrosis
COPS	Chronic Obstructive Pulmonary Syndrome
DAG	Diacylglycerol
DEPC	Diethylpyrocarbonate
DIC	N, N′-Diisopropylcarbodiimide
DMF	Dimethylformamide
DMSO	Dimethyl Sulfoxide
EDC	1-Ethyl-3-(3-Dimethylaminopropyl)-Carbodiimide
EDTA	Ethylenediaminetetraacetic acid
EndMT	Endothelial-Mesenchymal Transition
ERK 1/2	Extracellular signal-Regulated Kinase 1/2
ESI-MS	Electrospray Ionisation Mass Spectrometry
FBS	Foetal Bovine Serum
FITC	Fluorescein Isothiocyanate
FSC	Forward Scatter
GAG	Glycosaminoglycan
GalNAc	N-Acetyl-Galactosamine
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GlcA	Glucuronic Acid
GlcNAc	N-Acetyl-Glucosamine
GPCR	G-Protein Coupled Receptor
H&E	Haematoxylin and Eosin
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulphonic acid
HIF-1α	Hypoxia Inducible Factor-1 alpha
His	Histidine (His or H)
HIV	Human Immunodeficiency Virus
HMEC-1	Human Microvascular Endothelial Cell-1
HOBt	Hydroxybenzotriazole
HUVEC	Human Umbilical Vein Endothelial Cell
HPLC	High Performance (or Pressure) Liquid Chromatography
HPRT	Hypoxanthine guanine-Phosphoribosyltransferase
HRP	Horse Radish Peroxidase
HSQC	Heteronuclear Single Quantum Coherence spectroscopy

H_2O_2	Hydrogen peroxide
ICAM	Intercellular Cell Adhesion Molecule
IdoA	Iduronic Acid
IFN-γ	Interferon gamma
IL	Interleukin
IRI	Ischaemia-Reperfusion Injury
LAD	Leukocyte Adhesion Deficiency
LPS	Lipopolysaccharide
Lys	Lysine (Lys or K)
MALDI	Matrix-Assisted Laser Desorption/Ionisation
MAPK	Mitogen Activated Protein Kinase
MeCN	Methyl Cyanide
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinase
MS	Mass Spectrometry
MWCO	Molecular-Weight Cut Off
3NT	3-Nitrotyrosine
n-CCL2	nitrated CCL2
n-CXCL1	nitrated CXCL1
n-CXCL8	nitrated CXCL8
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NDST	N-Deacetylase/N-Sulphotransferase
NETs	Neutrophil Extracellular Traps
NEVP	Normothermic Ex Vivo Perfusion
NF-κB	Nuclear Factor-κB
NHK	Normal Human Kidney
NHS	National Health Service (e.g. NHS Blood and Transplant)
NHS	N-hydroxysuccinimide
NOS	Nitric Oxide Synthase
NMR	Nuclear Magnetic Resonance
ONOO-	Peroxynitrite
PAD	Peptidylarginine Deiminase
PBC	Primary Biliary Cholangitis
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
pI	Isoelectric point
PMN	Polymorphonuclear Neutrophil (also called Primary Neutrophils)
PPAD	Peptidylarginine Deiminase from *Porphyromonas gingivalis*
PSGL	P-Selectin Glycoprotein Ligand
PTM	Post-Translational Modification
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 or Response Units
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SOD	Superoxide Dismutase
SPPS	Solid-Phase Peptide Synthesis

SPR	Surface Plasmon Resonance
SSC	Side Scatter
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic Acid
TGF-β	Transforming Growth Factor Beta
TIPS	Triisopropylsilane
TLR	Toll-Like Receptor
TOF	Time of Flight
Trp	Tryptophane (Trp or W)
TW	Trans-Well chemotaxis
TNF-α	Tumour Necrosis Factor-alpha
Tyr	Tyrosine (Tyr or Y)
UFH	Unfractioned Heparin
UTI	Urinary Tract Infection
UOO	Unilateral Ureteral Obstruction
VCAM	Vascular Cell Adhesion Molecule

Acknowledgements

I would like to thank my supervisors Profs Simi Ali, Neil S. Sheerin and John A. Kirby for their continuing encouragement, ideas and critical guidance throughout the project at Newcastle University.

To the Chemistry Department at Durham University. Particularly to my supervisors, Dr Steven L Cobb and Dr Ehmke Pohl for their excellent training, ideas and invaluable help to learn all the biochemical and biophysical techniques applied in the project. Also, to Dr Elizabeth Bromley for her guidance during the CD studies. To the Cobb laboratory colleagues, particularly to Dr Sam Lear, Dr Hannah Bolt and Dr Alex Hudson. Implementation of chemical and biophysical techniques would have not been possible without them. Research and social group events were a continuous joyful learning process.

To Dr Helen Waller and Prof Jeremy Lakey at the Institute of Cellular and Molecular Biology (ICaMB) at Newcastle University for their brilliant training and guidance during the CD and Biacore studies.

To everyone who helped with my research at the Institute of Cellular Medicine (ICM). To the members of the Kirby/Ali laboratory including my amazing Marie Sklodowska-Curie ITN fellows, Gabriel Cantanhede, Laura Ferreras and Nina Jordan within the project POSAT (Prolong Organ Survival After Transplantation) for their help and support during my PhD research and for making life a joyful experience. Also, to Mrs Barbara Innes for her advice with staining, and various other technical tasks along the project, as well as always impressive cooking abilities, well-known and complemented with other colleagues' delightful shared treats within the Institute. I would like to acknowledge Mr Jonatan Scott for his assistance with the neutrophil isolation. To Dr Chris Lamb for his generous help with flow cytometry. Also, Jeremy Palmer for his help with protein purification and great technical advices.

To Prof Krishna Rajarathnam (Texas University Medical Branch) for his help and intellectual input with NMR studies of chemokines and post-translational nitration of chemokines.

To Cellix Ltd. and my supervisors, Dr Dmitry Kashanin and Dr Vivienne Williams for giving the POSAT project, and me particularly a brilliant chance to accomplish our research plan; also other side projects within the company during an intense, positive and fully enjoyable industrial PhD secondment period. And of course, to Cellix Ltd. team particularly Toby Paul, Francesco Dicorato and Javier Dominguez Gallardo. The excellent industrial dynamics, immersive experience and flow-based studies, as well as team meals, socials and Dublin life, I will never forget as decisive both in my personal life and career.

To Prof Paul Proost (KU Leuven, Belgium) and Dr Alison Tyson-Capper (Institute of Cellular Medicine) for evaluating this study.

To my funding body, European Commission FP7 Marie Sklodowska-Curie Actions, for the platform and financial support without which the project would have not been possible.

To Dad Ángel, Mum Mª de los Ángeles and Dr Yolanda Martínez Burgo, for their continuous invaluable encouragement, support and understanding throughout life.

Thank you.

1. General Introduction

1.1 Background to this study

Inflammation is a response to injury, but is often associated with tissue damage, and in this context, is an important clinical problem. Inflammatory diseases were estimated to be associated with 44% of global morbidity in 2008. Particularly vascular diseases, e.g. initiated by ischaemia-reperfusion injury (IRI) following transplantation are nowadays leading cause of death globally (World Health Organisation). In transplantation, more than 80% of kidney, heart, lung, and liver allograft survive for more than one year. However, most of these transplanted organs undergo a process of chronic rejection which, in the UK, results in the failure of 30% of transplanted kidneys, 40% of hearts and livers and more than 70% of lungs within 10 years (NHS Blood and Transplant). Leukocyte recruitment mediated by cytokines and chemokines is critical in inflammatory conditions, such as during IRI following transplantation. Chemokine function can be regulated by many factors such as by GAG-binding and GPCR-binding, and also by post-translational modifications. This study addresses the role of GAG-binding and the role of post-translational nitration on the chemokine regulation to offer a better understanding and to identify potential therapeutic interventions.

1.2 Transplantation

Transplantation is a well-established treatment option for patients with end-stage organ failure (kidney, lung, liver) (Storey and Storey, 2007; Abecassis *et al.*, 2008; Thompson *et al.*, 2017), and several forms of cancer (leukaemia, lymphoma or myeloma) (Kolb, 2017). Despite its success, it is limited by the number and quality of donor organs available relative to the number of patients on the waiting list ('Keeping kidneys,' 2012; Kaths *et al.*, 2015; Giwa *et al.*, 2017; Kaths *et al.*, 2017).

One of the major challenges in transplantation is to increase the number of high quality donor organs. Hence, the development of new strategies to repair and improve the quality of organs could have remarkable impact on the number of transplants performed and their outcome.

Early reperfusion is critical for improving immediate function of the organ after transplantation but reperfusion itself is responsible for increasing the injury that is initiated by ischaemia (Granger and Kvietys, 2015). Leukocyte recruitment to the injured tissue is a crucial component of damage after ischaemia. Heparan sulphate (HS), the most abundant glycosaminoglycan (GAG) on vascular endothelium, binds and presents pro-inflammatory molecules such as chemokines to the leukocytes, which can contribute to leukocyte recruitment and transmigration.

1.3 Kidney anatomy and physiology: brief overview

The kidneys purify toxic products of metabolism from the blood in thousands of functionally independent units called nephrons. This occurs by filtration, reabsorption and secretion to keep balance of essential elements and eliminate toxic waste substances. Each nephron consists of a glomerulus and a double hairpin-shaped tubule that drains the filtrate into the renal pelvis. The glomerulus is located in the kidney cortex and is surrounded by the Bowman's capsule (Figure 1-1). The glomerular filtration barrier consists of endothelial cells, the glomerular basement membrane and visceral epithelial cells (also known as podocytes) (Kurts *et al.*, 2013). All molecules below ~50kDa pass the filter and enter the tubule, moving through the proximal convoluted tubule, the loop of Henle and the distal convoluted tubule. High osmotic gradient is formed in the renal medulla by an intricate counter current system to concentrate the filtrate. The tubular epithelial cells reabsorb water, amino acids, small proteins, carbohydrates and electrolytes, this way regulating plasma osmolality, extracellular volume, blood pressure, acid–base and electrolyte balance. Non-reabsorbed compounds for secretion, pass from the tubular system into the collecting ducts to form urine. The space between tubules, the interstitium, contains most of the intrarenal immune system. It primarily consists of dendritic cells, and also macrophages, neutrophils and fibroblasts (Gluhovschi *et al.*, 2010; Kurts *et al.*, 2013). These cells are involved in different innate immune processes, or lead to adaptive immune responses, during kidney inflammation.

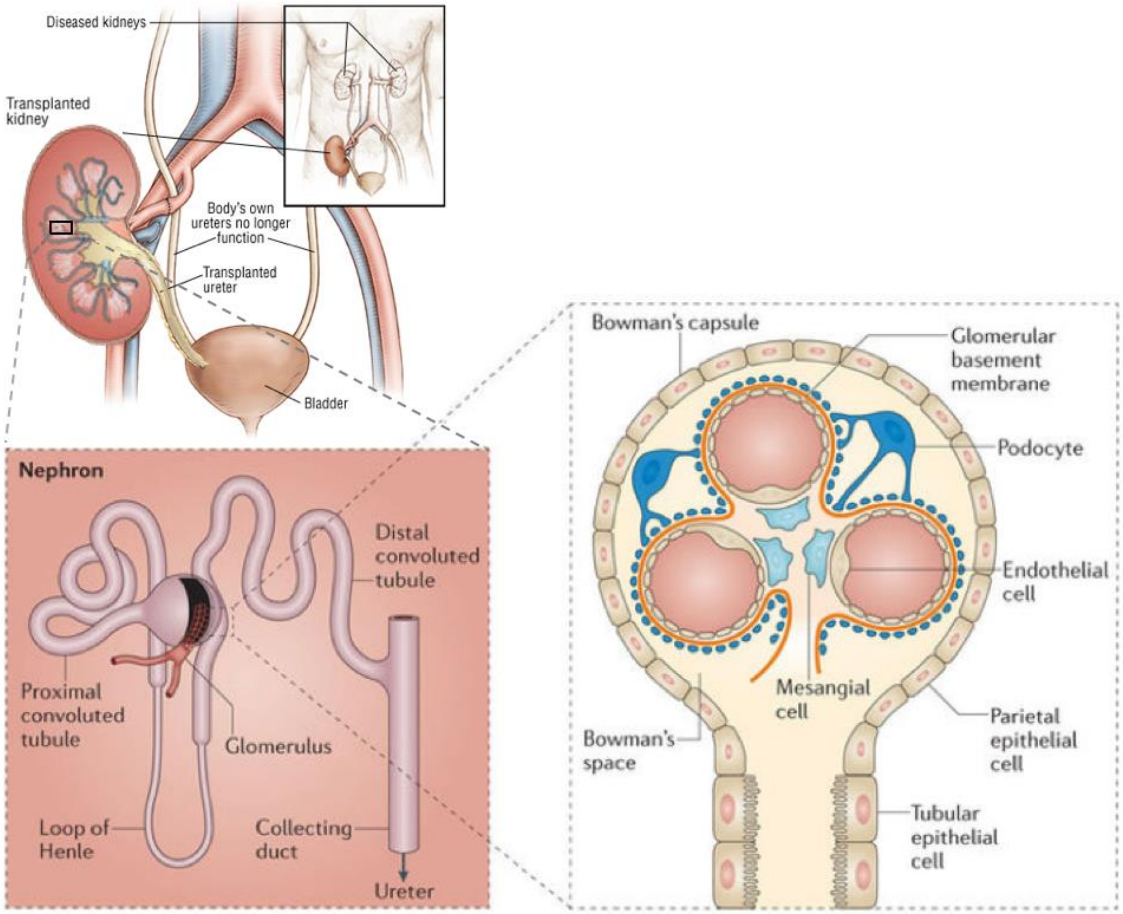


Figure 1-1. Kidney transplantation, anatomy and physiology.
Schematic representation of a kidney transplant and basic kidney anatomy and physiology (adapted from Harvard Health Publishing, 2012; Kurts *et al.*, 2013).

1.4 Organ transplantation after end-stage diseases

All organs transplanted suffer damage, either from the process of retrieval, transport or storage of organs prior to the surgery, from reperfusion after release of the vascular clamp and from immune responses generated post-transplantation. Each of these factors has a variable impact on the performance and survival of the organ.

Organ shortage has become one of the main factors limiting extension of deceased donor kidney transplantation worldwide. The reason for this shortage is complex (social attitudes, insufficient reimbursement for hospital and legal frameworks) (Breyer and Kliemt, 2007). To add patients on a waiting list who have no chance of receiving an organ is both senseless and highly questionable ethically.

This shortage has caused increasing use of deceased donors who would not have been considered previously, including ECD (Extended Criteria Donor) or DCD (Donation after Circulatory Death), organs which are marginal and may have less favourable outcomes for the recipient. Kidneys from such donors are exposed to much greater ischaemic damage before retrieval and have reduced chance of immediate, as well as long-term, function.

Although the immunological mechanisms of acute rejection are now well understood, the causes of chronic failure remain to be fully determined (Land, 1994; Cao *et al.*, 2017). The biggest challenge after organ transplantation is prevention of chronic deterioration of graft function (Ingulli, 2010). There is therefore an urgent need to prolong the life of a transplanted organ, including the continued development of preservation technologies and the early detection of any issues (O'Donnell *et al.*, 2011).

With coordinated and cross-disciplinary research, it is possible to dramatically accelerate progress in organ and tissue preservation using existing knowledge from a diverse array of fields. The past decade of progress allowed to understand and intervene in human physiology at the tissue and organ level as never before, with breakthroughs in nanotechnology, sequencing, imaging, omics approaches, and other areas (Giwa *et al.*, 2017). These technologies can be applied to organ preservation and include; organ cryopreservation (Fahy *et al.*, 2004; Arav *et al.*, 2005; Pomfret *et al.*, 2008; Campbell *et al.*, 2014; Lewis *et al.*, 2016), discoveries from organisms that can enter 'suspended animation' at sub-zero temperatures (Storey and Storey, 2004; Storey and Storey, 2010; Larson *et al.*, 2014), rapid advances in perfusion technologies (Guarrera *et al.*, 2010; Berendsen *et al.*, 2012; Cypel *et al.*, 2012; Moers *et al.*, 2012; Berendsen *et al.*, 2014; Machuca and Cypel, 2014; Messer *et al.*, 2015), and other recent advances, such as new organ allocation regulations aimed to maximise the efficiency of use of available organs (Abramowicz *et al.*, 2018); or development of standards for the combination of regenerative medicine and normothermic perfusion approaches during transplantation (Nasralla *et al.*, 2018).

Injury of the renal allograft after transplantation has major impact on long-term graft survival. Delayed graft function (DGF) is used to describe the need for dialysis after kidney allograft transplantation within the first week after transplantation. The frequency of DGF varies from 4–10% in living donor transplants to 5–50% in deceased donor transplants (Perico *et al.*,

2004). The early diagnosis of impaired allograft function after transplantation may help to improve post-transplant management with reduced morbidity and health care costs (Daemen *et al.*, 1999; Borst *et al.*, 2015).

1.5 Ischaemia reperfusion injury immediately after renal transplantation

Ischaemic Injury is damage caused due to the cessation of arterial blood flow with immediate oxygen deprivation of cells (e.g. hypoxia with accumulation of metabolic products). It is a major problem that can occur before, during or immediately after transplantation. It results in ATP depletion and calcium overload in the cytoplasm and mitochondria (schematic representation in Figure 1-2). These factors lead to a secondary burst of oxidative stress by cellular infiltration. The following category of tissue damage results from the reperfusion of the tissue after the ischaemic insult, and it is associated with endothelial inflammation. Repair and regeneration processes then occur (O'Donnell *et al.*, 2011; Kalogeris *et al.*, 2014) and the fate of the organ depends on whether cell death or regeneration prevails. The whole process has been described as the ischaemia-reperfusion injury (IRI). It has a profound influence on not only the early stages of inflammation but also on the late function of a transplanted kidney, such as during chronic rejection. Prevention of I-R injury could be started before organ recovery by donor pre-treatment. Low-dose dopamine infusion before organ retrieval; storage of the organ using hypothermic machine perfusion; or *ex vivo* normothermic perfusion to avoid cold ischaemic injury, particularly for marginal donors, and real-time quality assessment of kidney pre-transplant, are strategies that hold potential for further development and application to improve early renal graft function (Kosieradzki and Rowinski, 2008; Schnuelle *et al.*, 2009).

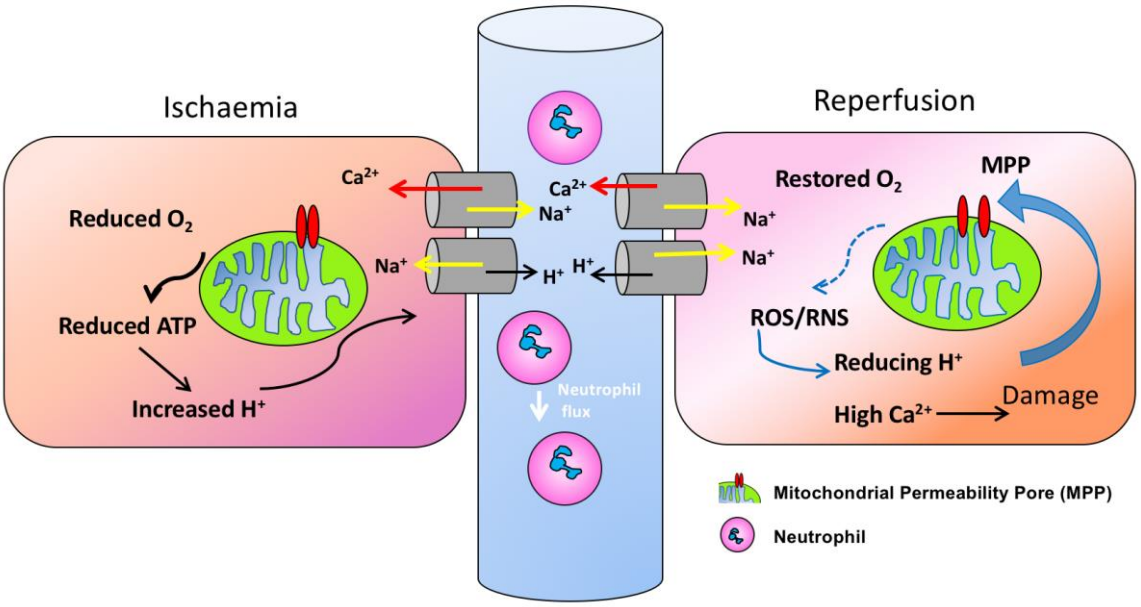


Figure 1-2. Cellular and molecular factors involved in Ischaemia-Reperfusion Injury (IRI).

During ischaemia, blood flow is interrupted triggering the reduction in oxygen levels (reduced O_2). Following ischaemia, blood flow is re-established in the affected organ (e.g. kidney, heart). The organ is subject to a number of abrupt changes during the transition from ischaemia to reperfusion. Both biochemical and metabolic alterations occur, including the generation of Reactive Oxygen Species and Reactive Nitrogen Species (ROS/RNS), reduction in ATP levels, an increase in inflammatory mediators (e.g. cytokines, chemokines), the rapid restoration of physiological pH, which in turn increases intracellular sodium (Na^+) and overload of intracellular and mitochondrial calcium (Ca^{2+}). These factors interact with each other to mediate reperfusion injury through the opening of the mitochondrial permeability transition pore (MPP) and initiation of cell death pathways, with consequent leukocyte recruitment in response to inflammatory mediators and ROS/RNS.

1.6 Types of graft rejection

Graft-related rejection can occur because the host's immune system can attack the graft, known as "host vs graft" response, or because immune cells in the graft can attack the host, "graft vs host". There are three main types of "host vs graft" rejection: hyperacute, acute (T-cell mediated or antibody-mediated) and chronic rejection (Moreau *et al.*, 2013; Gonzalez-Molina *et al.*, 2016).

Hyperacute rejection

Hyperacute Transplant Rejection occurs almost immediately and is often diagnosed while still in surgery. It is caused by presensitization to donor tissue, by circulating cytotoxic IgG

antibodies, and is most commonly due to a previous failed graft, blood transfusions or pregnancy (Tittelbach-Helmrich *et al.*, 2014). The host has pre-formed antibodies against the donated tissue. For example, a recipient with Type B blood would have pre-made antibodies targeted at the carbohydrates on the blood of a Type A donor. The presence of preformed antibodies is why the reaction takes places quickly. This results in thrombosis and occlusion of the graft vessels. This is an example or endothelial cell damage, complement activation and consequently inflammatory cell recruitment, particularly early responders as neutrophils.

Acute rejection

Acute Transplant Rejection is the most common type of rejection and usually has an onset between weeks and months of the transplant. It can be a T-Cell mediated response against foreign Major Histocompatibility Complex (MHC) in the donated organ. This process results in leukocyte infiltration of the graft. Particularly, neutrophil depletion has been shown to decrease acute rejection (Jones *et al.*, 2010). The risk of acute rejection can be diminished (but not eliminated) with prophylactic immunosuppression. When identified, early T-cell mediated acute rejection can be treated with immunosuppressants and corticosteroids. However, acute antibody-mediated rejection (ABMR) plays an increasingly critical role in allograft loss e.g. in kidney transplantation, and is considered, together with chronic antibody-mediated rejection, among the most important barriers that limit long-term outcomes (El-Zoghby *et al.*, 2009; Sellares *et al.*, 2012; Djamali *et al.*, 2014).

Chronic rejection

Chronic Transplant Rejection occurs months to years after the transplant. The exact mechanism is not well understood. Data has shown that neutrophil depletion, inhibition or DNase digestion of NETs worsens chronic tissue injury (Jones *et al.*, 2010; Schauer *et al.*, 2014). It results in intimal thickening and fibrosis of graft vessels as well as organ atrophy. Chronic rejection causes a slow progressive decline in organ function. There is no treatment available and these patients need to receive a new organ transplant. When chronic rejection is suspected a full workup is done to rule out “late onset” acute rejection which can be treated (Becker *et al.*, 2016).

1.7 Immune system: chemokine-mediated neutrophil trafficking and milieu

The immune system is an evolved and complex network of molecules, cells and organs that work together to protect the body from harmful substances or organisms, and defend against disease. It can be classified by distinctive responses: innate immune response and adaptive immune response (T cell-mediated or antibody-mediated response).

The innate immune response is non-specific, starts rapidly after damage and forms no memory of the pathogen/damage signal. The adaptive immune response is initiated by the innate immunity, a pathogen/damage signal-specific response is instigated, and memory is formed against future infection by the same pathogen/damage signal (Iwasaki and Medzhitov, 2015).

1.7.1 Innate immunity during ischaemia-reperfusion injury

Innate immunity is associated with the early stages of inflammation, such as during ischaemia and reperfusion injury following transplantation, and is characterised by the accumulation of neutrophils and monocytes/macrophages. A subsequent response is characterised by T cells, NK cells, and macrophages. B cells, dendritic cells, mast cells, platelet, endothelial cells and other parenchymal cells all contribute to these processes (Shimizu and Mitchell, 2008).

With regards to inflammation, its four cardinal signs are redness (rubor), heat (calor), swelling (tumour) and pain (dolor), first described in Roman times (Celsus, c. A.D.25). The inflammatory response is initiated mostly by tissue stress, e.g. trauma, hypoxia - Damage Associated Molecular Patterns (DAMPs), or by external damage - Pathogen Associated Molecular Patterns (PAMPs) that act through pattern-recognition receptors (PRR), such as Toll-Like Receptors (TLRs). Both dangers and strangers induce expression of several inflammatory markers, such as cytokines, chemokines, or compounds of the extracellular matrix. The expression of these factors is regulated by specific transcription factors, such as NF-κB, STAT, AP-1, key modulators of inflammation (Lutz *et al.*, 2010). Subsequently, innate immune markers are mediators for activation of an adaptive immune response, via effector cells such as neutrophils (Shimizu and Mitchell, 2008).

The main reasons for an inflammatory reaction of a transplanted organ are rejection episodes, infection or ischaemia reperfusion injury. The latter is particularly important as it affects

every solid organ during transplantation. It impairs acute as well as long-term graft function, and can further contribute to increase rejection episodes that again affect the long-term outcome.

Ischaemia or reperfusion injury following transplantation is associated with endothelial dysfunction, microvascular collapse, apoptosis, and infarction (Kalogeris *et al.*, 2012). Neutrophils primarily cause ischaemia/reperfusion injury via degranulation, releasing intracellularly stored cysteine and serine proteinases and neutrophil elastases and reactive oxygen species (ROS) (Witko-Sarsat *et al.*, 2000). Ischaemic ECs produce proinflammatory cytokines (e.g. TNF-α and IL-1) within minutes of organ reperfusion (Salvadori *et al.*, 2015). These cytokines in turn stimulate ECs, SMCs, cardiomyocyte, and parenchymal cell production of chemokines that are detected as early as 1 hour after transplantation. CC chemokines (CCL1, CCL2, CCL3, CCL4, CCL5) and the CXC chemokines (e.g. CXCL1, CXCL2, CXCL8, CXCL9, CXCL10) all appear during the first 24 hours, and quickly return to baseline by 48 hours (Shimizu and Mitchell, 2008). Hence, produced proinflammatory cytokines (e.g. TNF-α, IFN-γ, IL-1), complement C5a, as well as several chemokines (e.g. CXCL8) stimulate neutrophil degranulation as well as ROS production. Studies have shown that antagonism of CXCL8, using a dominant-negative CXCL8-based antagonist, is associated with reduction in neutrophil recruitment during acute renal rejection through increased affinity for GAG and decrease affinity for CXCR1/2 (Bedke *et al.*, 2010).

Cytokines likely affect all phases of transplantation injury by regulating intragraft leukocyte recruitment, as well as modulation of APC homing to secondary lymphoid organs and clonal expansion or tolerance induction of alloantigen-specific T cells. Production of cytokines by neutrophils, macrophages or dendritic cells can be modulated by cytokines such as IFN-γ, IL-4, IL-10, and IL-13, and potential associated phenotypic change of immune cells that could promote angiogenesis, regeneration and repair (Mocellin *et al.*, 2003; Hammer *et al.*, 2005). This suggests that T helper-1 (Th-1) or Th-2 cells may influence neutrophil cytokine production (Romagnani, 1994), which highlights the comprehensively complex control of cytokine-mediated immune response.

Strategies to prevent or treat IRI include blockade of cytokines/chemokines, adhesion molecules, NF-κB, specific MAP kinases, metalloproteinases, induction of protective genes, and modulation of the innate immune system (Eckle *et al.*, 2008; Grenz *et al.*, 2008; Lutz *et*

al., 2010). Furthermore, preconditioning of the organ is an area under intense investigation.

1.7.1.1 Neutrophils

Neutrophils are well known for their innate immune function as phagocytic cells. However, more recently a vast array of specialised functions has been recognised in health and disease. They can promote protective or pathological responses at different sites (Brinkmann *et al.*, 2004; Ley *et al.*, 2007; Soehnlein and Lindbom, 2010; Mantovani *et al.*, 2011; Phillipson and Kubes, 2011; Sadik *et al.*, 2011; Amulic *et al.*, 2012; Kolaczkowska and Kubes, 2013). When uncontrolled, neutrophil antimicrobial activity can lead to several autoimmune or inflammatory conditions, including systemic lupus erythematosus, rheumatoid arthritis or type I diabetes (Giaglis *et al.*, 2016). Therefore, their function must be regulated. Neutrophils have an approximate half-life of 1.5 and 8 hours in the circulation, in mice and humans respectively, and are continuously produced in the bone marrow from precursor cells (Kolaczkowska and Kubes, 2013). In humans, 50-70% leukocytes in circulation are neutrophils, whereas in mice they are 10-25%. Mature neutrophils in circulation have a diameter of 7-10μm, present a segmented nucleus and their cytoplasm is enriched in different granules and secretory vesicles (Soehnlein *et al.*, 2017). Essentially, they form three types of granules during maturation, filled with pro-inflammatory proteins: azurophilic (primary) granules, which contain myeloperoxidase (MPO), specific (secondary) granules which contain lactoferrin and tertiary granules which contain gelatinase B, also known as matrix metalloproteinase 9 (MMP9). Azurophilic granules and specific granules can be further subdivided. The reason for multiple types of granules is that some proteins cannot coexist in their innate form, such as neutrophil elastase and neutrophil-gelatinase-associated lipocalin (NGAL). Neutrophil can kill microorganisms both intracellular and extracellularly, responding to damage or infection through three main ways: phagocytosis, degranulation or using Neutrophil Extracellular Traps (NETs) (Phillipson and Kubes, 2011; Kolaczkowska and Kubes, 2013). When neutrophils encounter pathogenic microorganisms, they phagocytose them. Then, the cells kill the pathogen using NADPH-oxidase dependent mechanisms (reactive oxygen species) or antibacterial proteins (cathepsin, defensin, lactoferrin and lysozyme). These antibacterial proteins can also be released either into extracellular milieu. Highly activated neutrophils can eliminate extracellular microorganisms using NETs (Phillipson and Kubes, 2011; Soehnlein *et al.*, 2017).

In most tissues, the neutrophil recruitment cascade requires the following steps: tethering, rolling, adhesion, crawling, and transmigration (paracellular or transcellular) (Ley *et al.*, 2007; Sadik *et al.*, 2011) (Phillipson and Kubes, 2011; Kolaczkowska and Kubes, 2013) (Figure 1-3). The neutrophil recruitment is triggered by damage signals (IRI, trauma, infection, etc.) which induce the activation of leukocyte adhesion molecules (e.g. L-selectin) that interact with activated endothelial adhesion molecules, e.g. P-selectin glycoprotein ligand-1 (PSGL-1) allowing circulating leukocytes to tether and roll towards the site of injury. Cytokines (IL-1ß, TNF-α), chemokines (CXCL1, CXCL8, IL-6), components of the complement system, and other inflammatory players such as reactive oxygen species or nitrogen species (ROS/RNS) can be released by resident cells in the injured tissue to mediate the inflammatory response. Cell surface GAGs bind to chemokines at high concentrations and contribute to the generation of GAG-bound chemokine gradients. This leads to chemokine-leukocyte GPCR binding (e.g. CXCR1/2), a high-affinity interaction (Massena *et al.*, 2010). GPCR activation and mediated inside-out signaling induces conformational change of the leukocyte surface integrins from inactive to active form (e.g. CD11a/CD18, CD11b/CD18, CD11c/CD18). Chemokine-mediated integrin activation and binding to endothelial adhesion molecules such as ICAM-1, VCAM-1 induces neutrophil-endothelial adhesion. It allows subsequent neutrophil crawling and finally neutrophil migration (paracellular -between endothelial cells- or transcellular -through endothelial cell-) towards the injury. The process of neutrophil recruitment during early stages of inflammation, such as during ischaemia-reperfusion injury is represented below (Figure 1-3).

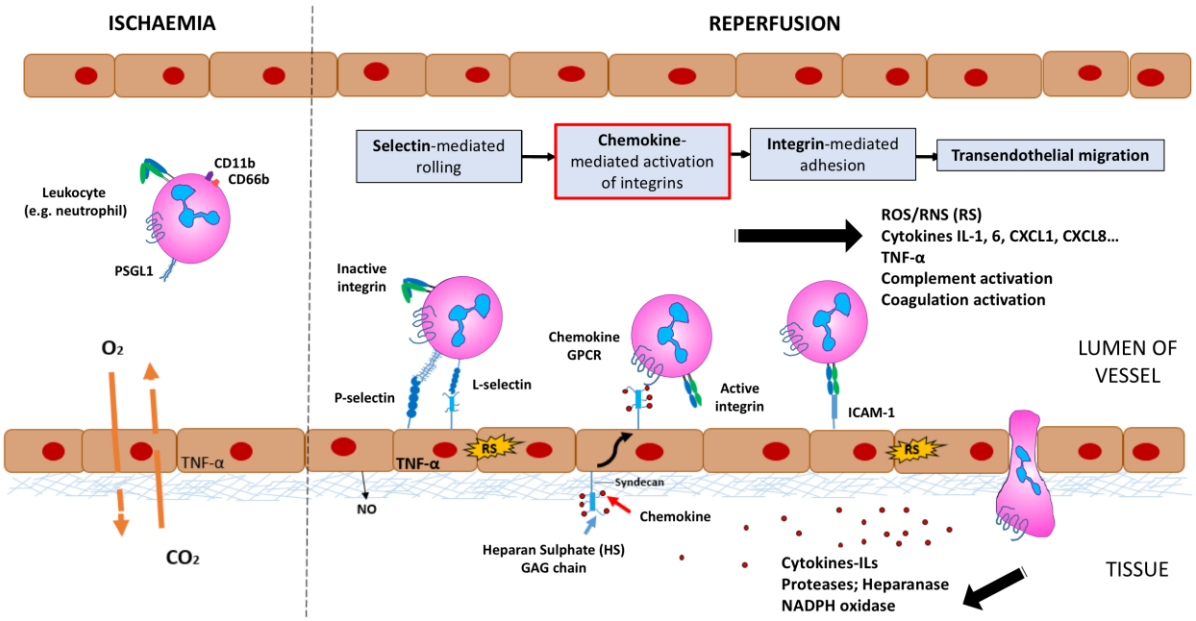


Figure 1-3. Neutrophil recruitment cascade e.g. during IRI.
Neutrophil recruitment is triggered by endothelial upregulation of adhesion molecules which results in interactions between selectins (e.g. P-selectin, E-selectin) and their ligands on neutrophils, leading to neutrophil tethering and rolling. Chemokines (e.g. CXCL1, CXCL8) sequestered on the lumen of the vessel through binding to glycosaminoglycans (GAGs), induce neutrophil binding to endothelium via a GPCR chemokine receptor. They trigger subsequent conformational changes of neutrophil β_2 integrins, which results in neutrophil adhesion and crawling. Mechanotactic and chemotactic guidance signals direct crawling of neutrophils to junctional transmigration sites (transcellular or paracellular) closer to the source of chemotactic agent.

Firm neutrophil adhesion to endothelial cells has been shown to involve critically the interaction of leucocyte β_2 integrins (CD11a/CD18, CD11b/CD18, CD11c/CD18) with ICAM-1, as shown by defects in CD18-deficient LAD patients and in ICAM-1 knock-out mice (Anderson *et al.*, 1984; Sligh *et al.*, 1993).

Evidence supports that during inflammation neutrophils are both target and source of various proinflammatory cytokines (e.g. IL-1, TNF-α), chemokines (e.g. CXCL8), and growth factors (e.g. GM-CSF). There is controversial literature on the role of neutrophils in inflammatory situations such as during renal ischaemia reperfusion injury after transplantation; otherwise, reports suggest this is due to the previous lack of high resolution imaging such as quantitative flow cytometry or *in vivo* labelling (Awad *et al.*, 2009; Bolisetty and Agarwal, 2009). Neutrophils exert their proinflammatory functions through an autoregulatory pathway (Witko-Sarsat *et al.*, 2000). Hence, tight regulation of the neutrophil

function is crucial for successful resolution of the inflammatory response (Witko-Sarsat *et al.*, 2000).

1.8 Chemokines

Chemokines are small cytokines (approximately 8 to 17 kDa) with chemoattractant properties that are involved in processes ranging from homeostasis to development and tissue repair. They also play essential roles in pathological conditions such as during tumorigenesis, cancer metastasis and inflammatory or autoimmune disorders, where their primary function is to direct the migration of leukocytes to the site of injury (Groves and Jiang, 1995; Mukaida, 2003; Meloni *et al.*, 2008; Lo *et al.*, 2011; Vogel *et al.*, 2011; Blanchet *et al.*, 2012; Salazar *et al.*, 2013). Chemokines are classified into four subfamilies: C, CC, CXC and CX3C in relation to the configuration of cysteine residues (Cys, C) within the N-terminal region (Scholten *et al.*, 2012; Bachelerie *et al.*, 2014a; Hughes and Nibbs, 2018) (Table 1). Many chemokines bind multiple receptors and most receptors bind more than one chemokine (Charo and Ransohoff, 2006). However, CC chemokine receptors exclusively bind CC chemokines and CXC receptors bind only CXC chemokines.

In addition, there are atypical receptors (ACKR) such as ACKR1/DARC or ACKR2/D6, that bind chemokines but do not induce G-protein signaling due to substitutions in the DRYLAIV domain, a highly conserved determinant of G-protein coupling (Bromley *et al.*, 2008; Bachelerie *et al.*, 2014c). They act as chemokine scavengers and are thought to be involved in the regulation of the immune response. For instance, DARC present on erythrocytes is known to induce adsorption and clearance of circulating CXCL8, affecting the chemokine's ability to stimulate neutrophil recruitment (Yoshimura *et al.*, 1987a; Yoshimura *et al.*, 1987b; Sekido *et al.*, 1993; Loos *et al.*, 2009), hence having a significant role limiting the inflammatory response.

CHEMOKINE RECEPTORS	CHEMOKINES
CXCR	
CXCR1	CXCL6, CXCL8
CXCR2	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8
CXCR3	CXCL4, CXCL4L1, CXCL9, CXCL10, CXCL11
CXCR4	CXCL12
CXCR5	CXCL13
CXCR6	CXCL16
CCR	
CCR1	CCL3, CCL3L1, CCL3L3, CCL5, CCL7, CCL14, CCL15, CCL16, CCL23
CCR2	CCL2, CCL7, CCL8, CCL13
CCR3	CCL2, CCL5, CCL7, CCL8, CCL11, CCL13, CCL15, CCL24, CCL26, CCL28
CCR4	CCL17, CCL22
CCR5	CCL3, CL3L1, CCL4, CCL4L1, CCL4L2, CCL5, CCL7, CCL11, CCL13
CCR6	CCL20
CCR7	CCL19, CCL21
CCR8	CCL1
CCR9	CCL25
CCR10	CCL27, CCL28
XCR	
XCR1	XCL1, XCL2

CX3CR	
CX3CR1	CX3CL1
DECOYRECEPTORS	
ACKR1/DARC/Duffy	CCL2, CCL5, CCL7, CCL11, CCL13, CCL15, CCL17, CCL18, CCL22, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, CXCL11
ACKR2/CCBP2/D6	CCL2, CCL3, CCL4, CCL4L1, CL4L2, CCL5, CCL7, CCL11, CCL13, CCL17, CCL22
ACKR3/CXCR7	CXCL11, CXCL12
ACKR4/CCRL1	CCL19, CCL21, CCL25
ACKR5/CCRL2	CCL19
FORMYL PEPTIDE RECEPTOR	
FPR2	CCL23

Table 1. **Chemokines and Chemokine receptors.**
Chemokines, organised in four main families, and associated chemokines receptors are shown. Chemokine functions can range from homeostasis to inflammation. Some chemokine receptors can act as chemokine scavengers involved in the regulation of the immune response, e.g. DARC or D6 (Blanchet *et al.*, 2012).

The migration of immune cells is mediated by the formation of chemokine gradients, which are generated by the anchoring of chemokines on glycosaminoglycans (GAGs) present on the surface of endothelial cells and extracellular matrix (Weber *et al.*, 2013). This creates an area of high chemokine concentration close to the site of injury, and aids presentation of chemokines to circulating leukocytes, which bind to the immobilised chemokine via their G-protein coupled receptors (GPCRs) (Figure 1-4) (Kolaczkowska and Kubes, 2013; Thompson *et al.*, 2017). This facilitates signaling through the GPCR, resulting ultimately in leukocyte rolling, adhesion, and transmigration into the tissue (as earlier shown in Figure 1-3). As shown in Figure 1-4, one of the possible scenarios, chemokines can exist as monomers or oligomers, with both forms playing an essential active role in modulating leukocyte trafficking and regulating other *in vivo* functions (Gangavarapu *et al.*, 2012).

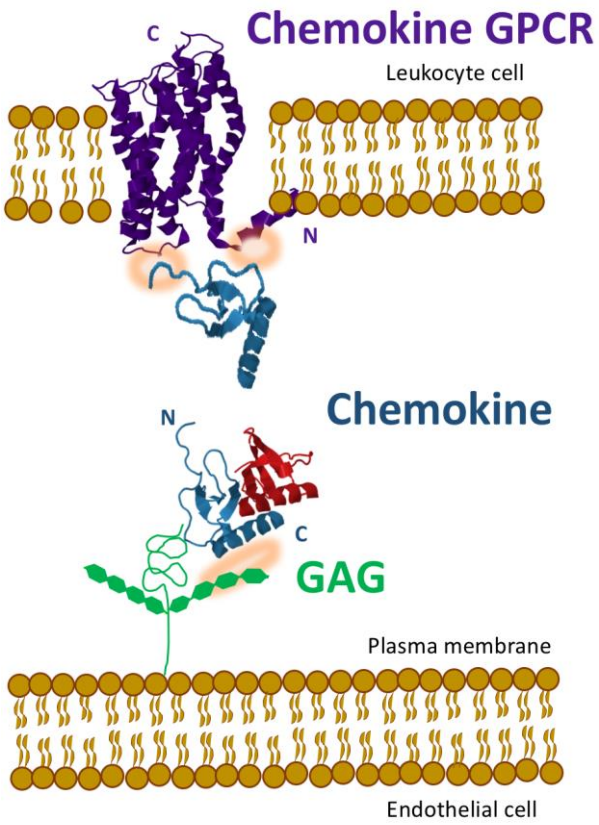


Figure 1-4. Chemokine interaction with endothelial GAG and with circulating leukocyte GPCR.

Schematic representation of chemokine interaction (e.g. CXCL8, PDB) with the endothelial surface through glycosaminoglycan (GAG) (via chemokine C-terminal and N-loop, highlighted in orange), which enables subsequent high-affinity chemokine binding to the leukocyte chemokine GPCR (2LNL, PDB) (via chemokine N-terminal region, also highlighted in orange). Chemokine monomer is shown in blue and the dimer is depicted with one molecule in blue and the other in red.

In general terms, monocytes are mainly attracted by CCL chemokines acting via CCR1, CCR2, and CCR5 receptors, whereas neutrophils are the target of CXCL chemokines such as CXCL1, CXCL2 or CXCL8. For instance, CXCL1 can only interact with neutrophil CXCR2. In fact, the murine CXCL1 and CXCL2/3, functional homologues of human growth-related oncogenes, and CXCL8, have been identified as primary chemokines mediating neutrophil recruitment in different models of acute and chronic inflammation (Lo *et al.*, 2011; de Oliveira *et al.*, 2013). Studies on a CXCL1 KO mice showed reduced neutrophil recruitment, which was reversed by LTB4 during *Klebsiella*-mediated lung infection (Batra *et al.*, 2012). CXCL8 and CXCL1 show an identity of 48% through BLAST alignment.

CXCL8 is the most abundant cytokine secreted by neutrophils, macrophages and endothelial cells. Neutrophils are the primary cellular target of CXCL8 (Gainet *et al.*, 1998). Targeting

CXCL8 in order to decrease inflammation has undergone early clinical studies (Campbell *et al.*, 2013). For instance, it showed improvement of shortness of breath in patients with COPD compared to placebo (Mahler *et al.*, 2004; Bachelerie *et al.*, 2014b). The presence of CXCL8 mRNA in freshly isolated neutrophils is widely reported. However, whether expression is constitutive or induced by isolation procedure is still debated. CXCL8 binding to the endothelial surface via GAGs such as heparan sulphate (HS), generating a haptotactic gradient concentration of surface-bound chemokine (Goger *et al.*, 2002), allows the subsequent neutrophil binding and recruitment via CXCR1/2 (K_D =1-2nM). Report showed that administration of anti-CXCL8 antibody to rabbits with an immune complex nephritis reduces proteinuria and neutrophil recruitment (Harada *et al.*, 1994; Wada *et al.*, 1994; Furuichi *et al.*, 2009). Although levels of urinary CXCL8 excretion are increased in patients in the acute phase of some forms of glomerulonephritis, a large number of CXCL1-positive neutrophils are also found in glomeruli and tubulointerstitium of patients with inflammatory glomerulonephritis (Segerer *et al.*, 2006; Segerer and Schlondorff, 2007).

1.8.1. Regulation of chemokine function

Regulation of chemokine function is essential to prevent excessive inflammation and optimise healing after injury or disease. This regulation can occur at many levels. It can involve different aspects of chemokine biology, including the concentration of chemokine, but also the steepness and duration of the chemokine gradient, the oligomeric state of the chemokine (monomer/dimer/oligomer) (Rot, 1993; Rot *et al.*, 1996; Lortat-Jacob *et al.*, 2002; Handel *et al.*, 2005; Taylor and Gallo, 2006; Rot, 2009; Bedke *et al.*, 2010; Das *et al.*, 2010; Weber *et al.*, 2013; Joseph *et al.*, 2015) and the ability of the chemokine to interact with GPCRs and GAGs (Kufareva *et al.*, 2015). Post-translational modifications (PTMs) such as proteolysis, nitration, glycosylation, or citrullination also play a critical regulatory role on chemokine function.

1.8.1.1. Chemokine interaction with GPCR chemokine receptor

Chemokines mediate their effects through G-protein coupled receptors (GPCRs), which all share a similar structure: an extracellular N-terminal domain, seven transmembrane-spanning segments, three extracellular loops, three cytoloops, and an intracellular C-terminal segment

(Rajagopalan and Rajarathnam, 2006). Binding of chemokine ligands to their receptors initiates a signaling cascade involving the influx of calcium, which ultimately leads to chemotaxis (Burg *et al.*, 2015; Qin *et al.*, 2015; Arimont *et al.*, 2017).

Targeting the interaction between chemokines and their receptors is one potential method to regulate the recruitment of leukocytes and modulate inflammation. However, this is limited by the high level of apparent redundancy displayed by chemokines and their chemokine receptors. While some receptor-ligand interactions are specific, e.g. CX3CL1-CX3CR1 or CCL20-CCR6 (Rajagopalan and Rajarathnam, 2006), chemokines can often bind multiple receptors, and receptors can be also activated by many chemokines, making it difficult to achieve a selective and specific response when targeting these interactions (Kunkel, 1999; Scholten *et al.*, 2012; Kleist *et al.*, 2016; Montague and Malcangio, 2016). For example, CXCL8 binds CXCR1 and CXCR2, but CXCR1 can also bind CXCL6 with low affinity and CXCR2 can also bind CXCL1/2/3/5/6/7 with high affinity (Baggiolini *et al.*, 1997; Rajagopalan and Rajarathnam, 2006). ELR+ chemokines (glutamic acid-leucine-arginine containing) are potent neutrophil chemoattractants and activators and, when administered exogenously, mediate neutrophil recruitment from the bone marrow into the circulation. CXCR2, and also CXCR1, are expressed not only by neutrophils, but also by eosinophils, basophils, mast cells and T lymphocytes. They are also found in endothelial cells where they contribute to chemotaxis. Also, CXCR2 is expressed in liver, kidney, and in cells of the central nervous system (Bromley *et al.*, 2008). CXCR1 and CXCR2 share 78% identity (Addison *et al.*, 2000).

Regarding CXCL8-GPCR interaction, regions within CXCL8 that contribute to receptor binding are the ELR motif, which is necessary but not sufficient for receptor binding (Lowman *et al.*, 1996), and the hydrophobic pocket within the N-loop, which consists of I10, Y13, F17, F21 and I22 (Williams *et al.*, 1996; Gschwandtner *et al.*, 2017) (Figure 1-5). Disulphide residues present within CXCL8 have been shown to be important, as modifying these disulphides results in reduced receptor binding (Groves and Jiang, 1995; Lowman *et al.*, 1996). The chemokine C-terminal is involved indirectly in signal transduction through conformational changes that allow the ligand to couple to the GPCR (Lowman *et al.*, 1996; Campbell *et al.*, 2013). All these residues are therefore potential candidates for modulation of CXCL8 signaling therapeutically. CXCR1/2 signaling occurs as the CXCL8 N-loop binds the receptor N-terminus (as earlier shown, Figure 1-4). Signaling through CXCL8 is commonly

under tight regulation, with minimal CXCL8 and CXCR1/2 expression in non-inflamed tissue (Hoffmann *et al.*, 2002). As earlier described, expression is known to be induced by inflammatory signals (e.g., TNF-α and IL-1), Reactive Oxygen Species (ROS) or different stresses, such as hypoxia. A variety of signaling pathways may be activated downstream of CXCR1/2, including MAPK, PI3K/Akt, PKC, FAK or Src. Reports from various groups have shown that CXCL8 signaling activates multiple transcription factors such as NF-κB, AP-1, HIF-1 and STAT3 (Waugh and Wilson, 2008) (Campbell *et al.*, 2013). Following ligand-induced activation, CXCR1/2 becomes desensitised by prolonged phosphorylation, internalisation and downregulation. Studies have shown that CXCR2 is internalised more rapidly than CXCR1, and its re-expression on cell surface occurs more slowly (Zaslaver *et al.*, 2001). The internalisation and recycling of the receptors plays a major role in the regulation of CXCR1/2 signaling (Campbell *et al.*, 2013). Better understanding of GPCR interactions is of focus within our group and others (Johnson *et al.*, 2005; Falsone *et al.*, 2013; Thompson *et al.*, 2017).

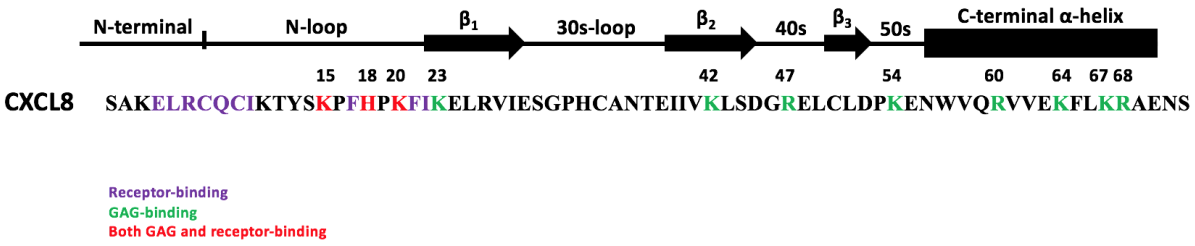


Figure 1-5. CXCL8 most common isoform (72 amino acids).
Relevant amino acids previously reported to be involved in GPCR/GAG binding are highlighted. Chemokine GPCR receptor-binding amino acid residues (purple). GAG-binding residues (green). Residues involved in both GPCR and GAG-binding (red).

1.8.1.2. Chemokine interaction with GAG and chemokine oligomerisation

Glycosaminoglycans (GAGs) are long linear polysaccharides that consist of a repeating disaccharide unit, frequently covalently attached to a core protein forming proteoglycans. GAGs are ubiquitously located on the surface of most cells or secreted/shed into the extracellular matrix (ECM). They bind and immobilise chemokines at high concentrations

towards the site of injury forming a chemokine gradient, to enhance their leukocyte chemotactic role. The highly sulphated and acidic GAGs bind to basic residues within chemokines through electrostatic interactions. This usually involves residues such as arginine, lysine or histidine, which typically form the BBXB or (B)BXX(X/B)BXXB(B) signature, where B is a basic amino acid and X a non-conserved amino acid, present in virtually all chemokines. In general, these GAG binding motifs are located at a site distant from the specific receptor-binding domain, often within the COOH-terminal region of the molecule, as occurs in CXCL8. Although chemokine-GAG binding largely depends on electrostatic interactions, a certain degree of specificity of this interaction has also been associated to the van der Waals forces and hydrogen bonding, which mainly contribute to the stability of the complex (Thompson *et al.*, 1994; Vanheule *et al.*, 2015).

For the CXCL8-GAG interaction the C-terminal α-helix region of CXCL8, while indirectly linked to receptor binding, is critical (Webb *et al.*, 2003; Andreoni *et al.*, 2014), primarily due to its considerable positive electrostatic charge, which has micromolar affinity for the negatively charged GAG. This binding is mediated by core residues H18, K20, R60, K64, K67 and R68, as shown in Figure 1-5.

1.8.1.2.1. Chemokine oligomerisation

Another mechanism that contributes to the regulation of chemokine function is oligomerisation. Chemokines interaction with GAGs present on the endothelial surface induces chemokine oligomerisation and can contribute to oligomer stabilisation, as reported for instance for CXCL8 (Salanga and Handel, 2011; Falsone *et al.*, 2013; Dyer *et al.*, 2016; Thompson *et al.*, 2017) (Figure 1-6) or CXCL1 (Sawant *et al.*, 2016). This could, in turn, affect receptor activation and protect chemokines from proteolysis.

In terms of monomer-dimer equilibrium, predominance towards dimeric CXCL8 being the higher-affinity GAG ligand has been observed, which indicates its potential as target therapeutic candidate (Das *et al.*, 2010). However, there are conflicting reports around this issue (Goger *et al.*, 2002; Krieger *et al.*, 2004; Gandhi and Mancera, 2011; Xu and Esko, 2014; Joseph *et al.*, 2015).

With regards to the chemokine-receptor binding, Nasser and colleagues (Nasser *et al.*, 2009; Gangavarapu *et al.*, 2012) demonstrated that while CXCL8 monomer/dimer equilibrium regulates CXCR1 and CXCR2 activation in a similar way (similar k_{off}), it differs in mediating receptor desensitisation and internalisation, because monomer is more active. CXCL8 monomer is more efficient than CXCL8 dimer in mediating PI hydrolysis and intracellular Ca^{2+} mobilisation. It has been hypothesized that function is determined not by K_d, but by the lifetime of the ligand-bound receptor complex ($1/k_{off}$).

Furthermore, chemokine receptors can interact with their chemokine as homo- or heterodimers, with both interacting possibilities in dynamic equilibrium, modulated by the receptor and ligand levels (Martinez Munoz *et al.*, 2009). In the case of CXCR1 and CXCR2, they interact constitutively, and selectively, and with equal apparent affinities (Wilson *et al.*, 2005).

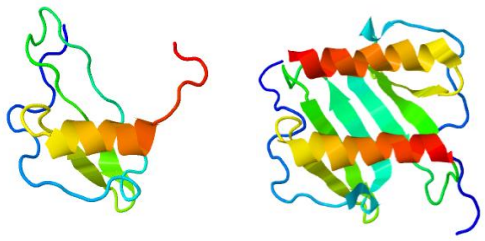


Figure 1-6. Scheme of CXCL8 monomer and CXCL8 dimer.

Both forms, monomer on the left (1KL, PDB) and dimer on the right (1CXCL8, PDB) are involved in the regulation of CXCL8 function.

1.8.1.2.2. Glycosaminoglycans

Glycosaminoglycans (GAGs), such as heparan sulphate (HS), are often covalently attached to a core protein forming proteoglycans, which can be classified according to their distribution, homologies, and function. Common examples of HS proteoglycans are syndecan, glypican, and perlecan. GAGs display varying patterns of sulphation, which in addition to carboxyl groups, confer a negative charge which is a critical determinant of chemokine binding (Handel *et al.*, 2005; Taylor and Gallo, 2006). They can be organised in four groups: heparin/heparan sulphate, chondroitin sulphate/dermatan sulphate, keratan sulphate, and hyaluronic acid (non-sulphated GAG that is non-covalently attached to proteins) (Figure 1-7).

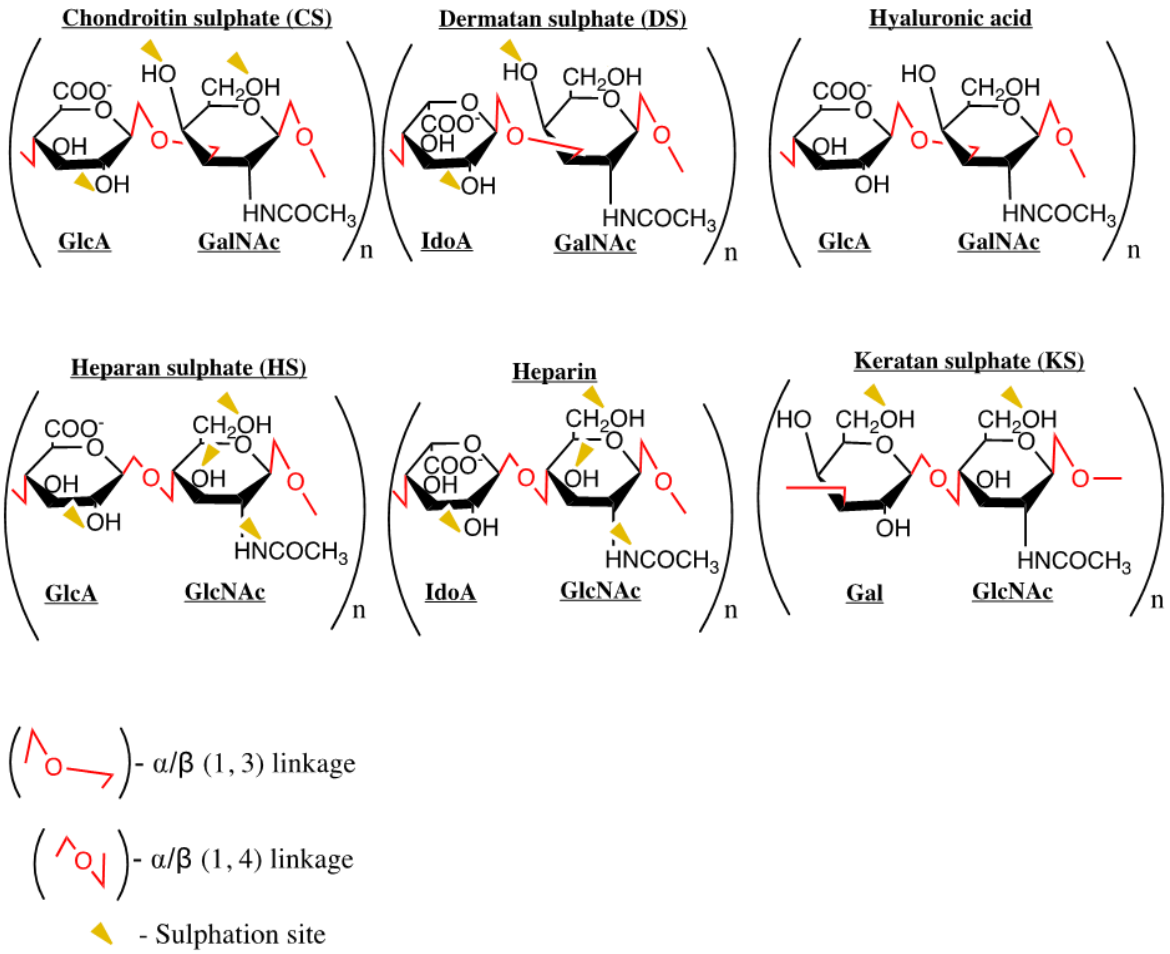


Figure 1-7. Structure and composition of GAGs.

Linkages are shown in red, and sites of sulphation indicated by yellow triangles. Disaccharide blocks composed of uronic acid (represented as GlcA: Glucuronic Acid or IdoA: Iduronic Acid) or Galactose (Gal), and the amino sugar (GalNAc: N-Acetyl-Galactosamine, GlcNAc: N-Acetyl-Glucosamine). Structure drawn with ChemDraw.

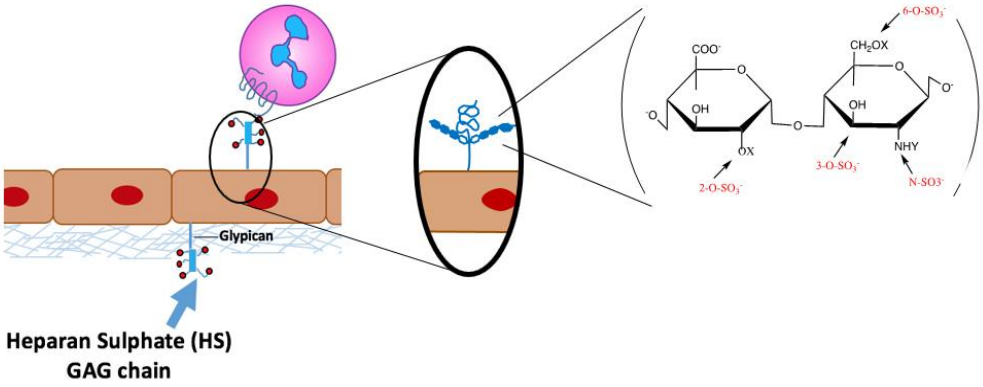
GAG binding has been identified as essential for the induction of chemotaxis, as studies using competent receptor binding, non-GAG binding, mutants showed these chemokines had impaired ability to recruit immune cells *in vivo* (Proudfoot *et al.*, 2003; Campanella *et al.*, 2006; Ali *et al.*, 2010). The diversity of GAGs (which vary notably in length, composition and sulphation pattern), the previously described oligomerisation state of the chemokine and the tissue microenvironment can all affect the chemokine-GAG interactions, and increase the challenge of targeting this aspect of chemokine biology (Gangavarapu *et al.*, 2012). It is hypothesised that structural plasticity through oligomerisation of chemokines on GAGs, or through GAG-induced folding of unfolded domains, are mechanisms of achieving functional

specificity. This way, through changes in oligomerisation or unfolded state structure, a given chemokine may recognise different GAGs (Kufareva *et al.*, 2015; Yan *et al.*, 2016).

1.8.1.2.2.1. Endothelial heparan sulphate (HS)

HS is an anionic glycosaminoglycan (GAG) component of proteoglycan molecules and the most abundant GAG on the surface of endothelial cells (Simon Davis and Parish, 2013). HS is initially synthesised as a repeating disaccharide composed of the monomeric units N-acetyl glucosamine (GlcNAc) and glucuronic acid. These units may or may not then be modified by a series of biosynthetic reactions within the Golgi. These give rise to N-sulphation of the glucosamine (GlcNS), 6-O-sulphation of the glucosamine and epimerisation and subsequent 2-O-sulphation of the glucuronic acid (Figure 1-8). The family of enzymes responsible for these modifications includes N-deacetylase/N-sulphotransferases (NDSTs), with four known human homologues (NDST 1-4); 2-O sulphotransferases (HS2ST), 6-O sulphotransferases (HS6ST), and 3-O sulphotransferases (HS3ST), which contributes to the rare addition of sulphate groups to C-3 of GlcNS (Parish, 2006). Mature HS can also be modified within the cell surface glycocalyx by specific sulphatases (including Sulph-2).

a)



b)

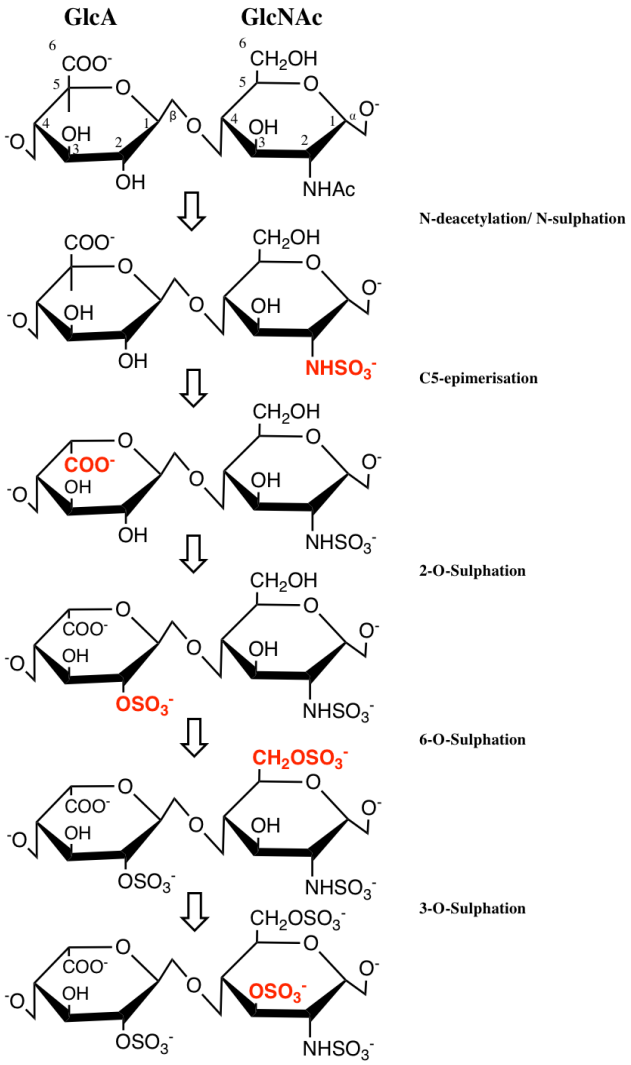


Figure 1-8. Heparan sulphate.

a) HS-GAG structure on vascular endothelium surface (adapted from (Ali *et al.*, 2005)). B) Modification steps in the biosynthesis of heparan sulphate, enhanced by proinflammatory cytokines. Structure drawn with ChemDraw.

On the apical surface of microvascular endothelium, the glycocalyx contains four times more HS than the next most commonly expressed GAG, chondroitin sulphate, and can reach a thickness of 500nm. Interestingly, the glycocalyx thickness has been shown to change during inflammatory situations such as in ischaemia reperfusion injury (Reitsma *et al.*, 2007). It can modulate the chemokine monomer-dimer equilibrium, thus the chemokine-GAG interaction. Particularly ischaemia can induce the release of some HS proteoglycans from the endothelial cell surface (Mulivor and Lipowsky, 2004). In addition, it is also known that the chemical composition of endothelial HS is variable, with proinflammatory cytokines being involved in increasing expression of NDST enzymes resulting in a corresponding increase in the extent of N-sulphation. Importantly, this enhances the potential of the endothelial surface of blood vessels to bind and present pro-inflammatory chemokines (Carter *et al.*, 2003), such as CXCL8 or CXCL1, which have been reported to be upregulated during ischaemia-reperfusion injury (Bertini *et al.*, 2004; Bedke *et al.*, 2010).

1.8.1.2.2.2 HS interaction with cytokines

HS serves homeostatic functions, including maintenance of the endothelial permeability barrier to small solutes and the activation of antithrombin III which inactivates thrombin and other proteases involved in blood clotting. It is also clear that endothelial HS can bind a range of cytokines, including chemokines. This binding occurs, as previously described, between appropriately sulphated, anionic domains on HS and consensus sequences of basic amino acids within the cytokine. CXCL8 can mainly interact with GAG (e.g. HS or Heparin) through a BBXXB motif. However, other residues, close or separated from this motif have also been described in CXCL8-GAG interaction. CXCL8-unfractioned-HS binding (K_D = 1.545 µM) and CXCL8-Heparin binding affinities (K_D = 2.710 µM) have been previously analysed through IFT (Gerlza *et al.*, 2014).

Several groups have shown elevated levels of soluble chemokines including CXCL8 (Chishti *et al.*, 2001), CXCL9, CXCL10 and CXCL11 (Ranjbaran *et al.*, 2006) in the blood of heparinised patients undergoing cardiopulmonary bypass during coronary artery bypass grafting. These levels return rapidly to near normal following the administration of protamine to reverse heparin after this procedure, suggesting that soluble chemokines are again sequestered by HS on the endothelial surface. Stress associated with ischaemia-reperfusion

injury induces a very rapid (3-10 minutes) release of pre-formed chemokines, such as CXCL8, and P-selectin molecules onto the apical surface of endothelial cells following the exocytosis of Weibel-Palade bodies.

1.8.1.2.2.3. Heparin

Heparin is a mixture of polysulphated oligosaccharides with a wide range of molecular weights from 5 to 30 kDa (Scholten *et al.*, 2012). It has an identical carbohydrate structure to HS but is more heavily sulphated, hence, more anionic. Unfractioned heparin (UFH) (with an average molecular weight of 13kDa), can interact with many proteins containing positively charged amino acids that yield a high charge density (Scholten *et al.*, 2012). This binding leads to a range of biological activities including well documented anti-coagulatory effects through the inhibition of Factor Xa and thrombin (Doster *et al.*, 2016). Studies performed *in vitro* have shown several additional molecular and cellular actions of heparins, suggesting their beneficial effects. These have previously been widely reported in inflammatory diseases, but are also proving potential influence in treating thrombosis in cancer patients (Kakkar, 2005). Li and colleagues determined that pre-treatment with UFH significantly inhibited lipopolysaccharide (LPS)-stimulated CXCL8 production in human pulmonary microvascular endothelial cells (Li *et al.*, 2015). Heparin was also shown to inhibit CCL5-mediated migration and invasion of human hepatoma cells (Sutton *et al.*, 2007).

Due to its uniform sulphation pattern, and the commercial availability of size-fractionated oligosaccharides of many different sizes, heparin is commonly used for structure–function and chemokine-GAG interaction studies.

1.8.1.3. Post-translational modifications (PTMs) of chemokines

Chemokine function can also be regulated by post-translational modifications (PTMs), which play a crucial role during inflammation. Some examples of PTM are truncation, glycosylation, nitration or citrullination. In this study, we focus on the role of post-translational nitration on the CXCL1 and CXCL8 function, which is associated with the

production of reactive species (RNS/ROS) and oxidative stress during inflammation (Molon *et al.*, 2011; Thompson *et al.*, 2017).

1.8.1.4. Other regulatory factors

Other mechanisms that contribute to chemokine regulation include genetic polymorphisms; mRNA splice variation; variation of expression, alternative signaling responses; chemokine concentration; steepness of the gradient; receptor homologous or heterologous desensitisation, internalisation, degradation and localization; down-regulation by atypical (decoy) receptors, or binding to natural or synthetically engineered inhibitors (Stone *et al.*, 2017).

1.8.2. Chemokines during IRI, acute damage and chronic damage

Chemokines are implicated in the pathogenesis of IRI as shown in kidney, heart or liver. During the pathogenesis of renal IR and transplant rejection in the mouse a role for CXCL5, CXCL8, CCL2, CCL3, CCL4 and CCL5 has been demonstrated (Furuichi *et al.*, 2008). In addition, CXCL9 and CXCL10, along with their shared receptor, have been detected in the renal interstitium after IR injury (Furuichi *et al.*, 2008). (Figure 1-9). During myocardial ischaemic injury, CCL2, CCL3, and CXCL2 and CXCL3 transcripts are elevated, peaking at 4 hours (Shimizu and Mitchell, 2008). Particularly, CXCL10, CXCL2, CXCL3, and CCL2 mRNA are found expressed in mouse cardiac isograft one day after transplantation (Yun *et al.*, 2000; Hancock *et al.*, 2001). CXCL9 and CXCL11 mRNAs are expressed in cardiac allograft not in isograft three days after transplantation. Also, mouse ECs expressed chemokines CXCL9, CXCL10 and CXCL11 in liver ischaemia/reperfusion injury; these chemokines also recruit activated T lymphocytes, NK cells, and monocytes/macrophages by binding to CXCR3 receptors (Zhai *et al.*, 2006; Merhi *et al.*, 2015).

NK cells and CD8+ T cells serve as potent effectors of the innate and adaptive immune response, respectively. They use the FasL and the perforin/granzyme pathway to kill target cells. CCL2, CCL3, CCL5, and CXCL10 activate NK cells and induce degranulation. A combination of direct ischaemia and the secondary effects of degranulating neutrophils and NK cells and activated macrophages leads to further endothelial dysfunction with impaired

vascular function, an increased procoagulant activity, and augmented inflammation and vascular permeability.

The role of the CXCL chemokine family in AKI was clearly shown by the finding that treatment with CXCR2 inhibitor or neutralising antibodies to CXCL1 or CXCL2 during renal IRI stops interstitial neutrophil infiltration, reduces renal damage and improves survival (Chung and Lan, 2011). Studies have also emphasised the importance of CXCR2 in the innate defence against urinary tract infections (UTIs). In mCXCR2 KO mice, neutrophil recruitment into the kidneys was slow and there was a delay in the subsequent neutrophil exit from the mucosa into the urine (Frendeus *et al.*, 2000). Consequently, neutrophils were trapped underneath the epithelium, where they caused a dysfunctional inflammatory response leading to tissue damage and renal scarring (Hang *et al.*, 2000; Topley *et al.*, 2005). Thus, the chemokine-receptor interaction has been shown to control resistance to both pyelonephritis and renal scarring in mice (Svensson *et al.*, 2011). In addition, studies in mCXCR1 KO mice showed increased hepatic damage by reduction of exosome release, which reduced sphingosin-1-phospate formation and liver regeneration after IRI. In contrast, mCXCR2 KO showed higher exosome release than wild type (Nojima *et al.*, 2016).

Other cytokines involved in chemokine function regulation during AKI are IL-23, IL-17A, or IL-17 receptor, as deficiency attenuates neutrophil infiltration in IRI (Li and Nord, 2009). IL-17A is known to act synergistically with TNF-α to induce expression of CXCL8, CXCL1 and CCL20 (Lee *et al.*, 2008). Therefore, in addition to pro-inflammatory effects, IL-17 and IL-23 pathways are also modulators of chemokine-mediated neutrophil infiltration during the innate immune response to IRI. Thus, targeting this pathway has been suggested as an approach for treatment of IRI (Gelderblom *et al.*, 2012). Furthermore, IL-13 a potent Th2 cytokine, has been demonstrated to inhibit the interstitial infiltration of neutrophils and macrophages, which associates with reduced expression of CXCL2, CXCL8 and CCL2. This supports the involvement of CXCL chemokines in immune cell recruitment during AKI (Cugini *et al.*, 2005).

Chemokines and chemokine receptors are also critical during chronic renal injury for the recruitment of T cells, macrophages, and dendritic cells, as supported from clinical studies (Holdsworth and Tipping, 2007) and animal models (Bonventre and Yang, 2011; Merhi *et al.*, 2015).

Overall, chemokines are important regulators of leukocyte recruitment during kidney injury. Although interference with their biology holds promise to ameliorate renal damage, chemokine function in different models of disease shows promiscuity and high complexity, hence further understanding of their function is required in order to develop targeted chemokine therapies for human renal disease.

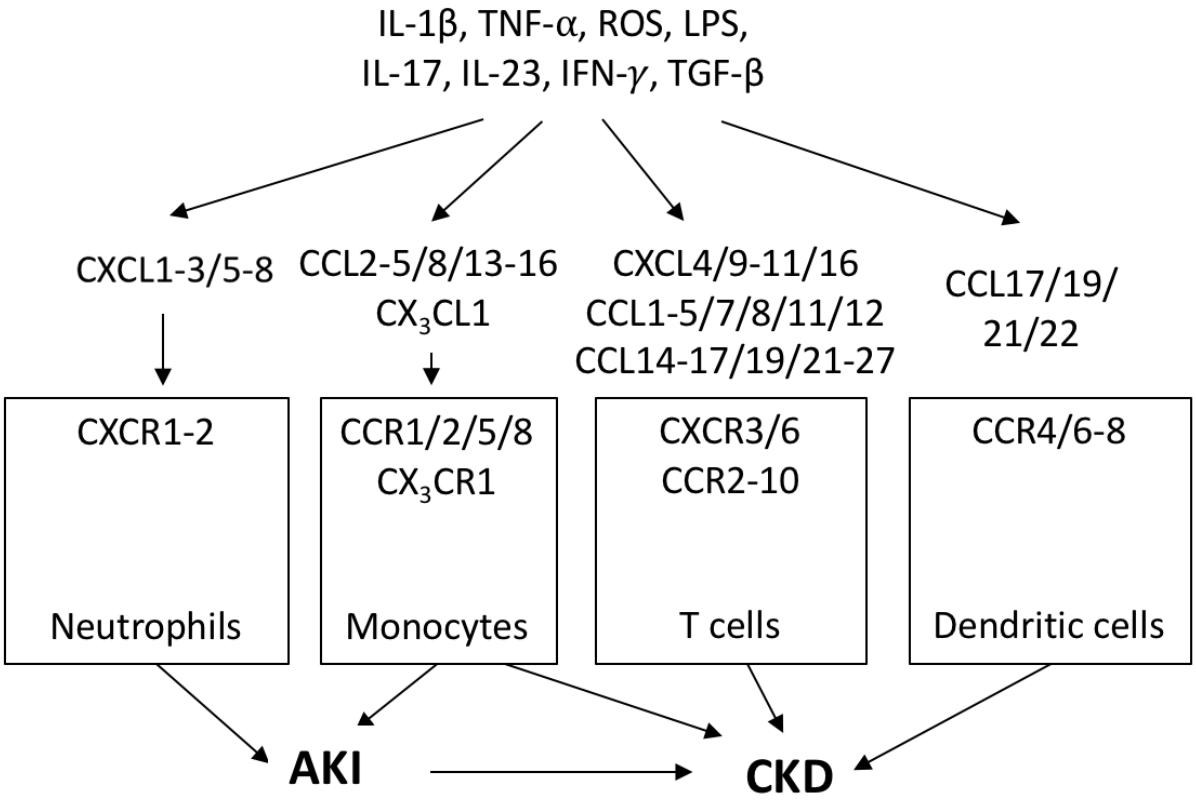


Figure 1-9. Chemokines and associated leukocyte subtypes during acute kidney injury (AKI) and chronic kidney disease (CKD).
(adapted from (Chung and Lan, 2011))

1.8.3. Strategies to target the inflammatory response during IRI

1.8.3.1. Studies of GAG and chemokine interaction

1.8.3.1.1. Competitive Displacement of Chemokines with GAG mimetics

Although it is not possible therapeutically to modify endogenous chemokines, our group and others have shown that chemokines can be displaced from the endothelial cell surface by competitive binding. Administration of a GAG, usually heparin, is thought to act through disruption of pre-formed chemokine gradients present on cell surface GAGs. Examples of low molecular weight heparins (LMWHs) currently used in anticoagulation therapy for haemodialysis patients are tinzaparin (Palamaner Subash Shantha *et al.*, 2015), enoxaparin (Robinson *et al.*, 2014) or dalteparin (Wu *et al.*, 2015). Heparin in various forms has been shown to inhibit leukocyte recruitment in mouse models of arthritis, traumatic brain injury and LPS treatment (Nagata *et al.*, 2016; Riffo-Vasquez *et al.*, 2016; Al Faruque *et al.*, 2017; Kozek-Langenecker *et al.*, 2017), although its effectiveness depends upon the dose given, the duration of inflammation and inflammatory mediators involved (Arimateia *et al.*, 2015). These studies show the potential of GAG mimetics on chemokine-mediated immunomodulation when administered either local or systemically. However, it is of note that, heparin can interact with broad range of cytokines due to its highly anionic nature, hence has potential to interfere with their function. Thus, more specific disruptive strategies targeting chemokine gradient formation with an effect on leukocyte recruitment could be beneficial.

Chemokine-GAG interactions play a crucial role in the antiviral immune response. Viruses can evade the chemokine-mediated immune response by expression of viral chemokine binding proteins (vCKBPs). They are able to interfere with the GAG-binding, GPCR-binding, or both, thus modulating chemokine-mediated leukocyte migration to the site of infection or tissue damage *in vitro* and *in vivo* (Gonzalez-Motos *et al.*, 2016).

1.8.3.1.2. Chemokine mutants with altered GAG binding

Investigations have also focused on development on non-GAG binding mutants by substitution of basic residues for alanine residues in the GAG binding domain. This way, mutants normally bind their cognate receptors and competitively inhibit binding of their wild type analogue, thus preventing migration along a chemokine gradient, which inhibits

chemotaxis. This has been reported for CCL2, CCL5, CCL7 and CXCL12 among others (Johnson *et al.*, 2004; Ali *et al.*, 2010; Gerlza *et al.*, 2015). For instance, our group developed a non-GAG-binding mutant form of CCL7 which maintains its affinity for chemokine receptors (Ali *et al.*, 2010). Similarly, our group found that a non-heparan-sulphate-binding CXCL12 mutant was also unable to stimulate leukocyte migration (O'Boyle *et al.*, 2009). These effects can be solely attributed to the loss of GAG-binding ability of CCL7 and CXCL12 biology, as both mutants were found to have unaffected receptor binding abilities. Both these mutants were able to inhibit the effects of their wild type counterparts *in vitro* and *in vivo*. Thus, mutant chemokines may represent a novel therapeutic tool to improve management of inflammation associated with ischaemia and reperfusion.

A non-GAG binding mutant of CXCL8 showed to efficiently reduce neutrophil recruitment into the peritoneum (Gangavarapu *et al.*, 2012), but did not reduce migration to the lung *in vivo* (Tanino *et al.*, 2010). This suggests that better understanding of the mechanisms of action in different tissues is crucial. Taken together, studies suggest that synthesising non-GAG binding mutants and using them to therapeutically antagonise the wild type chemokine is a viable method to modulate chemokine function.

PA401, a variant of CXCL8 (Δ6, F17K, F21K, E70K, N71K), which has no ability to bind GPCRs but does show increased GAG binding affinities has been shown to inhibit transendothelial migration of neutrophils by displacing CXCL8 already bound to endothelial surface GAGs (Adage *et al.*, 2015; Gschwandtner *et al.*, 2017). Therefore, administering higher affinity mutant chemokines to out-compete wild type molecules is another potentially successful method of regulating chemokines.

1.8.3.1.3. Chemokine peptide studies

In addition to whole chemokine mutants, short-length peptides of some chemokines have also been reported to modulate chemokine GAG binding (Gross *et al.*, 2015; Vanheule *et al.*, 2016). Several CXCL9 C-terminal peptides were created with and without mutations substituting in positively charged lysine residues to increase the affinity for GAGs. The longest peptide, CXCL9 (74-103) aa, was shown to successfully compete with CXCL8, CXCL11 and CCL2 for binding to heparin by Isothermal Fluorescence Titration (IFT) (Vanheule *et al.*, 2015; Vanheule *et al.*, 2017). In this study, we examine the potential of

CXCL8 C-terminal peptide [CXCL8 (54-72)], and variants, corresponding to the core GAG-binding CXCL8 region, to modulate CXCL8-mediated neutrophil function.

1.8.3.2. Studies of CXCR1/2 and chemokine interaction

1.8.3.2.1. Small molecule chemokine receptor antagonists

Acute and chronic inflammatory conditions involving CXCR2 include renal ischaemia/reperfusion injury, chronic obstructive pulmonary disease and fibrosis. In CXCR2−/− mice, neutrophil migration to sites of inflammation was severely disturbed (Frendeus *et al.*, 2000). In experimental approaches, CXCR2 antagonism showed to attenuate tissue damage and disease progress, e.g. in renal damage, arthritis, or sepsis (Hang *et al.*, 2000; Topley *et al.*, 2005). Modulation of the function of CXCR2 is therefore considered as a possible therapeutic strategy in the treatment of several inflammatory conditions.

Repertaxin, a non-competitive allosteric blocker of CXCL8 receptors CXCR1/2 has been used in murine models of IRI after renal transplantation. It was developed to lock CXCR1/2 in an inactive conformation, preventing receptor signaling and neutrophil chemotaxis (Wang *et al.*, 2016). Data showed that it potently and selectively blocked neutrophil adhesion to fibrinogen and CD11b up-regulation induced by CXCL8. Reduction of CXCL8-mediated neutrophil adhesion by repertaxin (K_D= 1nM) was followed by inhibition of neutrophil activation including secondary and tertiary granule release and pro-inflammatory cytokine production, whereas neutrophil phagocytosis of *Escherichia coli* bacteria was unaffected. Repertaxin also selectively blocked CXCL8-induced T lymphocyte and natural killer (NK) cell migration. The data has suggested that repertaxin is a potent and specific inhibitor of a wide range of CXCL8-mediated activities related to leukocyte recruitment and functional activation in inflammatory sites (Souza *et al.*, 2004; Casilli *et al.*, 2005; Cugini *et al.*, 2005; Wang *et al.*, 2016).

1.8.3.2.2. Blocking antibodies

CXCR1 blocking antibodies have been reported to reduce migration of neutrophils as well as their inflammatory functions (Hammond *et al.*, 1995; Quan *et al.*, 1996; Dyer *et al.*, 2014). Therapeutic antibodies targeting CXCR1/2 have been proven in preclinical studies of COPD and cancer.

Monoclonal antibodies targeting the CXCL8 ligand, rather than its receptors, have also been shown to block its biological activity (Leong *et al.*, 1994; Verleden *et al.*, 2006; Bao *et al.*, 2010). Clinical trials in COPD with a fully humanised CXCL8 antibody (ABX-IL8) showed some positive results as it decreased shortness of breath in patients with COPD compared to placebo (Lowman *et al.*, 1996; Mahler *et al.*, 2004; Scholten *et al.*, 2012; Campbell *et al.*, 2013; Bachelerie *et al.*, 2014a; Ha *et al.*, 2017; Thompson *et al.*, 2017). Also, this antibody showed potential in preclinical studies of melanoma by downregulation of matrix metalloproteinase-2 (MMP2), inhibition of angiogenesis and increased apoptosis (Ha *et al.*, 2017; Thompson *et al.*, 2017).

1.8.3.2.3. Mutation affecting CXCR1/2 and chemokine interaction

Mutation studies are useful both to study function and to determine which exact amino acids are essential for different aspects of chemokine biology. For instance, CXCL8 mutations targeting the Y13 residue have also resulted in altered functionality of CXCL8. A Y13E CXCL8 mutant showed reduced binding to CXCR1 by 150-fold and to CXCR2 by 43-fold (Lowman *et al.*, 1996), and a Y13T variant showed a modest 6-fold decrease in affinity for both receptors (Clark-Lewis *et al.*, 1994). Conversely, a Y13L mutant variant of CXCL8 showed a slightly increased affinity for CXCR1 only (Hammond *et al.*, 1996).

Tyrosine has also been shown to be an important residue within the receptor CXCR1, as a Y188A mutant showed decreased affinity for CXCL8 ($K_D \geq 25nM$) in comparison to the wild type receptor (Leong *et al.*, 1994).

1.8.3.2.4. Chemokine peptide studies

Peptide synthesis targeting chemokine-receptor interaction has also been addressed with successful inhibition of chemokine-receptor interaction, as shown by a CXCL8 peptide with ability to inhibit CXCL8 binding to CXCR1 (Jiang *et al.*, 2015), which might be of use for the development of therapeutic drugs.

1.9. Free-radical-mediated tissue injury

1.9.1. Oxidative stress

Oxidative stress is the accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Finkel and Holbrook, 2000). It results from an imbalance in reactive species (RS) producers 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, as it is the case during IRI (Brown and Griendling, 2015; Ghezzi *et al.*, 2016).

Infiltration of immune cells able to perform an oxidative burst, for example neutrophils and macrophages, is a further source of oxidative stress during inflammation by the release of multiple reactive species. This is an innate immune effector mechanism to aid the killing of pathogens. Although protective, it can contribute to inflammation and tissue injury due to excess release of reactive species. Sufficient antioxidants are crucial to prevent damage by oxidative stress (Barker *et al.*, 2014).

Oxidative stress can alter the protein covalent bonding by changes in amino acid content and protein conformation by changes in protein folding. It can also alter the oligomerisation states of proteins. Reactive species such as peroxynitrite ($ONOO^-$) 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 in order to maintain protein homeostasis. If repair is unsuccessful, there are two mechanisms of degradation: the ubiquitin-proteasome system, and the autophagic or lysosomal system (Filomeni *et al.*, 2015).

1.9.2. Peroxynitrite and related species

Peroxynitrite is produced by the rapid reaction of nitric oxide ($NO^{\cdot}$) and superoxide ($O_2^{\cdot -}$) at a rate approaching the substrate diffusion limit (Huie and Padmaja, 1993). It is 3 to 8 times faster than superoxide degradation by SOD, or other biomolecules (Dedon and Tannenbaum, 2004) and therefore can evade decomposition by the antioxidant enzyme. Figure 1-10 represents peroxynitrite and related species, including nitric oxide which is formed from the oxidation of arginine by NOS, with the stable end product of L-citrulline (Marletta, 1993; Pacher *et al.*, 2007). *In vivo* production of peroxynitrite is estimated to occur at 50 to

100µmoles per minute. Because of its short half-life of only 10ms at physiological pH, peroxynitrite is commonly spatially associated with sites of superoxide production such as the plasma membrane (Szabo *et al.*, 2007). However it can affect cells up to 20µm away (Szabo *et al.*, 2007). Peroxynitrite is found in cis form, more stable; and trans, which has greater reactivity, thus isomerisation creates complex kinetics (Beckman et al., 1992).

Due to the short half-life of peroxynitrite, the reactions and exact biological effects attributed to peroxynitrite or RNS in this study, are likely to be caused by breakdown products and related radicals of peroxynitrite.

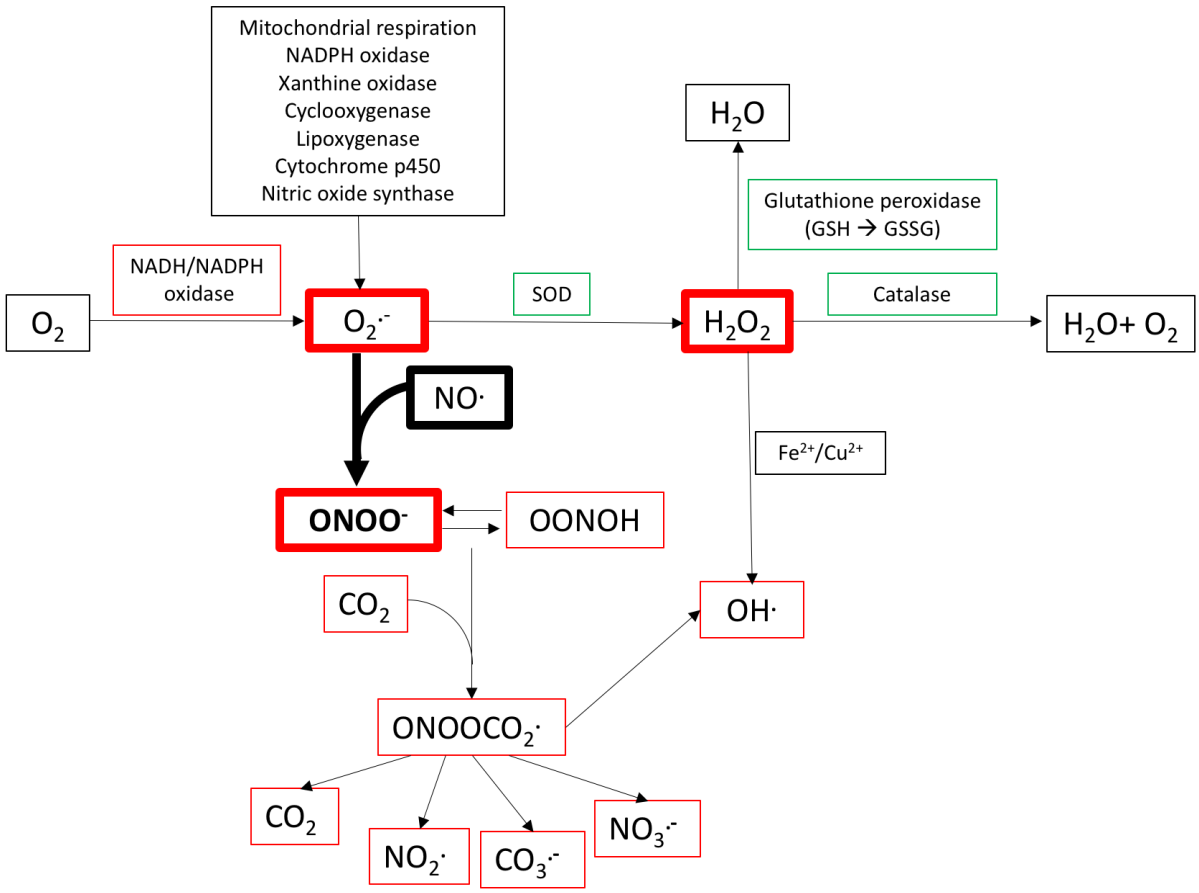


Figure 1-10. Reactive species that can be involved in oxidative stress
Reactive oxygen species (ROS) and reactive nitrogen species (RNS) can both be generated from superoxide, itself a ROS and a byproduct of aerobic respiration (NADH/NADPH oxidase). Antioxidant enzymes reduce the oxidative stress. Superoxide dismutase turns the highly reactive superoxide into the slightly less damaging hydrogen peroxide. ROS are turned to water to further remove damaged species. This is for example by the NADH dependant glutathione peroxidase or catalase. A representative list of involved species includes: $O_2^{\bullet-}$ -superoxide, H_2O_2 – hydrogen peroxide, $OH^{\bullet}$ - hydroxyl radical, $ONOO^-$ - peroxynitrite, $NO^{\bullet}$ - nitric oxide, ONOOH - peroxynitrous acid, $ONOOCO_2$ - nitrocarbonate ion, $NO_2^{\bullet}$ – nitric dioxide, $NO_3^{\bullet-}$ - nitrate, $CO_3^{\bullet-}$ – carbonate radical. Antioxidant enzymes (green): superoxide dismutase (SOD). Glutathione (GSH) (reduced). Glutathione disulphide (GSSG) (oxidised) (adapted from (Barker *et al.*, 2014; Biswas, 2016)).

The oxidative stress caused by the reoxygenation of ischaemic myocardium also causes injury by decreasing the intracellular NO concentration. Cardioprotective effects mediated by NO include inhibition of neutrophil accumulation, inactivation of superoxide radicals and improvement of blood flow through vasodilation (Jones and Bolli, 2006; Pagliaro and Penna, 2015).

1.9.3. Effects of peroxynitrite at a molecular level

Peroxynitrite, or derivatives, can selectively oxidise or nitrate many molecules with broad ranging effects. In DNA, peroxynitrite reacts mainly with guanine to form 8-nitroDG by nitrosation (incorporation of NO instead of NO_2), or 8-oxoDG (Dedon and Tannenbaum, 2004). It can also attack the DNA backbone causing strand breaks and deoxyribose oxidation (Dedon and Tannenbaum, 2004).

Particularly in proteins, peroxynitrite can nitrate aromatic amino acids, such as tyrosine and tryptophan. It is also of note that SOD and Fe can act as catalysts of nitration (Beckman *et al.*, 1992; Alvarez *et al.*, 1996), with the reported optimal pH of 7.5 (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*, hence it is generally accepted that the biomarker 3-nitrotyrosine (3NT) is indicative of peroxynitrite presence. Antibodies have been raised against 3-nitrotyrosine and are used to identify nitrated proteins indirectly and the presence of peroxynitrite or related species (Szabo *et al.*, 2007).

3NT can be generated in a protein sequence both co-translationally and post-translationally by free-radical reactions (Figure 1-11) (Souza *et al.*, 2008). Nitration is not a silent modification and can have profound effects in protein function (Molon *et al.*, 2011; Barker *et al.*, 2017). When alteration of protein function occurs and this leads to reduction 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 and Crow, 2011).

Peroxynitrite inactivation of CuZnSOD, however, is due to oxidation of a histidine residue in the active site (MacMillan-Crow *et al.*, 1996; Souza *et al.*, 2008).

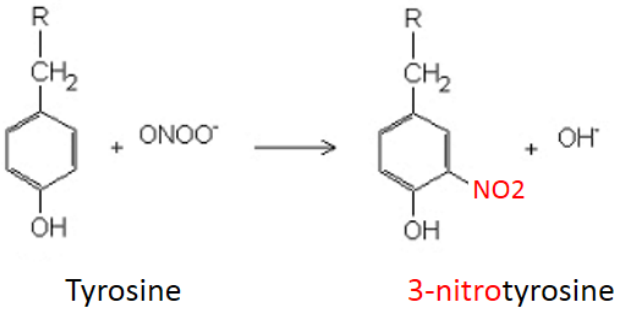


Figure 1-11. Tyrosine nitration
Nitration of tyrosine by peroxynitrite ($ONOO^-$) results in the formation of 3-nitrotyrosine. The reaction can occur on both peptidyl and free-tyrosine residues.

1.9.4. Effects of peroxynitrite at a cellular level

The effects of peroxynitrite, or RNS, can be both damaging and beneficial, synergising with or antagonising the effects of ROS. It depends on the peroxynitrite concentration and surrounding microenvironment, and 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-dependent activation or inhibition of multiple signaling biomolecules such as NF-κB, 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), and it is also involved in senescence induction, although at low concentrations it initiates survival signals (Urtasun *et al.*, 2008). 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, whereas macrophages are resistant to NO-induced apoptosis (Jang and Van Remmen, 2009; Shaw *et al.*, 2011).

In addition to affecting protein function, tyrosine nitration can also be involved in tyrosine phosphorylation, altering intracellular signaling. Generally, high peroxynitrite concentrations inhibit phosphorylation, possibly via cross-talk mechanisms and direct competition between nitration and phosphorylation (Lopez-Otin and Hunter, 2010), but low concentrations can

lead to an increase in phosphorylation either by activation of tyrosine kinases or irreversible inhibition of phosphatases (Pacher *et al.*, 2007). It has also been suggested that nitration can act similarly to phosphorylation (Souza *et al.*, 2008) through inhibition of phosphatases, therefore increasing phosphorylation levels (Takakura *et al.*, 1999).

Peroxynitrite has been suggested to be protective in early stages of injury, although this is likely to depend on the cell type and the situation (Urtasun *et al.*, 2009). Balance of the mediators of stress and the related inflammatory pathways is therefore critical in the cell outcome, for instance during cellular proliferation or fibrogenesis.

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, contributing to decrease fibrosis. It can also inhibit tissue inhibitor of metalloproteinase-1 function and therefore further increase the effects of MMPs (Frears *et al.*, 1996). However, anti-fibrogenic effects are only observed if cells are treated with peroxynitrite before being activated. Activated cells do not respond to peroxynitrite treatment as they undergo an increase in levels of glutathione, TGFβ and ROS.

1.9.5. Involvement in disease

Peroxynitrite is toxic to pancreatic β-cells, and there is increased 3NT in the autoimmune attack of type 1 diabetes (Szabo *et al.*, 2007). In renal transplantation, peroxynitrite can further increase oxidative stress by inactivating MnSOD, even though low levels of peroxynitrite are suggested to be protective, activating cell survival signals (MacMillan-Crow and Crow, 2011; Marine *et al.*, 2014). Peroxynitrite toxicity increase by depletion of its scavenger glutathione has been also associated with neurodegenerative disorders such as Parkinson's disease (MacMillan-Crow *et al.*, 1996; Pacher *et al.*, 2007). High levels of 3NT have also been shown to contribute to the development of numerous cancers including colon, liver and prostate (Kasic *et al.*, 2011; Molon *et al.*, 2011).

1.9.6. Citrullination

The post-translational conversion of arginine to citrulline is referred as citrullination (Figure 1-12**)**. Citrulline has $C_6H_{13}N_3O_3$ in the end of its lateral chain, as opposed to $C_6H_{14}N_4O_2$ in arginine. This arginine to citrulline conversion, can be catalysed by a nuclear enzyme called Peptidyl Arginine Deiminase 4 (PAD4). This enzyme is increased during kidney ischaemia-reperfusion injury (Ham *et al.*, 2014). It is also known that PAD can citrullinate CXCL8, alter receptor usage, prevent proteolysis, and dampen neutrophil extravasation during acute or chronic inflammation (Proost *et al.*, 2008; Moelants *et al.*, 2011; Moelants *et al.*, 2013; Moelants *et al.*, 2014).

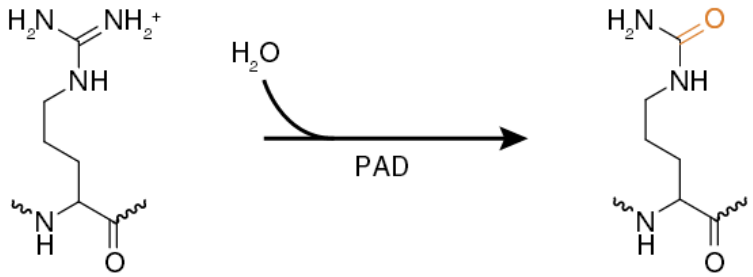


Figure 1-12. Conversion of arginine residues into citrulline residues by PAD enzymes through a hydrolysis reaction.
(Mowen and David, 2014)

Overall, this project aims at overcoming early inflammation during transplantation by identifying biomarkers associated with early-stage damage, hasten repair, and by enhancing post-ischaemic organ preservation. It focuses on improving our understanding of chemokine function, including the role of post-translational modifications (PTMs) during inflammation, by investigating potential anti-inflammatory mechanisms, such as GAG binding chemokine mutants, heparin-like drugs, small molecule chemokine receptor antagonists or chemokine modifications, to improve tissue function and avoid chronic progressive organ disease.

1.10 Hypothesis

Leukocyte recruitment mediated by CXCL8 function is critical in early stages of inflammation, such as during ischaemia reperfusion injury (IRI) following transplantation. Better understanding of the chemokine regulation with focus on GAG binding and on post-translational modifications could improve current patient treatment and organ preservation.

1.11 Aims

- Synthesise, purify and biophysically characterise CXCL8 C-terminal peptides
- Evaluate the biological function of CXCL8 peptides in endothelial GAG-binding and the potential role in modulation of CXCL8 function
- Investigate the role of post-translational nitration in the regulation of chemokine function

2. General Materials and Methods

Laboratory Safety Procedures and Risk Assessment

Good Laboratory practice was used during the conduct of all experiments in a manner that was compliant with Newcastle University safety policy, which included reading and signing of all relevant regulation on Control of Substances Hazardous to Health (COSHH) and Biological Control forms (BIOCOSHH). Tissue culture was carried out in compliance with the regulations for containment of class II pathogens.

Unless otherwise stated, all reagents used were from Sigma.

2.1 Cell culture

Cells were grown in a humidified incubator at 37°C with 5% CO_2 and cell culture was carried out in a class II containment cabinet. Cells were grown in 25 and 75 cm^2 tissue culture flasks or 6 well plates (Greiner Bio-One). Suspension cells were washed with phosphate buffered saline (PBS; Sigma), and adherent cells were washed with PBS and detached by incubation with trypsin-EDTA (Sigma). If cells were to be used for assays requiring immediate cell surface protein expression, cells were detached from the flask using EDTA-PBS (Sigma). Then, centrifuged (5min, 500xg) using MSE Mistral 2000 centrifuge, resuspended in tissue culture medium, counted using a haemocytometer if necessary, and seeded into new flasks or plates.

2.1.1 Culture Media

All media were supplemented with 2mM L-glutamine (Sigma), 10% foetal bovine serum (FBS; Biosera) and 100U/mL of penicillin and 100μg/mL streptomycin.

RPMI 1640 media

RPMI 1640 medium (Sigma, 5886) was used to support the growth of suspension cell lines such as THP-I monocyte cell line and HL-60 neutrophil-like cell line, also for primary neutrophils. For migration experiments, medium was supplemented with 1% BSA instead of FBS as specified.

MCDB-131 media

MCDB-131 is the media that has been optimised to support the growth of Human Microvascular Endothelial Cells (HMEC-1). This medium (Sigma, M8537-1L) was purchased in a powder form containing L-glutamine. The content of each vial was dissolved in 900 ml of sterile water containing 15.7ml of 7.5% sodium bicarbonate (Sigma, S8761). Complete medium was prepared by adding 10ng/ml Epidermal Growth Factor (EGF) (Peprotech), 1µg/ml of hydrocortisone (Sigma), 10% FBS, 100U/mL penicillin and 100µg/mL streptomycin. To sterilise the media, it was passed through sterile 0.2µm filters using a vacuum pump. Also, MCDB-131 (Thermo Fisher, 10372019) was used.

HUVEC media

Human Umbilical Vein Endothelial Cells (HUVECs) were grown in Basal Endothelial Cell medium (C-22210, Promocell) supplemented with Endothelial Cell Growth Medium Supplement Pack (C-39210, Promocell), with contained 7.5% sodium bicarbonate, 10ng/mL epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (Long R3 IGF), basic fibroblast growth factor (bFGF), 10% FCS and 1µg/mL hydrocortisone.

DMEM hybridoma media

C1 and C4 hybridoma cell lines were cultured in 75cm^3 flasks in DMEM-F12 medium (Sigma), supplemented with 5% stripped FBS and 1ng/mL IL-6.

2.1.2 Cell Storage

Cells were washed and detached as indicated previously, and resuspended in 1ml freezing media (10% dimethylsulphoxide (DMSO; Sigma) in FBS), or 500µL of 20% DMSO freezing media and 500µL of the cell media 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. For long-term storage, cells were transferred to liquid nitrogen. 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.

2.1.3 Cell counting and viability

To determine cell number and concentration, 10µl cell suspension was counted using a Neubauer improved 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 of viability calculated.

2.1.4 Mycoplasma testing

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

2.1.5 Cell line

HL-60 neutrophil cell line

HL-60 is a human cell line of promyelocyte origin, established from a 36-year old woman with acute promyelocytic leukaemia (Collins, 1987). HL-60 cells grow in suspension, with doubling times from 20-45 hours. Morphologically, they consist of large, blast-like cells with large rounded nuclei containing 2-4 distinct nucleoli, and a basophilic cytoplasm with azurophilic granules. HL-60 cells can be induced to differentiate either to granulocyte-like or to monocyte/macrophage-like cells depending on the nature of the inducing agent. Polar-planar compounds such as dimethyl sulfoxide (DMSO) and dibutyryl cyclic AMP, and other compounds such as retinoic acid and actinomycin D induce differentiation to granulocytes. This can be observed as a progressive decrease in cell size and nuclear/cytoplasmic ratio, as well as appearance of kidney-shaped nuclei and later lobed nuclei, characteristic of banded and segmented neutrophils. On the other hand, compounds such as 1,25-dihydroxy-vitamin D_3, phorbol esters like phorbol-12-myristate-13-acetate (PMA), and sodium butyrate induce differentiation to monocytes or macrophages. Within 24hs of compound addition, the cells start to clump and adhere to the substrate, then spread out and acquire pseudopodia, whereas the nuclei remain rounded (Birnie, 1988). In this study, HL60 cell line was grown in RPMI

1640 complete media. DMSO was used as differentiation inducing reagent to HL-60 neutrophil-like cell line, incubating cells in 1.25% DMSO-complete RPMI for 6 days before analysis.

THP-I monocyte cell line

This is a human monocytic cell line established from the peripheral blood of a 1-year-old male with acute monocytic leukaemia (Tsuchiya *et al.,* 1980). THP-1 cells express Fc and C3b receptors and lack the expression of surface and cytoplasmic immunoglobulin. They were grown in 75 cm^3 flasks vertically in complete RPMI 1640.

HMEC-1 cell line

This cell line is a model for microvascular endothelial cells generated from the transfection of human dermal microvascular endothelial cells (HMEC) with a PBR-322-based plasmid containing the coding region for the Simian Virus 40 A gene product, large T antigen (Ades *et al.*, 1992). These cells were cultured in horizontal 75cm^3 flasks using complete MCDB-131 media in a humidified 5% CO_2 atmosphere at 37°C. SV40 transformed endothelial cells retained the characteristic cobblestone morphology of the confluent primary endothelial cells. The modulation of the expression of adhesion molecules by inflammatory cytokines makes HMEC-1 cells a good model to study endothelial-leukocyte interaction occurring in micro-blood vessels. Confluent monolayer cells were routinely sub-cultured every 3-4 days. Adherent cells were washed twice with sterile phosphate buffered saline (PBS), detached using trypsin-EDTA and split in the ratio 1:3 or 1:4 depending on cell density.

HUVECs

This cell type is a model of primary human umbilical vein endothelial cells (HUVECs) and was grown in complete HUVEC media. Cells were cryopreserved (C-12203, Promocell), or proliferating (C-12253, Promocell), from pooled donors.

Hybridoma cell lines for production of nitrotyrosine-CXCL8 antibody

These hybridoma cell lines were obtained from mice treated with CXCL8-derived peptides at Abmart Inc., Shanghai, with the aim to produce a specific nitro-tyrosine CXCL8 antibody. The cells were grown in complete DMEM-F12 media as previously indicated.

2.1.6 Primary neutrophils from healthy volunteers

Neutrophil isolation protocol 1 (Institute of Cellular Medicine, Newcastle University)

Polymorphonuclear human neutrophils (PMN), also called Primary 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 20minutes, 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 (Figure 2-1). Neutrophil isolation protocol was performed by Mr Jonathan Scott from Prof John Simpson's laboratory (Institute of Cellular Medicine).

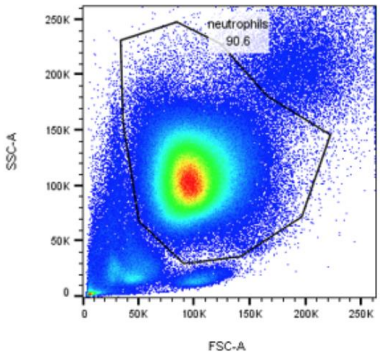


Figure 2-1. Isolated neutrophils were analysed for their purity by flow cytometry.
An example of primary blood neutrophil preparation is shown.

Neutrophil isolation protocol 2 (Cellix Ltd. Dublin, Ireland)

Polymorphonuclear human neutrophils (PMN) were isolated from whole blood, taken from healthy volunteers. 6mL of whole blood minimum were collected in a sodium citrate tube (blue) or heparin tube (green). 3mL of Ficoll hystopaque 1119 density were pipetted in a falcon tube, followed by 3mL of 1077 Ficoll hystopaque, and 6mL of whole blood. Then, tube was centrifuged at room temperature (23°C) for 30 minutes, 700xg. At the end of centrifugation, only the second layer was collected (granulated cells) and layer was transferred to a new falcon tube. The other layers were discarded with a different Pasteur pipette. The granulocyte layer was obtained with a clean Pasteur pipette avoiding contamination of other cells. The granulocyte layer obtained was washed with 1mL PBS (pH 7.4), and centrifuged at 350xg for 10 minutes at 4°C. Then, supernatant was discarded, and tube inverted, observing pellet formation. 10mL lysis buffer (11814389001, Sigma) were added, and cells were kept in ice for 10-15 minutes for lysis of Red Blood Cells (RBCs). After 10-15 minutes, cells were centrifuged again at 350xg for 10 minutes at 4°C, in 6 of acceleration and break. After the lysis, cells were washed again with PBS, for 10 minutes at 350xg at 4°C. Finally, PBS volume was discarded by inverting the tube and cells were resuspended in 500µL RPMI media. Cells were counted before being used in assays. Neutrophil isolation was performed by Toby Paul at Cellix Ltd., Dublin, Ireland.

Main cells applied in this study are shown in Figure 2-2.

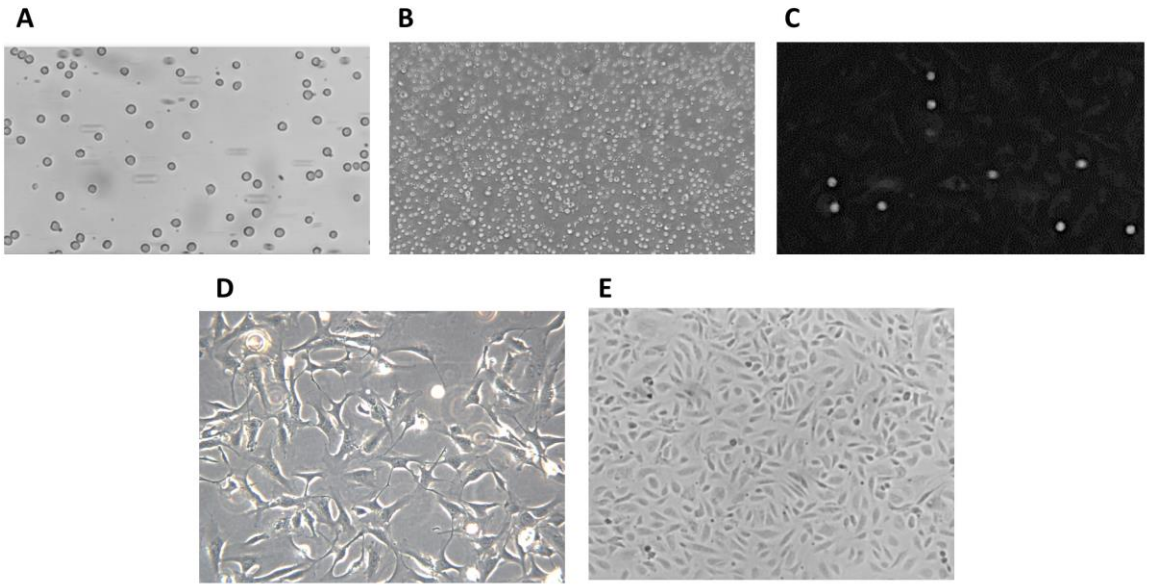


Figure 2-2. Cell types used in this study.
A) THP-I monocyte cell line during perfusion. B) HL-60 neutrophil-like cell line. C) Primary neutrophils (white cells) during perfusion over HUVEC layer (elongated cells). D) HMEC-1 endothelial cell line. E) HUVEC primary endothelial cells. Images were taken with bright field microscope (A, B, D, E) or fluorescence microscope (C). 20x magnification.

2.2 FACS

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 to 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.

In this study flow cytometry was 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.

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 assessed.

Antigen	Clone	Isotype	Label	Manufacturer
hCXCR1	42705	mIgG2a	FITC	R&D
hCXCR2	48311	mIgG2a	FITC	R&D
hCD11b	ICRF44	mIgG1	PE	R&D
hCD66b	G10F5	mIgM, κ	FITC	Biolegend
hCD45	2D1	mIgG1	PerCP	R&D
ICAM-1	HA58	mIgG1, κ	PE	Biolegend

Table 2. List of antibodies used in flow cytometry.

Instrument and analysis

All flow experiments were performed using BD FACSCanto II flow cytometers, and FACS-Fortessa flow cytometer for calcium flux studies, and data recorded using BD FACSDiva software. Data were analysed using FlowJo software v10 (Tree Star).

2.3 Molecular biology

All reagents used for this procedure were of a high degree of purity and designed for molecular biology use. These reagents are also RNase free which provide protection during the RNA isolation process. All reagents used for RNA isolation in this project were molecular biology grade available commercially. All areas used for RNA isolation where decontaminated by using RNase removal spray (Sigma). All pipettes were decontaminated by exposure to UV light for 30 minutes prior to use. Filter sterile tips and autoclaved sterile eppendorfs were used during the whole process.

2.3.1 qRT-PCR

Real-time quantitative Reverse Transcription Polymerase Chain Reaction

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.

2.3.1.1 RNA isolation

RNA was isolated from cells using RNeasy Plus Mini Kit (Qiagen). Briefly, biological samples were first lysed and homogenized in a highly denaturing guanidine-isothiocyanate–containing RLT buffer plus ß-mercaptoethanol, which immediately inactivates RNases to ensure isolation of intact RNA. For neutrophils, a minimum of 20-30 million cells were required. The lysate was then passed through two gDNA Eliminator spin column, or more depending on the number of cells, using full speed centrifugation. This column, in combination with the optimised high-salt buffer, allows efficient removal of genomic DNA.

Ethanol was added to the flow-through to provide appropriate binding conditions for RNA, and the sample was then applied to an RNeasy spin column where total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA was then eluted in 30 µl, or more, of water.

RNA concentration and purity was determined using a Nanodrop spectrophotometer. The machine measures the absorbance at wavelengths 230, 260 and 280 nm and use these values as a guide to calculate nucleic acid purity and concentration. 1µl of extracted RNA was tested with Nanodrop after blanking with 1µl of RNase-free water. The concentration was obtained in ng/µL. RNA absorbs light at 260 nm and the optical density relates proportionally to the amount of RNA in samples. Contaminant proteins and organic chemicals in isolated RNA samples absorb light at 280 and 230 nm, respectively. Therefore, the purity of isolated RNA sample was evaluated by the 260/280 and 260/230 ratios. Reading around 2 for each ratio is considered as a pure sample free from protein and phenol contamination and suitable for cDNA synthesis. Figure 2-3 shows an example of measurement of RNA purity and concentration.

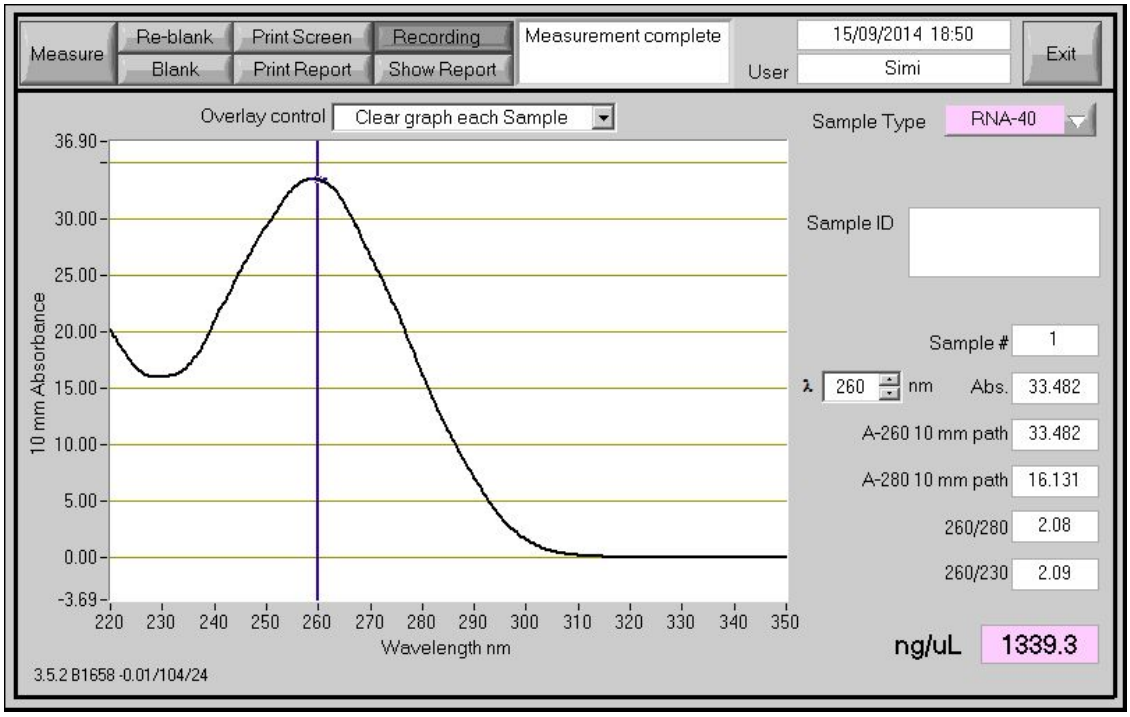


Figure 2-3. Examination of RNA purity and concentration using Nanodrop.

2.3.1.2 First strand complimentary DNA synthesis

Complimentary DNA (cDNA) was made from the RNA suspension using Tetro cDNA Synthesis Kit (Bioline) and a thermocycler (T100™ Thermal Cycler, Bio-Rad) according to the manufacturer's instructions (Bioline - 30min at 45°C, 5min at 85°C and 10 min at 4°C). 2µg RNA was used per reaction. Following synthesis, short-term storage of cDNA samples was at 4°C until PCR amplification.

Components	Tetro cDNA synthesis
RNA	2µg
5x RT buffer	4 µL
10mM dNTP mix	1 µL
50 µM Primer oligo-(dT)	1 µL
RNase inhibitor	1 µL
Tetro RT (enzyme)	1 µL
RNase free water	Variable
TOTAL VOLUME	20 µL

2.3.1.3 Taqman real-time PCR

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 initial template, the more product will be created. Conventional PCR is semi-quantitative as levels of product are visualised by gel electrophoresis and compared; real-time PCR is quantitative, qRT-PCR. Both techniques require comparison to a housekeeping gene, such as GAPDH or HPRT, which serve as endogenous control.

PCR is performed in a thermocycler which repeatedly heats samples to a set range of temperatures for the reaction to happen. First, an initialisation step is needed to activate the polymerase, and then a series of steps are repeated, usually for forty cycles. Cycles follow: denaturing step – which creates disruption of hydrogen bonds, generating single stranded DNA, annealing step – for annealing of the primers to the single stranded template,

elongation step – synthesis of a new strand of DNA at the optimal temperature for polymerase activity. There is exponential amplification in which the amount of product doubles, followed by levelling off and plateauing in which no more product is formed by consumption 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 2-4. If the probe is intact, when the reporter is excited by light it does not emit fluorescence as energy is transferred to the quenching molecule (fluorescence resonance energy transfer, FRET). 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, proportionally to the accumulation of PCR products. A key parameter for quantification is the threshold cycle (C_t), the cycle number at which the fluorescence level passes a fixed limit or threshold, set within the lower third of the exponential phase of increasing fluorescence (see Figure 2-5). The lower the C_t, the higher the initial amount of template.

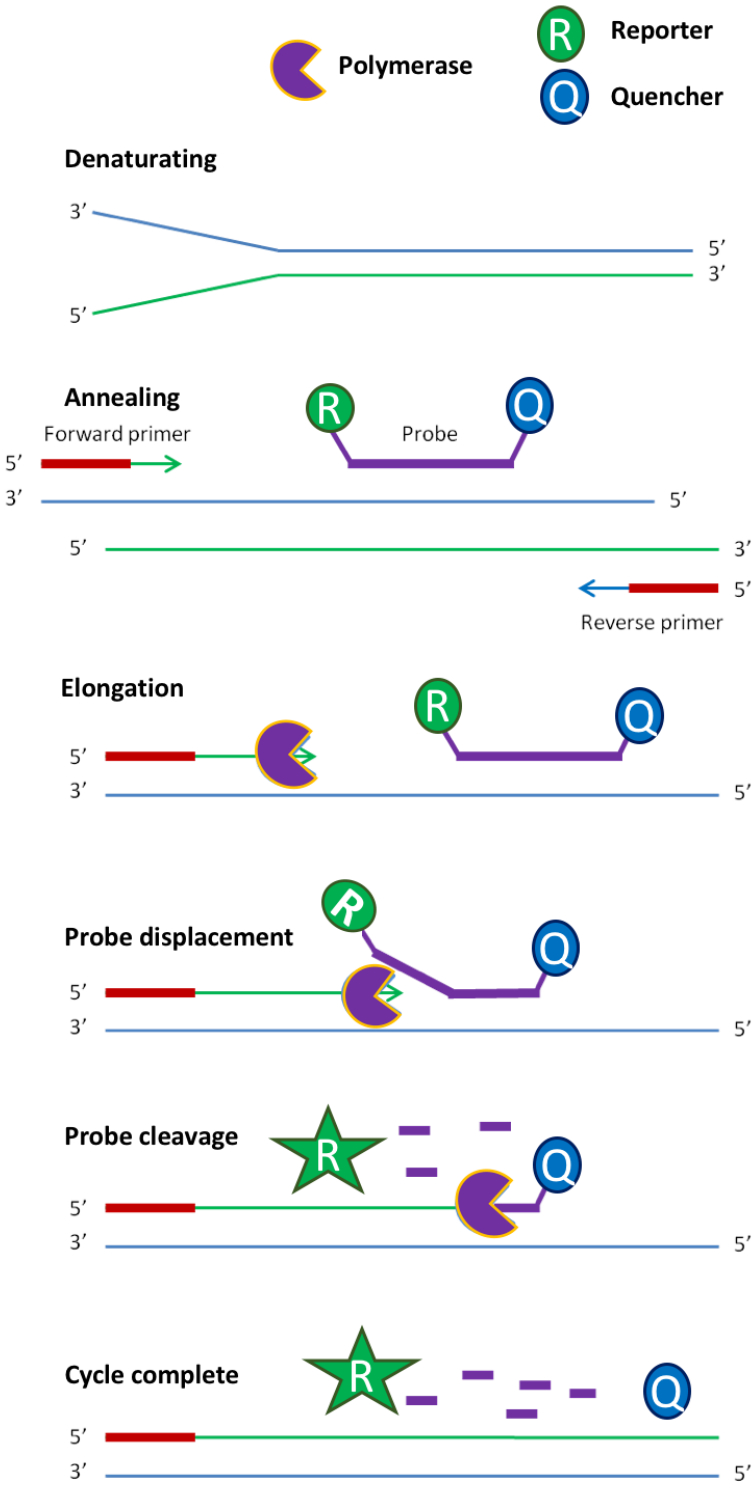


Figure 2-4. Taqman qPCR.

Mains steps of qPCR - initial denaturation of the cDNA, 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 quantity of PCR product.

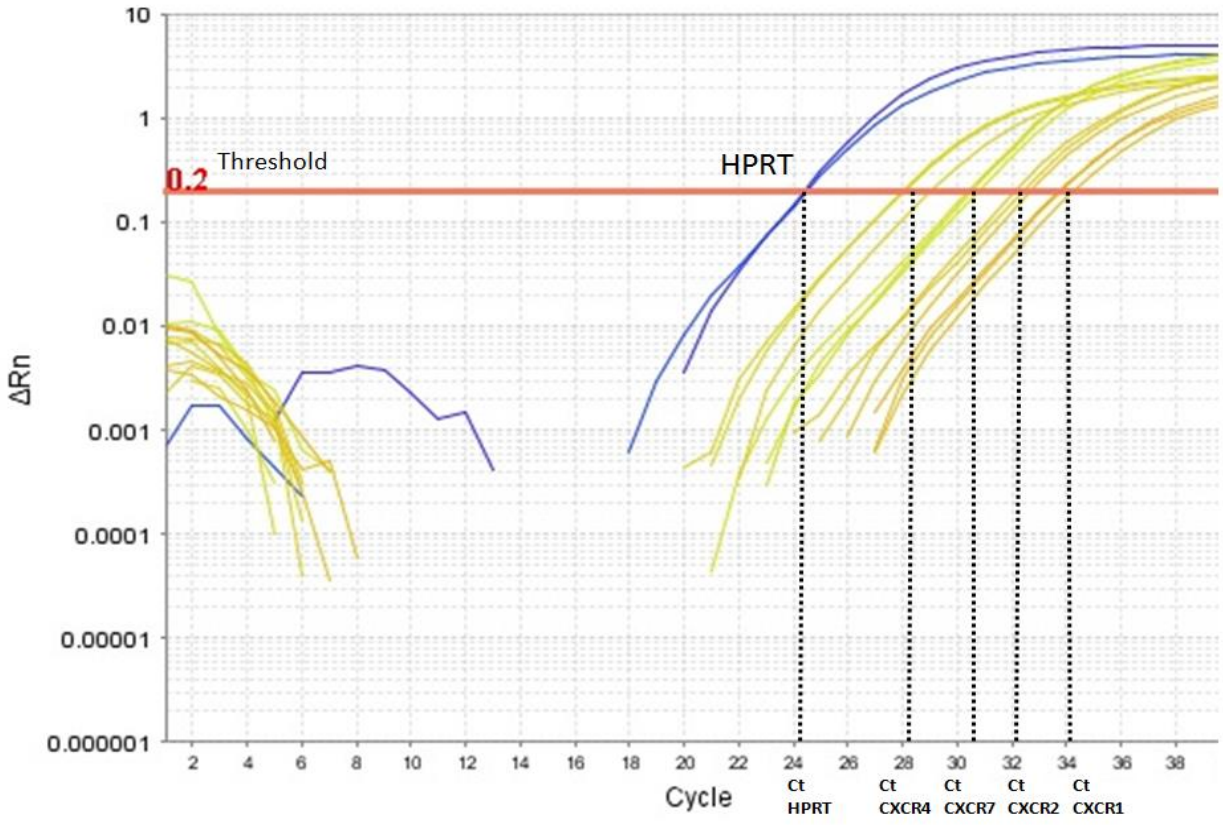


Figure 2-5. Real-time PCR amplification plot
The threshold is placed in the lower third of the linear phase of the amplification curve, establishing the threshold cycle (Ct) for each sample. This allows calculation of the fold increase relative to the housekeeping gene HPRT using dCt. The plot shows CXCR receptors expression for differentiated HL-60. The lower the Ct value, the higher the starting level of gene specific mRNA.

Method

Real-time PCR (qRT-PCR) was performed to quantify the mRNA expression of various chemokine receptors for cell characterisation. 20µl reactions were performed in MicroAmp Optical 96 well reaction plates (Applied Biosystems). Each reaction contained: 2µl cDNA, 7µl sterile water, 1µl TaqMan primer-probes (shown in Table 3**)** and 10µl 2x SensiFast Probe Hi-ROX Mix (Bioline). Reactions were carried out in triplicate in a StepOnePlus real-time PCR machine (Applied Biosystems). In order to activate the *Taq* polymerase, the reaction was first heated to 95°C for 5min. Then a step of 95°C for 10sec followed by 60°C for 20sec was repeated 40 times.

Components	Volume (µL)
2x SensiFast Probe Hi-ROX Mix	10 µL
cDNA	2 µL
10mM Primer	1 µL
DPEC water	7 µL
TOTAL VOLUME	20 µL

Target	Assay ID
CXCR1	Hs01921207_s1
CXCR2	Hs01011557_m1
CXCR4	Hs00607978_s1
CXCR7	Hs00604567_m1
HPRT	Hs02800695_m1
GAPDH	Hs02758991_g1

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

The housekeeping gene used was HPRT. ΔΔCt value, error and significance were calculated as described by Yuan *et al.* (Yuan *et al.*, 2006) and shown below (Figure 2-6).

ΔCt = Ct target gene – Ct housekeeping gene

ΔΔCt = ΔCt group of interest (e.g.: treated) – ΔCt control group

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

Figure 2-6. Equations for fold change calculation in real-time PCR.

2.4 Western Blot

2.4.1 General principle

Western blotting or protein immunoblotting is a widely used method to detect a specific

protein in a mixture, for example, a tissue homogenate or cell lysate. This analytical technique is based on the separation of proteins by gel electrophoresis followed by protein transfer onto a suitable membrane to be accessible and detectable by specific-antibodies. The separation can be performed on the basis of isoelectric points, molecular weight, electric charge or a combination of these factors. In this project, the separation was based on the molecular size by using a reducing agent and a detergent, Sodium Dodecyl Sulphate or SDS. β-mercaptoethanol is a reducing agent that cleaves the disulphide bonds between cysteine residues converting the tertiary structure of the proteins into linear polypeptide chains, while SDS maintains the denatured form of the proteins and coats the proteins with negative charge enabling the separation according to the molecular size. Separated proteins are transferred into a membrane where the specific protein is determined by indirect immunochemistry. Polyvinylidene fluoride or PVDF is the most hydrophobic membrane that is used to immobilize the separated proteins in the transfer process. This membrane is characterised by its capacity to bind various amino acids non-specifically and can tolerate probing with different antibodies. The non-protein bound areas of the membrane are blocked before probing with primary protein specific antibody. After incubation, a secondary antibody conjugated with an enzyme, fluorophore or isotope is added. The chemiluminescence method depends on the label on the secondary antibody. Horseradish peroxidase (HRP) is one of the most common and safe conjugates, and used in this project. The detection of this enzyme, which correlates with the abundance of the examined protein, is determined indirectly by the addition of a peroxide-luminol based reagent. The peroxidase enzyme catalyzes the oxidation of the luminol, resulting in the emission of the light. The emitted light can be captured by the exposure to X-ray film.

2.4.2 Preparation of cell lysate

For analysis of cell culture proteins, cells were stimulated as required and lysed immediately using lysis buffer consisting of cell lytic solution (Sigma) supplemented with protease inhibitor tablets (Roche) and kept in aliquots in (-20°C). The phosphatase inhibitor solution (Thermo scientific) was added in the experiments determining the presence of phosphorylated proteins. Around 40µl of lysis buffer was added per million cells with continuous mixing. Cell lysates were incubated for 10 minutes on ice with gentle hand shaking every 5 minutes. To ensure complete cell lysis and protein extraction, samples were

sonicated using an MSE Soniprep 150 sonication. The sonication was performed twice separated by cooling in ice for one minute to avoid heating causing protein denaturation. The samples were centrifuged at 15,000xg for 15 minutes to pellet cells debris and either used immediately or stored at -80°C.

2.4.3 Determination of protein concentration

The protein concentration of each sample was estimated colorimetrically using a BiCinchoninic Acid (BCA) protein assay kit (Pierce, USA) in accordance with the manufacturer's instructions. This assay is based on the biuret method which determines production of Cu^{1+} ions by a reduction of Cu^{2+} ion under an alkaline condition of sample proteins using the BCA reagent. The addition of BCA reagent forms a coloured, water-soluble complex with Cu^{1+} ions that shows a strong absorbance at 562 nm. The extent of the colour of sample is proportional to the amount of the protein presence. The concentration of the unknown protein was evaluated from a bovine serum albumin standard curve, run in parallel with unknown samples. A range of 10µL 125-2000µg/ml standard samples or unknowns were mixed with 200µl of a working solution in a 96 microwell plate. The working solution was prepared before use by mixing reagent A (BCA in alkaline buffer) with reagent B (4% cupric sulphate) in a ratio of 50:1. The plate was incubated at 37°C for 30 minutes before recording the absorbance at 490 nm. A linear regression analysis of the standard curve was calculated, and the unknown protein concentration was determined by interpolation. Figure 2-7 shows an example of standard curve used to determine the protein concentration of unknown samples.

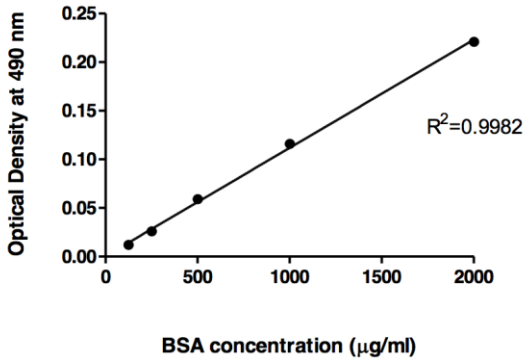


Figure 2-7. BCA standard curve for calculation of protein concentration.
Different known concentrations of BSA were mixed with the BCA reagent provided in BCA assay kit as explained in the methods. Absorbance was measured at 490 nm after 30 minutes. The linear regression was determined between the concentration and the absorbance. The standard curve was used to determine the protein concentration of unknown samples.

2.4.4 SDS-PAGE electrophoresis

SDS-PAGE is Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis. SDS-PAGE gels consist of a stacking gel on the top of a resolving gel. The amount of acrylamide in the resolving gel determines the percentage of that gel and its pore size. In this project, 10% gel was used to separate large proteins such as an antibody, and 12-15% was used for smaller proteins with 60-10 kDa molecular weight. The following constituents, all from Sigma, were mixed to prepare 5 ml resolving gel solution and 3 ml stacking gel solution sufficient to cast one gel and added to glass and alumina plates:

Resolving gel (12%)	**Stacking gel (5%)**
2ml of 30% acrylamide solution	0.5ml of 30% acrylamide solution
1.3ml of 1.5M Tris-HCl, pH 8.8	380µl of 1 M Tris-HCl, pH 6.8
1.6ml deionised water	2.1ml deionised water
50µl of 10% (w/v) SDS	30µl 10% (w/v) SDS
50µl of 10% (w/v) ammonium persulphate	50µl 10% (w/v) ammonium persulphate
2µl of N,N,N,N – tetramethylethylenediamine	3µl of N,N,N,N – tetramethylethylenediamine

The resolving gel was left to polymerize for around 30 minutes after the addition of a butanol layer on the top of the gel to prevent air disturbance. After polymerization, the butanol was washed by deionized water and the stacking gel was added on the top. The stacking gel was left to polymerize after insertion of the combs. To prepare cell lysates, samples were mixed with laemmli loading buffer (12% sodium dodecyl sulphate, 60% (v/v) of 20% glycerol, 30% (v/v) β-mercaptoethanol, 0.0012% bromophenol blue, 0.375 M Tris-base pH 6.8) and boiled for 5 minutes before loading on the gel. The gels were immersed in running buffer consists of 3g/L Tris base, 14.4g/L Glycine and 1g/L SDS at pH 6.8. In parallel, prestained protein marker (Fementas, SM0671) or (Thermo Fisher, 26616) was run on the same gel each run as a guide for the molecular weight of the studied proteins. Gels were run at room temperature at 30 mA for each gel using mini vertical electrophoresis unit (SE260, GE Life Sciences).

2.4.5 Wet protein transferring

After protein separation, the proteins were transferred to PVDF membrane. This membrane was equilibrated in absolute methanol for ten seconds followed by washing with distilled water twice for 5 minutes each. After washing, the membranes were soaked in transfer buffer consisting of 3g Tris base, 14.4g glycine and 100ml methanol made up to 1L in distilled water 10 minutes before performing the transfer sandwich. The gels and transfer electroblotting cassette were soaked in transfer buffer before being assembled. Protein transfer was performed either overnight at 30 V or for 1 hour at 220 V using a transfer tank (TE22, GE Life Sciences).

2.4.6 Immunoblotting

After transfer, the PVDF membrane was washed once with PBS containing 0.1 % tween 20 (TPBS) for 5 minutes to remove the transfer buffer before blocking. To block the non-specific binding, the membrane was blocked with 3 % BSA for phosphorylated proteins or 5% milk for other proteins in 0.1 % TPBS at room temperature for 1 hour before probing with primary antibody. Primary antibody was diluted in the blocking buffer at a concentration recommended by a manufacture and added to the membrane. This was incubated overnight at

4°C with continuous shaking on a rocker. After incubation with primary antibodies, the membrane was washed three times with 0.1 % TPBS for 5 minutes each. HRP-conjugated secondary antibody was added for 1 hour at room temperature with continuous shaking. After three washes with TPBS, peroxidase activity was detected by using Pierce enhanced chemiluminiscent (ECL) substrate (Thermo-Scientific) for 5 minutes. The detection of bound antibodies was dependent on the oxidation of the luminol in the substrate by conjugated peroxidase to the secondary antibody and consequent emission of light. The resulting bands were visualized by exposure of the membrane to Kodak film (Sigma) using ready to use developer and fixer (Tentenal, Germany). The exposure time was varied according to the expression of sample. As a loading control, membrane was stripped at room temperature for 30 minutes using stripping buffer consisting of 1.5 % glycine, 0.1 % SDS and 1 % Tween 20 (v/v) followed by washing with TPBS and reprobed with loading control specific antibody by the same procedure.

2.5 Chemotaxis

Chemotaxis allows evaluation of the ability of chemokines to induce cell movement in response to a chemotactic stimulus or chemokine concentration gradient. In this study, *in vitro* leukocyte migration was assessed by two ways: chemotaxis induced by diffusion gradient (also called transwell chemotaxis), and by transendothelial chemotaxis (TEC). Diffusion gradient chemotaxis defines the level of chemokine-mediated leukocyte migration mainly dependent on chemokine-leukocyte receptor interactions. Transendothelial chemotaxis, a more physiological approach, evaluates the level of chemokine-mediated leukocyte migration in presence of endothelial surface glycosaminoglycans.

2.5.1 Diffusion gradient chemotaxis

Diffusion gradient chemotaxis was carried out using a transwell system (see Figure 2-8). Prior to chemotaxis, 24 well companion plates (BD Falcon) were blocked with 1ml 1%BSA/RPMI per well for 1hour 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, at different concentrations,

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 used as a negative control. The plate was then incubated at 37°C for at least 90 minutes. Afterwards, migrated were counted by haemocytometer or by flow cytometry.

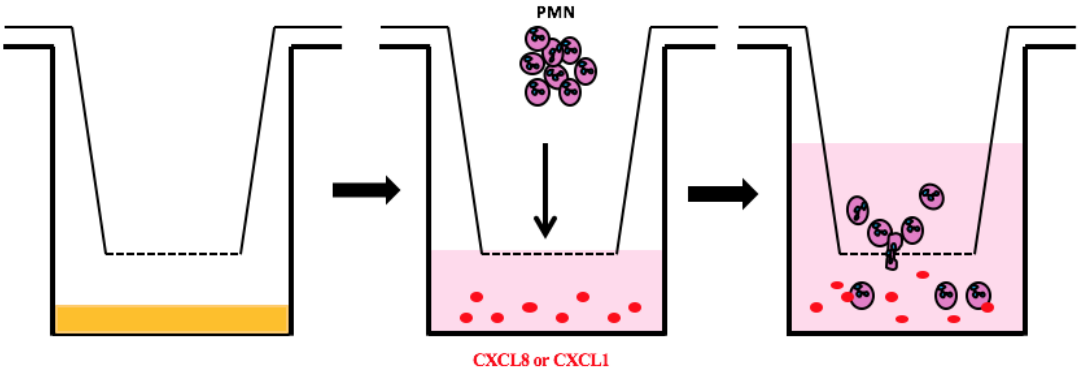


Figure 2-8. Schematic representation of a diffusion gradient neutrophil migration assay. 300,000 neutrophils (PMN) were placed on upper chamber onto a 3µm-pore size insert, once chemokine (or chemokine combined with chemokine peptide) was added to the bottom chamber (red dots). After incubation at 37°C for 90min, cells that have fully migrated to the lower well are counted by haemocytometer or flow cytometry as a ratio to known number of counting beads. Migrated neutrophils adhere to the underside of the insert, at low level, hence can be counted using a microscope.

2.5.2 Transendothelial chemotaxis

To create more physiological conditions *in vitro*, transendothelial chemotaxis assays were performed. This was as above, except in addition HMEC cells were cultured in HMEC media in cell culture inserts three days before the assay (Figure 2-9). Media with HMEC cells 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.

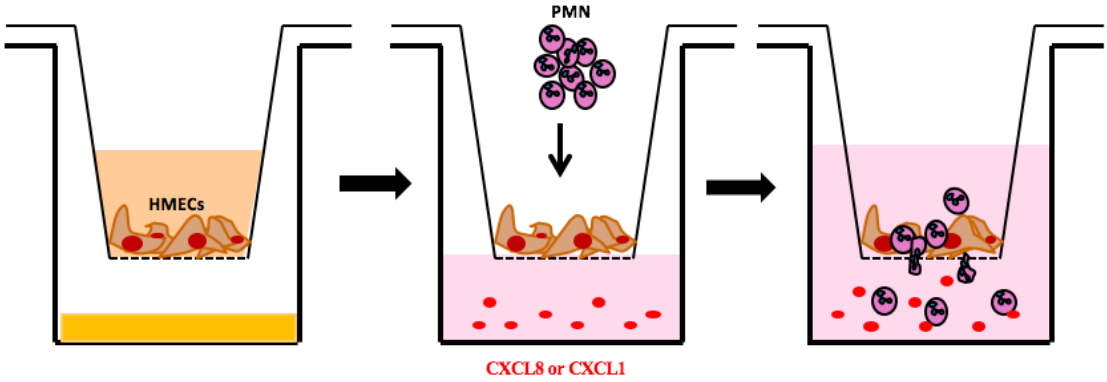


Figure 2-9. Schematic representation of a transendothelial neutrophil migration assay.
In order to obtain confluent endothelial layer, about 100,000-200,000 endothelial cells are seeded on the insert three days before the assay. Chemokine (or chemokine combined with chemokine peptide) was then added to the bottom chamber (red dots). Then, 300,000 neutrophils (PMN) were placed on the upper chamber. After incubation at 37°C for 90 min, cells that have fully migrated to the lower well are counted by haemocytometer or flow cytometry as a ratio to known number of counting beads. Migrated neutrophils adhere to the underside of the insert only at low level, thus migrated cells can be counted using a microscope.

Counting migrated cells

Flow cytometry

Cells that fully migrated through the filter into the lower chamber below, were counted by flow cytometry. 200µL of cell suspension was transferred into a FACS tube. 8µL of counting beads (CountBright Absolute Counting beads, Life Technologies), containing a known number of beads, were mixed, and this was then added to the tube, vortexed, and used for flow cytometry to determine the number of migrated cells as a ratio to beads (Figure 2-10). The bead excitation wavelength is UV to 635nm, and emission at (385-800) nm.

(number of cells/number of beads) x (total number of beads x volume of beads/total volume)

=

Migrated cells per volume

Figure 2-10. Calculation of migrated cells using flow cytometry beads.
8µL of fluorescently labelled beads (approx. 7,920 beads) were mixed with 200µL cells of interest for cell counting. As beads mix with cells, the number of beads and number of cells is determined by flow cytometry, so the final number of migrated cells per volume is calculated.

2.6 Synthesis of chemokine peptides and biophysical characterisation

Studies were carried out at Durham University Chemistry Department under joint supervision of Dr Steven Cobb and Dr Ehmke Pohl (see section 3).

2.7 Dot Blot (DB)

Initial validation of clones from ascites, obtained for production of monoclonal antibody, was performed by Dot Blot. Briefly, 2µL sample (chemokine peptide, chemokine or nitrated chemokine) was printed onto PVDF membrane. Concentrations of 2.5ng/mL or 0.5ng/mL of chemokine peptide (Abmart Inc.), 25ng/mL of chemokine (CN-09, Almac) and 50ng/mL of nitrated chemokine were used. Samples were allowed to air dry on membrane for one hour. Then membrane was blocked for one hour with 5% nonfat milk/TBS-T at room temperature. Next, the membrane was washed once with TBS-T. It could then be frozen and stored for future use, or directly incubated with primary antibody (1:1000) (Abmart Inc.) in 5% nonfat milk/TBS-T, for 1 hour at room temperature. The membrane was washed with TBS-T for 5 minutes three times, then incubated with HRP-linked secondary antibody in 5% nonfat milk/TBS-T for 1 hour at room temperature. The membrane was washed again with TBS-T for 5 minutes three times and developed by ECL.

2.8 Leukocyte flow-based adhesion assay (industrial secondment at Cellix Ltd)

2.8.1 General principle-Cellix Venaflux platform

Cellix Venaflux platform is a microfluidic system used to examine cell-cell interactions and the adhesion of leukocytes to recombinant proteins (ICAM-1, VCAM-1, fibronectin) or monolayers of endothelial cells under physiological *in vitro* flow conditions. The system can be used to analyse cell morphology and track different steps of leukocyte migration including rolling and adhesion in a manner that is more physiological than the static chemotaxis assays. Different types of fluids can be used with the Cellix platform such as media and blood. Therefore, the system can be used to examine platelet adhesion, aggregation and thrombi formation by using whole blood samples. The efficiency of a particular drug can be also examined by this system in order to manage drug toxicity and development.

The system consists of bright field and fluorescence microscopy with a motorised stage, microscope cage incubator, fluorescence and temperature controllers and, importantly, an adjustable syringe pump that is all controlled by Venaflux software, as shown in Figure 2-11. The syringe pump accurately maintains the flow rates of a desired fluid ranging from 5 picoliter/minute to 10 microliter/minute producing a wide range of shear stresses, reaching

450 dyne/cm^2, depending on the syringe type. These characteristics facilitate the study of cell-cell interactions in an equivalent manner to that which occurs in the blood vessels *in vivo*. The Vena8 biochips are designed to mimic the blood vessels. Each chip consists of 8 channels which can be coated with different recombinant proteins for adhesion assays. Each channel is 28 mm length and (400-800) µm in width plus (100-120) µm in depth.

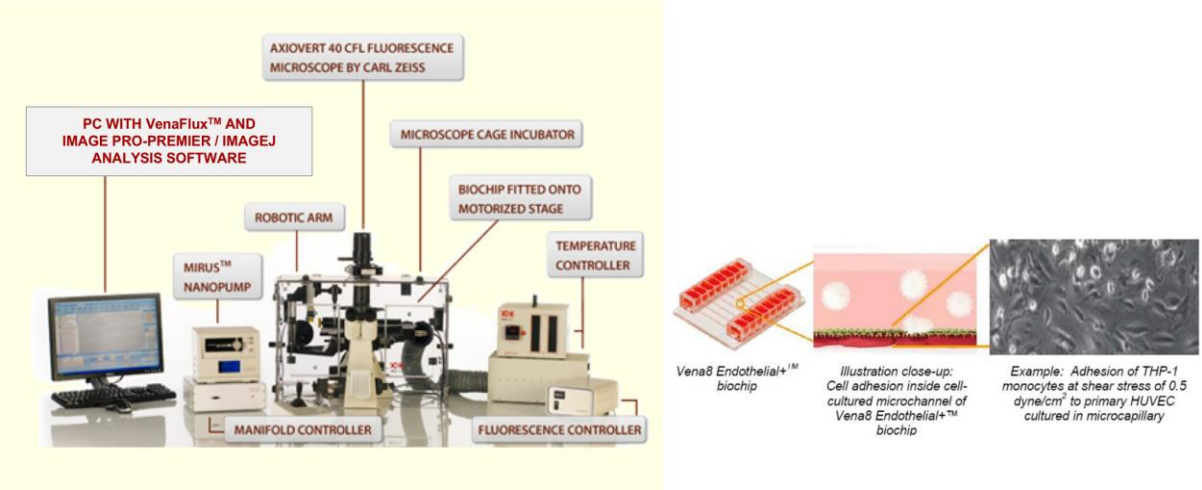


Figure 2-11. VenaFlux Platform (Cellix Ltd.).

2.8.2 Neutrophil flow-based studies

In order to evaluate the neutrophil adhesion in response to chemokine or chemokine peptides under physiological *in vitro* flow-based conditions, Venaflux flow-based platform was used, similarly to previous studies (Lamberti *et al.*, 2014; Shetty *et al.*, 2014; Zhou *et al.*, 2014).

For optimisation purpose, Vena8 Fluoro+ chip (400x100µm) was initially coated with 10µL of 100µg/mL VCAM-1 adhesion molecule. Second day, VCAM-1-coated Vena8 Fluoro+ biochip was used for analysis of THP-I cell line perfusion and adhesion, using 5 million THP-I/mL at 0.5 dynes/cm^2.

For the present study, in order to accommodate an endothelial layer for neutrophil perfusion, the Vena8 Endothelial+ chip (800x120µm) was used. It was initially coated with 10µL 100µg/mL fibronectin. The coated biochip was stored in a closed humidified chamber O/N at 4°C. In order to mimic microcirculation, capillary cells, or endothelial cells can be a good model. Otherwise, Human Umbilical Vascular Endothelial Cells (HUVECs) (Promocell, C-12203) suited the study. HUVECs were treated with (0.1, 1 or 10) ng/mL TNF-α (R&D, 210-TA-010), O/N at 37°C. On the second day, fibronectin-coated Vena8 Endo+ biochip was

seeded. 10µL of 1.5million HUVECs per 100µL was used as negative control. TNF-α-treated HUVECs was positive control. Then, about 10 minutes after HUVEC seeding, 40µL of extra culture media were carefully added on to the two reservoirs of channel, to humidify it for generation of an endothelial layer within 1-1.5 hours after seeding.

Afterwards, treatment of the biochip was performed. The biochip channel was treated with chemokine (10-2000ng/mL), chemokine peptide (50ng/mL) (WT, E70K or scrambled); heparin (100µg/mL); or Low Molecular Weight Heparin (LMWH) tinzaparin (50ng/mL). To assess GPCR binding, neutrophils were treated with CXCR1/2 antagonist (50ng/mL). 10µL were carefully inserted into each channel, followed by slow addition of 40µL into each channel reservoir, and incubated for 1 hour at 37°C. Next, neutrophil flow-based adhesion was evaluated using the Venaflux platform (Cellix Ltd). Primary neutrophils were isolated from blood from healthy volunteers, as previously described and used for the assay. Neutrophils were stained with 1µM $(DIOC_6)_3$ right before the assay, cell-permeant green-fluorescent lipophilic, mitochondrial-selective dye, to facilitate visualisation, and cells were maintained at 37°C throughout the assay. 300,000 neutrophils per mL were perfused in each channel and analysed. Cell adhesion analysis was done using Image-Pro Premier, or ImageJ Analysis Software. Cell adhesion count for each treatment was calculated from the average of five fields of view (FOV) of adhered cells.

2.9 Calcium flux assay

Intracellular calcium (Ca^{2+}) was measured by loading cells with Indo-1, AM, a ratiometric and sensitive indicator dye. Indo-1, AM is excited by the UV laser, and its emission depends on whether it is bound to calcium (420nm) or free (510nm). The ratio of these two wavelengths can indicate changes in intracellular calcium. For each tube, about 3million neutrophils were used. Neutrophils received were first left to rest in incubator for about 15minutes, and then used for the experiment. Cells were washed in HBSS and resuspended at 10 million cells per mL. Then, were washed in supplemented HBSS with 1mM $CaCl_2$, 1mM $MgCl_2$, 1%FBS (v/v). In order to confirm quality of isolation, cells are analysed by flow cytometry; and can also be analysed using cytospin at 500,000 cells/mL at 500xg for 5 minutes, then fixed in ice-cold acetone for 10minutes and air dried. Once cells are washed, they are loaded with 3µM indo-1, AM, and incubated for 30minutes at 37°C covered in foil.

After the 30minutes of indo-1, AM incubation, cells are washed with supplemented HBSS at 400xg for 5minutes, then resuspended at 3million per 1.5mL in their corresponding FACS tube and left to rest for 30minutes at 37°C before analysis. For optimisation purpose, 200,000 cells can be initially used as unstained control to set gate. Calcium flux was measured by FACS-Fortessa flow cytometry, using UV filter 530/30. Once settings were adjusted with unstained cells at low flow rate, the stained cells were run. As baseline, stained untreated cells (HBSS only) are first run for 1minutes at medium flow. Then 1µL HBSS or chemokine treatment was added for 4minutes, and then 8µL ionomycin were added for 2minutes. To secure equipment is well-cleaned after each test tube, FACS-Fortessa was cleaned at high flow rate using clean solution for 30secs, then HBSS with disengaged arm for 1min to wash out dead volume of syringe, and then with HBSS with engaged arm for 40secs to wash out ionomycin. Same process was repeated with all treatments. Cells were studied for the effect of CXCL8 on calcium flux, as opposed to the effect of CXCL8 combined with the synthesised CXCL8 WT peptide, CXCL8 E70K peptide, or scrambled peptide. The light emission was calculated as a ratio of fluorescence intensities at 340 and 380 nm (Figure 2-12).

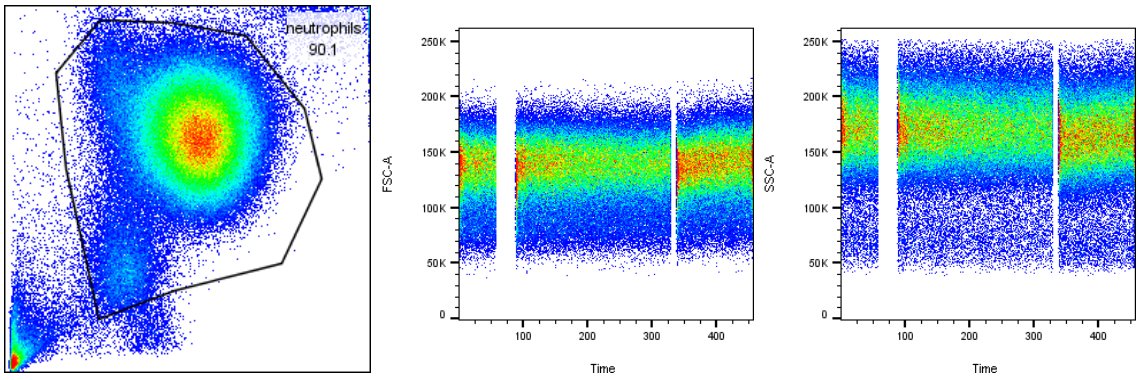


Figure 2-12. Flow cytometry analysis of neutrophil Ca^{2+} flux. SSC vs FSC (a), FSC vs time (b), SSC vs time (c).

2.10 Data analysis

Data for most experiments were graphed and analysed using Prism 7.0c (GraphPad Software Inc). For each graph, column denotes mean (M) and bar indicates standard error of mean (SEM). P values were calculated using two-tailed student t-test for comparison between two groups, or one-way ANOVA followed by Bonferroni post hoc test for comparison among

different groups, with significant differences denoted by asterisks as indicated: *=$p<0.05$, **= $p<0.01$, ***= $p<0.001$.

3. Synthesis and Biophysical Characterisation of truncated CXCL8 C-terminal Region

3.1 Introduction

The expression of chemokines, key mediators during inflammation, is highly complex (Mortier *et al.*, 2012). In order for chemokines to play vital biological functions such as directing leukocyte recruitment towards the injured tissue during the inflammatory response, tight chemokine regulation is crucial. A better understanding of chemokine regulation and the specificity of chemokine function, despite their apparent redundancy, is still required (Bennett *et al.*, 2011; Thompson *et al.*, 2017).

With a focus on the chemokine GPCR binding, several neutralising ligands, antibodies, modified chemokines and antagonists have been developed. However, only two chemokine receptors antagonists are validated and currently used as therapeutics, Maraviroc (a CCR5 antagonist) (Lieberman-Blum *et al.*, 2008; Horuk, 2009) and AMD3100 (a CXCR4 antagonist) (Cashen *et al.*, 2007; Roy *et al.*, 2014). Remarkably, these two antagonists are not used as anti-inflammatory drugs, but rather as an HIV inhibitor and as a stem cell mobiliser during transplantation, respectively. The challenge of using chemokines as anti-inflammatory therapy arises primarily from the known apparent redundancy within the chemokine system, and the differences between the human and mouse chemokine systems (Anders *et al.*, 2004; Vielhauer *et al.*, 2004).

In addition to the well-characterised, high affinity interaction of chemokines with G-protein coupled receptors (GPCR), a major role in chemokine activity has been established for the low affinity and less studied interaction with glycosaminoglycans (GAGs) (Kufareva *et al.*, 2015), which are thought to inhibit chemokine diffusion away from the site of damage. GAGs bind and immobilise chemokines at high concentration to the injured tissue enabling the formation of chemokine gradients towards the site of injury (Murphy, 1997; Bedke *et al.*, 2010). The low affinity of chemokines for GAG can induce chemokine cooperativity by competitive binding, essential for early stages of inflammation, which contributes to chemokine binding specificity. This allows the synergy between chemokines (for different receptor or for the same receptor in heterocomplexes). In addition, this competitive

chemokine binding, instead of enhancing cooperativity, can also induce the inhibition of activity by displacing other ligands (Verkaar *et al.*, 2014; Proudfoot and Uguccioni, 2016). For instance, CCL18 is known to be present in the circulation at considerably higher concentrations than other chemokines, and in this way is able to displace certain chemokines bound to heparin *in vitro*. This finding suggests that CCL18, which is upregulated in many pathological conditions, could prevent the leukocyte recruitment by displacing chemokines, removing them from the endothelial surface (Islam *et al.*, 2013; Krohn *et al.*, 2013).

Chemokines are initially translated with signal sequence, approximately 23 amino acids long, which is cleaved prior to secretion of the mature protein. However, biochemical analysis of biological samples containing chemokines has shown that they may be further processed by either N-terminal or C-terminal truncation. Often both the full-length form and one or more truncated forms are observed (Moelants *et al.*, 2013). Truncation occurs through the catalytic action of proteases, some of which have been identified (Mortier *et al.*, 2008), for example, matrix metalloproteinases can process monocyte-directed chemokines near their N- or C-terminal (Starr *et al.*, 2012).

Structure–function studies have emphasised the crucial role for the N-terminal regions of chemokines in receptor activation. N-terminal truncation can either increase or decrease the activity of chemokines at their receptors or can alter their selectivity across receptors. For example, the neutrophil chemoattractant CXCL8 exists in two forms (−2–77 and 1–77) as a result of alternative signal peptide cleavage (Mortier *et al.*, 2011). These two forms have different susceptibility to subsequent cleavage by aminopeptidases, giving rise to two additional forms (2–77 and 3–77), which have enhanced affinity for heparin. Moreover, further proteolytic processing catalyzed by coagulation proteases in the blood gives a shorter form (6–77) with increased chemotactic activity. It is of note, that CXCL8 (6–77) is also formed under the action of a bacterial protease in cultures of pathogenic *Porphyromonas gingivalis*, apparently a mechanism to elicit an initially enhanced host response against this pathogen, although chemokine then undergoes slow degradation (Moelants *et al.*, 2014).

Truncation of chemokines at their C-terminal is much less likely to influence receptor activation, as this region of chemokines is not directly involved in interaction with receptors. The C-terminal region is, however, involved in GAG binding and/or oligomerisation. For example, a splice variant of CXCL12 known as SDF-1α undergoes removal of a single C-

terminal residue in human serum. This truncation diminishes its ability to bind heparin or to cell surfaces, and therefore to stimulate cell proliferation and chemotaxis, although the truncation has no effect on receptor activation *in vitro* (Eckhard *et al.*, 2016). The chemokines CCL3 and CCL4, can be cleaved internally nearby their C-termini and then further degraded and inactivated. Cleavage of these chemokines by the protease cathepsin D is highly selective and has a regulatory role affecting the invasive capacity of breast cancer cells, and the generation of tumour-associated immune response (Wolf *et al.*, 2003).

CXCL8 levels have been shown to significantly increase during the inflammatory response associated with ischaemia-reperfusion injury (Sekido *et al.*, 1993; Bertini *et al.*, 2004), which is one of the earliest changes seen in graft damage, and it can lead to the development of acute kidney injury (Cugini *et al.*, 2005; Elmoselhi *et al.*, 2016). CXCL8 contributes to the neutrophil and monocyte arrest by activation of integrins (Gerszten *et al.*, 1999). Due to the interaction between innate and adaptive immunity CXCL8 antagonism might not only be involved in ameliorating IR injury but might also lead to reduced transplant rejection (Bertini *et al.*, 2004; Cugini *et al.*, 2005; Bedke *et al.*, 2010).

This chapter aims to improve the understanding of the role of CXCL8-GAG binding and how chemokine function can be modulated during inflammation using chemokine peptides.

3.2 Specific aims

- Synthesise CXCL8 C-terminal peptides as a tool to interfere with CXCL8 function
- Biophysically characterise the GAG binding ability of CXCL8 C-terminal peptides

3.3 Materials and methods

3.3.1 Synthesis of CXCL8 peptides: tools to study CXCL8 function regulation

It is known that chemokines can undergo cleavage, for instance due to matrix metalloproteinases, during tissue oxidative stress. Here we aimed to design and investigate the role of synthetically engineered chemokine peptides. According to previously identified GAG binding amino acid residues and GPCR binding residues in CXCL8 (Figure 3-1),

CXCL8 peptides were designed to investigate their potential to interact with GAG or with GPCR, with the aim to define whether they can modulate or compete with CXCL8 function.

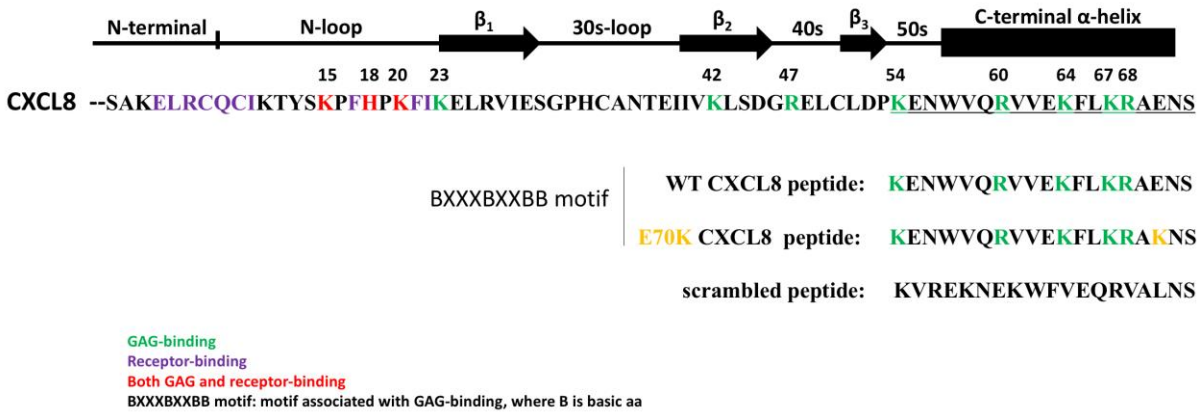


Figure 3-1. CXCL8 amino acid sequence and selected region for GAG binding studies.
CXCL8 most common active form, with 72 aa (P10145, PDB). Relevant amino acids are highlighted. GAG-binding residues (green). Receptor-binding residues (purple). Residues involved in both GAG and Receptor binding (red). Underlined amino acids: C-terminus region selected for synthesis. Below, note the WT peptide and the E70K mutant peptide assessed in this study, which present the BXXXBXXBB signature associated with GAG binding (Baldwin *et al.*, 1991), and scrambled peptide used as control.

Truncated CXCL8 C-terminal peptides were chemically synthesised and biophysically characterised for their structure and ability to interact with GAGs. Synthetic peptide chemistry studies were carried out during my research secondment at Durham University Chemistry Department, as part of our Marie Sklodowska-Curie Actions - Initial Training Networks (MSCA-ITN) project, an Innovative Development Platform (IDP), under joint supervision of Dr Steven Cobb and Dr Ehmke Pohl.

3.3.1.1 Peptide design

A specific CXCL8 region, 19-aa long, was selected for synthesis according to the following structural rationale; GAG-binding residues are mainly present in the C-terminus α-helix (19 aa), except two residues, H18 and K20 located in the β-sheet. A C-terminal peptide, reproducing the GAG-binding region was hypothesised to bind GAG thus competing with chemokine for GAG binding. The truncated version of C-terminal region of CXCL8 (54-72)

was synthesised by Solid-Phase Peptide Synthesis (SPPS). Then, it was further biophysically characterised for GAG binding. In parallel, we attempted to generate CXCL8 peptide corresponding to CXCL8 N-loop and first β-sheet (13-30) where most of the receptor-binding amino acids are located. However, low yield was obtained for the N-region pure peptide. Overall, six different truncated peptides were synthesised: CXCL8 N-region (13-30) peptide, WT CXCL8 (54-72) corresponding to the C-terminal region, three peptide variants of the C-terminal region (R68A, R68Citrulline and E70K), and a scrambled peptide of the C-terminal. Once synthesised, biological characterisation was performed for WT peptide, and E70K mutant peptide hypothesised to have increased binding to GAG compared to WT peptide due to the increased positive charge (Adage et al., 2012).

PEPTIDE	SEQUENCE
CXCL8 N-region	**Y(13)SKPFHPKFIKELRVIES(30)**
CXCL8 WT C-terminal	**K(54)ENWVQRVVEKFLKRAENS(72)**
CXCL8 E70K C-terminal	**K(54)ENWVQRVVEKFLKRA<u>K</u>NS(72)**
CXCL8 R68A C-terminal	**K(54)ENWVQRVVEKFLK<u>A</u>AENS(72)**
CXCL8 R68Cit C-terminal	**K(54)ENWVQRVVEKFLK<u>Cit</u>AENS(72)**
Scrambled peptide	**(54) KVREKNEKWFVEQRVALNS(72)**

Table 4. Sequence of the synthesised peptides. Cit: Citrulline

3.3.1.1.1 Detailed peptide synthesis and purification

All reagents used were from Sigma-Aldrich unless otherwise specified. Peptide synthesis grade (Dimethylformamide) DMF was acquired from AGTC Bioproducts (Hessle, UK) and amino acid derivatives were acquired from CEM, Novabiochem (Merck) or AGTC. Rink Amide resin was acquired from Novabiochem. Fmoc Solid Phase-Peptide Synthesis (SPPS) procedures are detailed in the following sections. Fritted polypropylene or PTFE reaction vessels were used for all manual reactions and resin swelling, with DMF as the reaction solvent.

For peptide couplings, N, N′-Diisopropylcarbodiimide (DIC) in DMSO and Hydroxybenzotriazole (HOBt) were used as activators unless otherwise indicated. Amino acid side chain functionality was protected as follows: Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(tBu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(tBu)-OH, Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Trp(Boc)-OH and Fmoc-Tyr(tBu)-OH. Fmoc deprotections were carried out using a 20% (v/v) solution of piperidine in DMF. Pre-swelling of the resin was carried out in DMF for a minimum of 1 h (overnight preferred), followed by washing with DMF. The peptide-resin was transferred from the SPPS equipment to a new column with bottom cap, through gentle washes with DMF. Then, capping with acetic anhydride of the peptide was performed.

3.3.1.1.2 Automated Fmoc solid-phase peptide synthesis (SPPS)

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 30 mL PTFE reaction vessel, with microwave heating and agitation by bubbling nitrogen. Rink Amide Resin was used, at 0.10mmol scale, dissolved in DMF in the Liberty1 column that was introduced into the system. Couplings were carried out using a 5-fold excess of Fmoc-protected amino acid; a 0.8 M solution of DIC in DMSO was used in the activator base position, and a 0.5 M solution of HOBt in DMF was used in the activator position. For double couplings, the reaction vessel was drained after each cycle and fresh reagents were added. DMF and 20% piperidine in DMF were used as deprotectants.

Couplings were carried out at room temperature for 60 min. The Fmoc group was removed by two successive treatments with piperidine solution (5 min then 10 min).

3.3.1.1.3 Capping

Peptide-resin was agitated twice with a 20% solution of acetic anhydride in DMF at room temperature (RT) for 30 minutes, doing a washing with DMF in between. The solvent was removed, and the resin washed with 5 portions of DMF and 2 portions of ether. Acetylation increases peptide stability. Also, it can be used to block peptide impurities, thus enhance

obtained peptide yield.

3.3.1.1.4 Cleavage of peptides from acid-labile resins

Peptide-resin was treated with 0.95 mL Trifluoroacetic Acid (TFA) and 0.05 mL deionized water, with 0.05 mL Triisopropylsilane (TIPS) as a scavenger for 3-4 h at room temperature (values were tripled when the mass of resin was greater than 100 mg). Longer cleavage times were used for peptides containing Pbf or benzyl protecting groups. The resin was then removed by filtration and the filtrate concentrated *in vacuo*, using the rotary evaporator at 50°C, before precipitation using ether and decanting of the liquid (followed by subsequent ether washes). The resulting solid peptide was dissolved in deionized water and lyophilized. For hydrophobic sequences, a small amount of acetonitrile was added (< 50% v/v) in the event of poor solubility (water and MeCN containing 0.1% TFA or triethylamine were also used to improve solubility for particularly insoluble peptides).

3.3.1.2 Analytical chemistry and biophysical characterisation

Matrix-assister laser desorption/ionization-Time of flight (MALDI-TOF)

Fundamentally, MALDI/TOF is an analytical technique for mass spectrometry (MS) that allows rapid identification of proteins or changes to proteins without the financial cost of sequencing, or the skills or time required to solve a crystal structure in X-ray crystallography. MALDI refers to ionization of a molecule using a matrix that absorbs laser energy creating ions from large molecules with minimal fragmentation. In MALDI-TOF, an ion's mass-to-charge ratio is determined by measurement of time required for the ion to reach a detector at a known distance, after being accelerated by an electric field of known strength.

In this study, MALDI-TOF mass spectra was collected using an Autoflex II ToF/ToF mass spectrometer (Bruker Daltonik GmBH) equipped with a 337nm nitrogen laser. Peptides were dissolved in 1:1 deionized water/MeCN for MS analysis (solvents containing 0.1% TFA or triethylamine were used for particularly insoluble analytes). Sample solution (1 mg/mL) was mixed with matrix solution (α-cyano-4-hydroxy-cinnamic acid, 50 mg/mL) in a ratio of 1:9, and 1 μL of the resulting solution spotted onto a metal target and placed into the MALDI ion

source. Reflectron mode was used for molecules with m/z > 4000. MS data was processed using FlexAnalysis 2.0 (Bruker Daltonik GmBH) thanks to Durham University mass spectrometry facility service manager Jackie Mosely.

3.3.1.2.1 High performance liquid chromatography (HPLC)

Analytical liquid chromatography mass spectrometry (Analytical LCMS) was carried out using an Acquity HPLC system (Waters Ltd, UK) equipped with a 200 LC pump, and Waters 486 UV tunable absorbance detector, set to 220nm. Samples were injected onto an SB Analytical C8 column (5 μm, 10 mm) and a gradient of 5–95% B (solvent A = H_2O, 0.1% formic acid; B = MeCN) was run over. The flow of solvent, from the fractions of interest, was lyophilized. Then, introduced into the Autoflex II ToF/ToF mass spectrometer.

Batches of the CXCL8 C-terminal peptides (WT, E70K and scrambled peptides) obtained following the same protocol in collaboration with ISCA Biochemicals at 95% purity, were used for biophysical and biological studies.

3.3.1.2.2 Biophysical characterisation of C-terminal peptides for GAG binding

3.3.1.2.2.1 Circular dichroism (CD) spectroscopy

Circular dichroism (CD) spectroscopy was conducted using a Jasco J-810 spectropolarimeter, with a 0.1 cm path length and 500 μL quartz cuvette. Samples were made diluted (5-100) μM in a phosphate buffered solution (PBS, 1 mol dm^{-3}). 300 μL of each sample solution was transferred to a cuvette for the measurements. All data was collected at room temperature as an accumulation of 5-10 measurements (N=5 to 10), adjusted by the background (baseline) spectrum of the PBS buffer, and when required also by spectrum of the heparin. Scans were conducted at 50 nm/min, between (240-197) nm wavelength, at 1 nm data pitch, 5 mdeg sensitivity and a 2s response.

Mean residue molar ellipticity [θ] was obtained using the equation:

$$[\theta] = \frac{100 \times \theta_{obs} \times M}{n \times l \times c}$$

where θ_{obs} is measured ellipticity (degrees), M is the molecular weight (g mol^{-1}), n is the number of residues, l is path length (cm), and c is the concentration of peptide (mg mL^{-1}). Samples were processed and data analysed thanks to guidance from Dr Beth Bromley (Physics, Durham University), and from Dr Helen Waller and Prof Jeremy Lakey (ICaMB, Newcastle University).

3.3.1.2.2.2 Surface plasmon resonance (SPR)

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. The interface must be in conditions of total internal reflection for SPR to occur. Alterations in binding can therefore be readout in resonance units (RU) (Figure 3-2). The running buffer used was HBS-P (10mM HEPES pH7.4, 150mM NaCl, 0.005% P20). Unless otherwise stated all reagents were from GE Healthcare.

Training and guidelines to use Biacore X100 and for data analysis was kindly provided by Dr Helen Waller (ICaMB, Newcastle University) and Tim Fagge (Biacore, Edinburgh).

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.

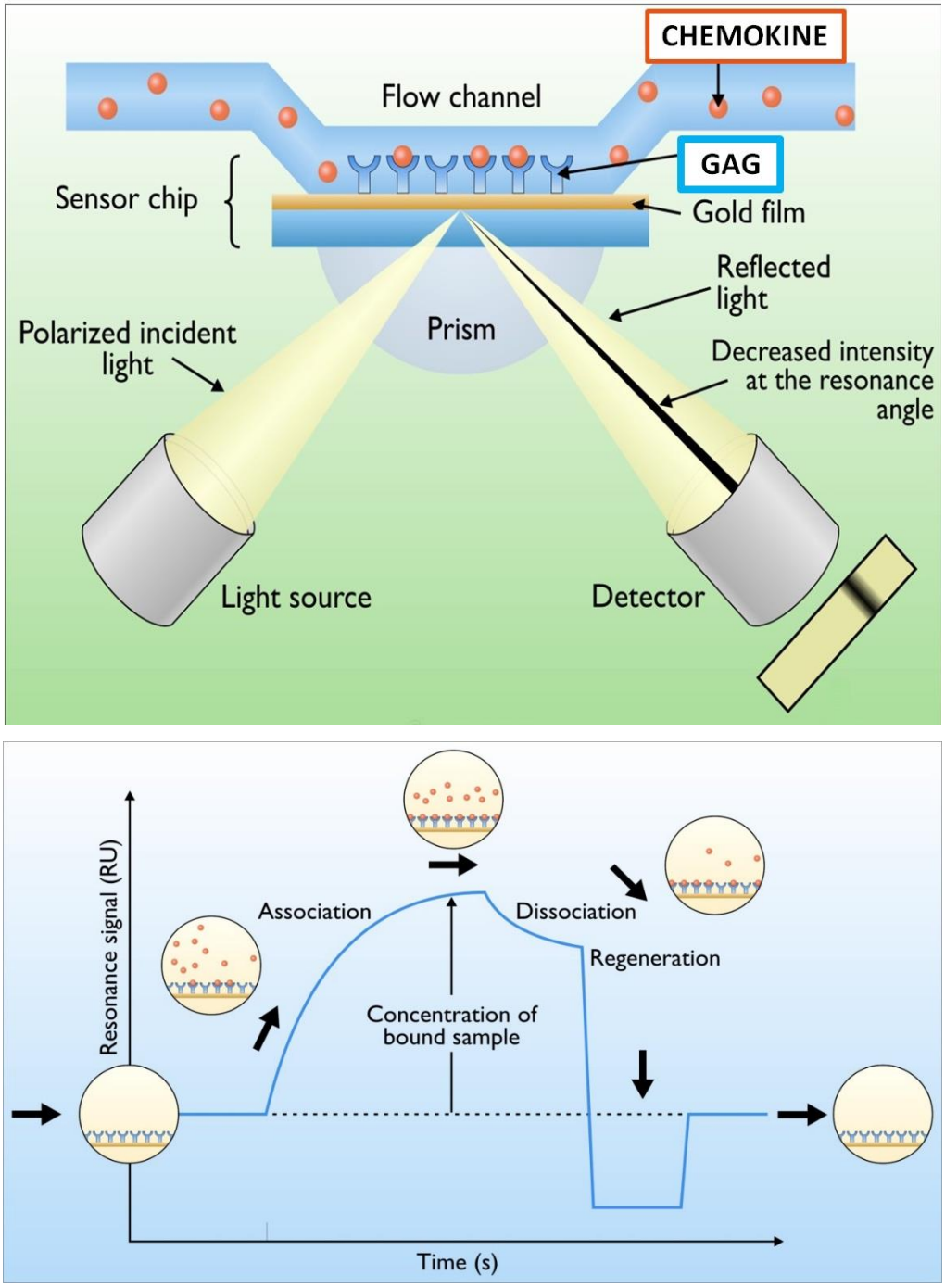


Figure 3-2. Surface plasmon resonance
Upper panel - schematic of surface plasmon resonance. GAG (e.g.: Heparin) is immobilised to the gold coated sensor surface using dextran and streptavidin. As the chemokine (analyte) binds to the GAG, the refractive index shifts and the SPR angle alters. The movement 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 (adapted from EU ELTE TTK, 2013).

In this study, CM4 chip and SA chip were used. CM4 chip has a lower level of carboxymethylation than 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 are optimal. SA chip is also suitable for our approach and allows simpler application, as it does not require previous manual streptavidin coating.
For CM4 chips, there are several methods for covalent attachment to the chip surface, here streptavidin amine coupling was used. This is stable, allowing the chip to be reused multiple times. For coupling the CM4 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.

For the streptavidin immobilisation, 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 isoelectric point (pI) 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 was immobilised, after two incubation rounds with a 20mM acetate buffer, pH 4.2, washing in between, 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. The RU value should increase a minimum of (2500-3000) RU units to consider a sufficient chip activation.

GAG immobilisation through NaIO4 oxidation and iodination

GAG biotinylation

Heparin was biotinylated to allow immobilisation on to the chip by streptavidin. Mono-biotinylation at the reducing end of the GAG is important for correct presentation when immobilised.
Biotinylation was performed as described previously (Sadir *et al.*, 2001; Saesen *et al.*, 2013). First, it is necessary to oxidise the GAG to generate free aldehydes (carbonyls), making the GAG accessible to react with the hydrazide group of the activated hydrazide-LC-biotin (Figure 3-3). Oxidation was performed using 10mM sodium periodate (NaIO4) in 0.1M

sodium acetate buffer pH=5.5. GAG was reacted for 30 minutes at RT, followed by overnight dialysis in 8KDa Molecular Weight Cut-Off (MWCO) dialysis tube, to remove NaIO4 from the solution, in order not to interfere in the subsequent biotinylation. Then, briefly, 1mM oxidised heparin (9kDa; Sigma) or heparan sulphate (12kDa; Sigma) in PBS was reacted with 10mM biotin-LC-hydrazide for 24hr at RT. This then underwent three rounds of dialysis buffer, 2 for 4hs and a third time overnight, against 1L water to remove any unreacted biotin, and kept at 4°C, as explained in (Thakar *et al.*, 2014).

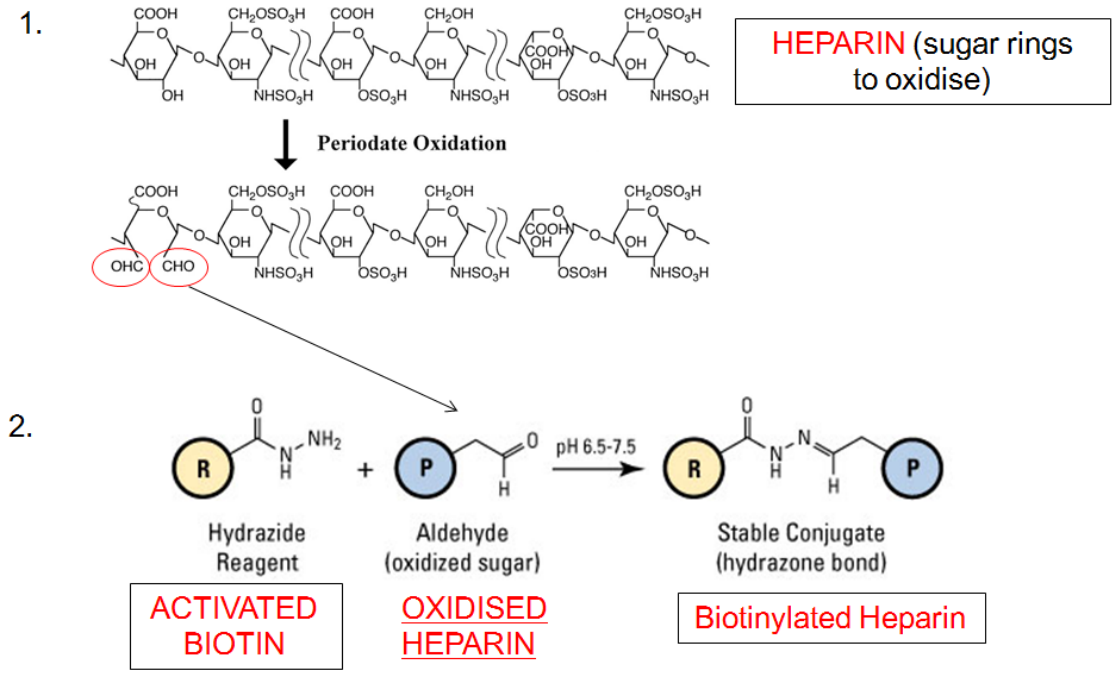


Figure 3-3. Biotinylation of Heparin requires two main steps.
1. Oxidation of Heparin by Sodium Periodate (NaIO4) generates free aldehydes (CHO) in Heparin (or another GAG, such as HS). This is key step for subsequent reaction 2, where the activated biotin (hydrazyde-LC-biotin) can react with the opened rings (free aldehydes) on Heparin.

Immobilisation

Biotinylated heparin was 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. Biotinylated heparin was automatically immobilised by setting Biacore wizard to about 200RU.

Chemokine SPR

Following preparation of the chip surface, SPR was performed to compare the GAG binding properties of CXCL8, CXCL8 C-terminal peptides, and nitrated CXCL8.

A range of chemokine concentrations (50-1000) nM, or chemokine peptide concentrations (2.5-10000) µM, were flowed across the chip at 5 or 30µl/min for 2minutes followed by a 300sec dissociation phase. After every chemokine measurement, chemokine was removed from chip surface using regeneration buffer (2M NaCl, HEPES, EDTA, 0.005% P-20) for 2minutes, and then chip was left on running buffer for complete regeneration for next study cycle. RU from a flow cell coated with streptavidin only was subtracted from the results of GAG coated flow cells and analysis was performed using BIAevaluation 4.1.

3.4 Results

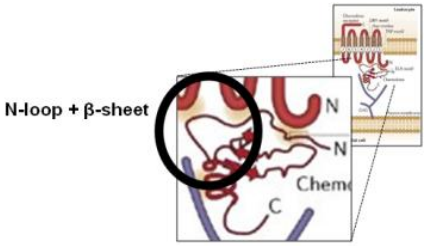
3.4.1 Synthesis and purification of CXCL8 C-terminal WT and mutant peptides

Firstly, the region corresponding to the CXCL8 N-terminus (N-loop and first β-strand) was selected for peptide synthesis, because it contains the residues H18 and K20 which are known to be involved in GAG-binding (Kuschert *et al.*, 1999), and also two residues involved in GPCR-binding (Baldwin *et al.*, 1991). Unfortunately, CXCL8 N-terminal peptide was not obtained with sufficient purity, which could be explained by the presence of two types of secondary structure within this region, hence a complex peptide coupling, or destabilisation. Hence, in order to attempt to increase stability of N-terminus region, the protocol was modified to couple one more residue to the previous sequence, Tyrosine 13, the only tyrosine in CXCL8 (Figure 3-1). Tyrosine 13 in CXCL8 is involved in receptor binding (Bertini *et al.*, 2004), and can also be modified by peroxynitrite through nitration (Barker *et al.*, 2014), so this peptide could be used as potential probe for binding studies. However, synthesis of this N-terminus region did not yield pure peptide, consistent with the hypothesis that this region would require further stabilisation for its synthesis and biological investigation (Figure 3-4). Therefore, our peptide synthesis studies focused on CXCL8 C-terminal, region which corresponds to the α-helix within CXCL8 (truncated WT C-terminal peptide region in Figure 3-5; E70K mutant peptide in Figure 3-6; R68A mutant peptide in Figure 3-7; R68Cit mutant peptide in Figure 3-8) and a scrambled peptide (Table 5).

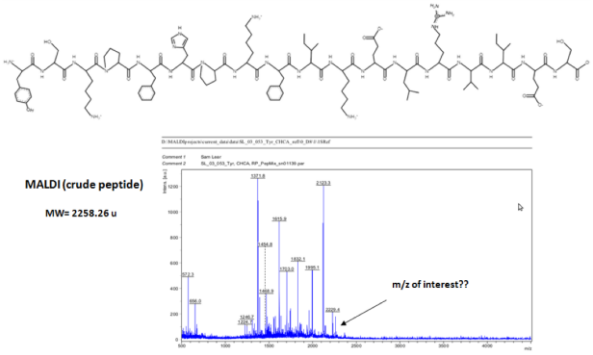
3.4.1.1 Chemical synthesis of CXCL8 N-loop + β-sheet

acetylated-YSKPFHPKFIKELRVIES-amide

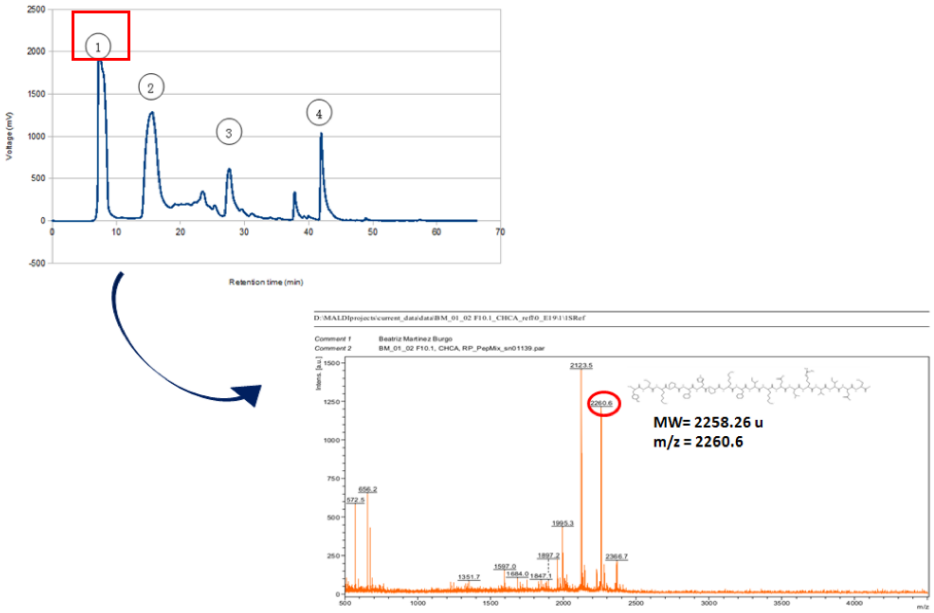
a) Truncated Chemokine Region



b) Peptide Sequence and MALDI of crude peptide



c) HPLC of crude peptide, followed by MALDI of positive peptide fraction



d) Analytical HPLC shows fraction is not pure

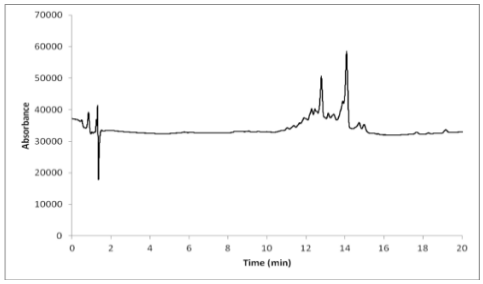


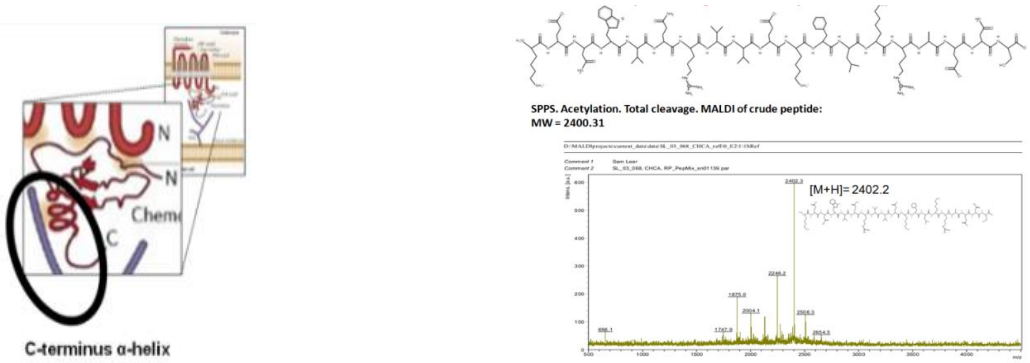
Figure 3-4. Synthesis trial of N-terminal region peptide (N-loop + β-sheet), acetylated-YSKPFHPKFIKELRVIES-amide

a) CXCL8 tertiary structure and selected peptide (within black circle). (Mantovani *et al.*, 2006). b) Peptide sequence and MALDI of crude peptide, c) RP-HPLC of crude peptide and MALDI of a positive fraction for analysis of its purity (red). d) Analytical HPLC shows fraction is not pure.

3.4.1.2 Chemical synthesis of CXCL8 C-terminal WT peptide

acetylated-KENWVQRVVEKFLKRAENS-amide

a) Truncated Chemokine Region **b) Peptide Sequence and MALDI of crude peptide**



c) HPLC of crude peptide, and MALDI and analytical HPLC of pure peptide fractions

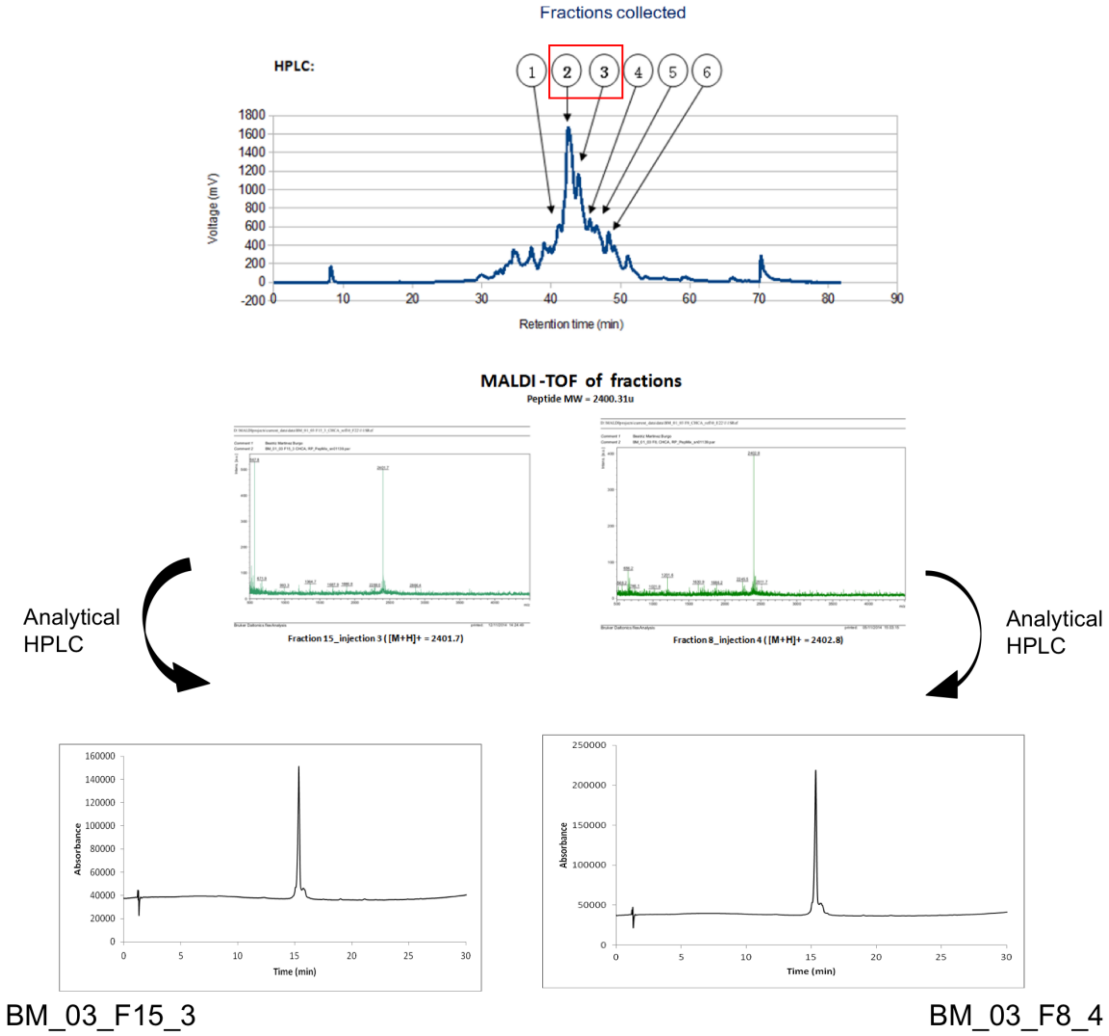
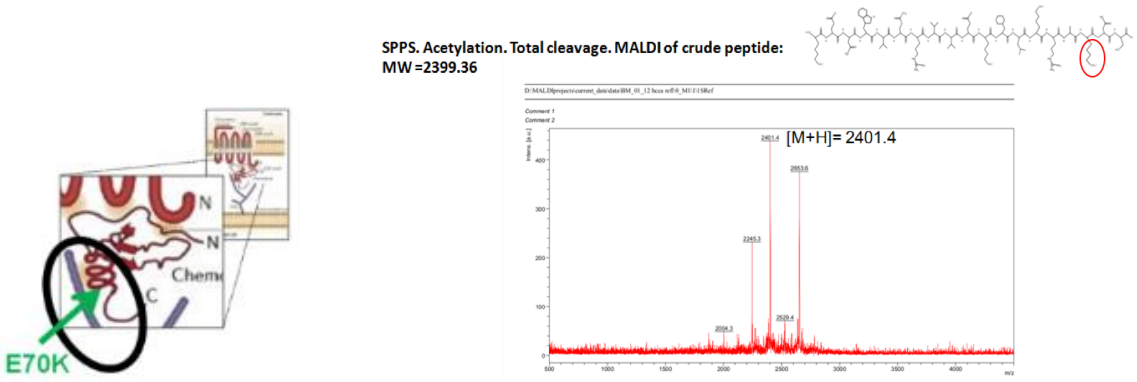


Figure 3-5. Synthesis of WT CXCL8 C-terminal peptide acetylated-KENWVQRVVEKFLKRAENS-amide.
CXCL8 tertiary structure and selected peptide (circled in black). Peptide charge is +2 (5 positive charges, 3 negative charges). b) Peptide sequence and MALDI of crude peptide. c) RP-HPLC. Below, MALDI of positive peptide fractions. Analytical HPLC shows 90% pure product.

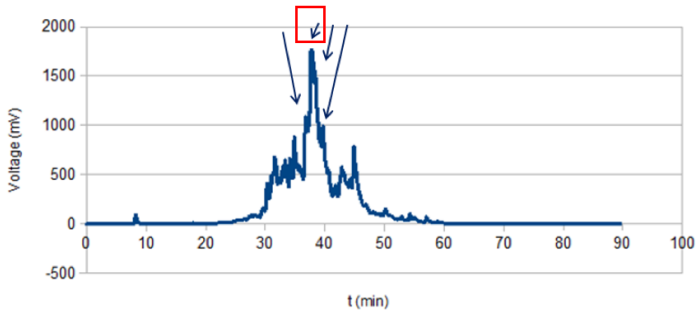
3.4.1.3 Chemical synthesis of CXCL8 C-terminal E70K peptide

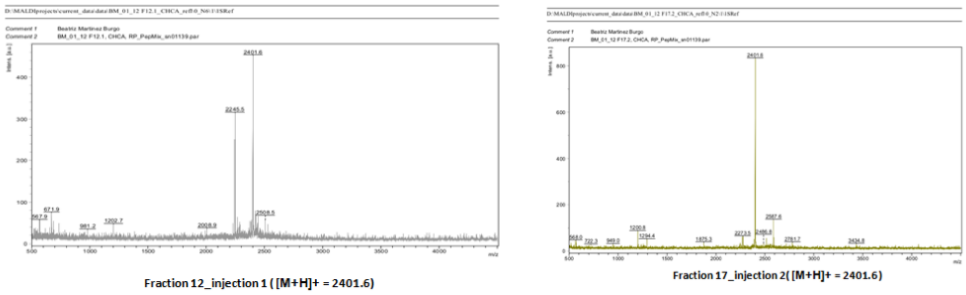
acetylated-KENWVQRVVEKFLKRAKNS-amide

a) Truncated Chemokine Region **b) Peptide Sequence and MALDI of crude peptide**



c) HPLC of crude peptide, and MALDI and analytical HPLC of pure peptide fractions





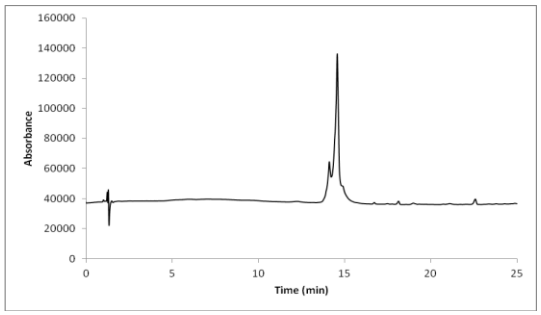
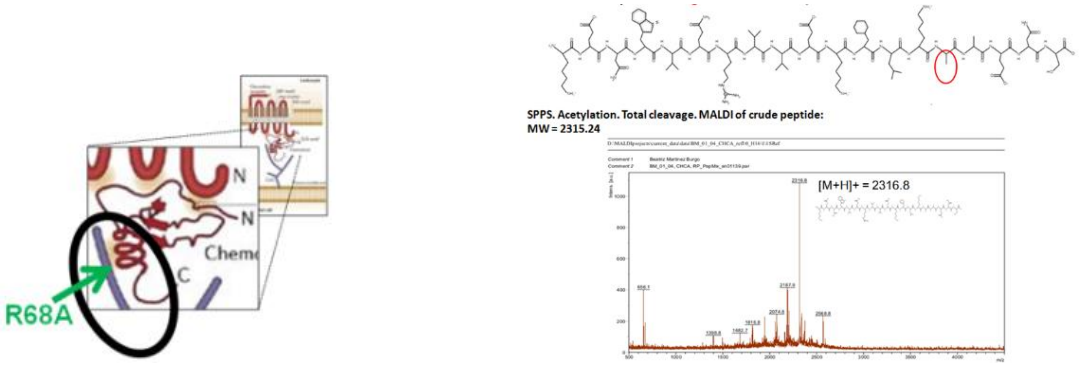


Figure 3-6. Synthesis of E70K CXCL8 C-terminal peptide acetylated-KENWVQRVVEKFLKRAKNS-amide.
a) CXCL8 3D structure and selected peptide (circled). Peptide charge is +4 (6 positively charged residues, 2 negatively charged residues). b) Peptide sequence and crude MALDI. c) RP-HPLC. Below, some positive peptide fractions. Analytical HPLC shows 85% pure product.

3.4.1.4 Chemical synthesis of CXCL8 C-terminal R68A peptide

acetylated-KENWVQRVVEKFLKAAENS-amide

a) Truncated Chemokine Region **b) Peptide Sequence and MALDI of crude peptide**



c) HPLC of crude peptide, followed by MALDI and analytical HPLC of pure fractions

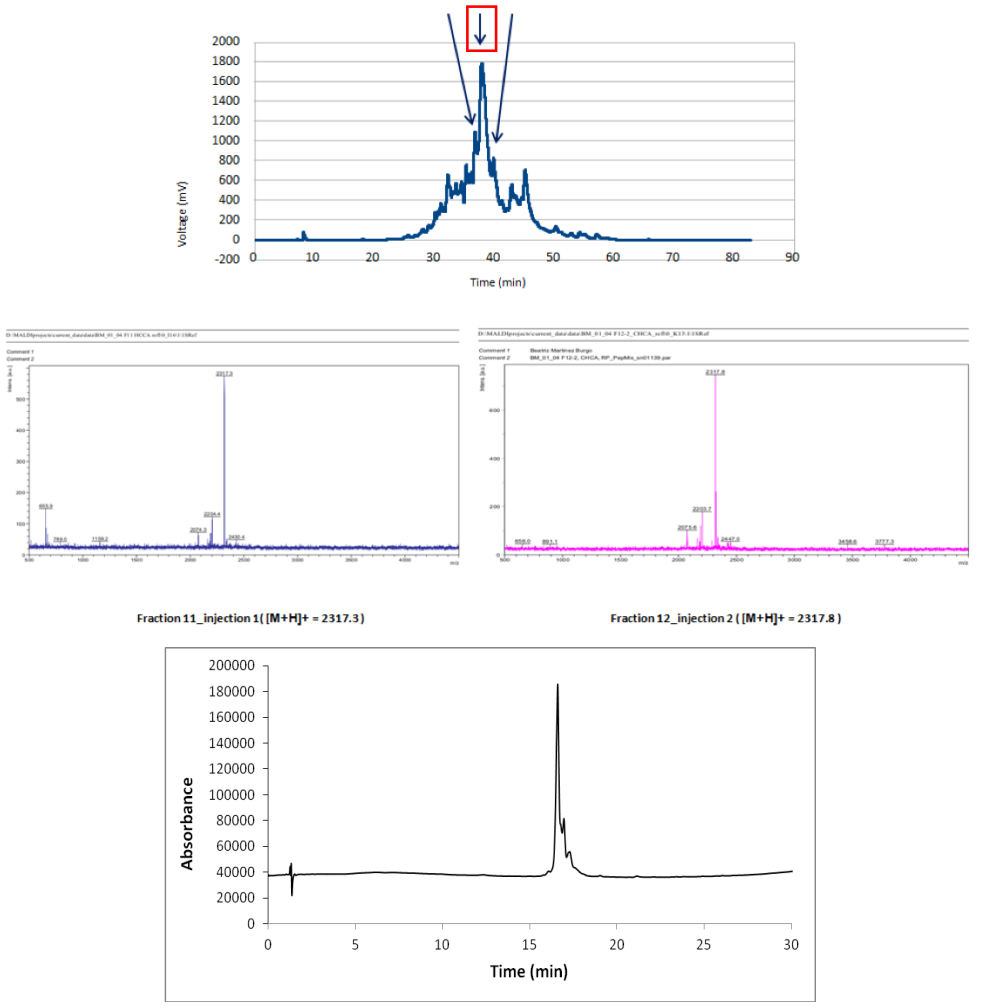
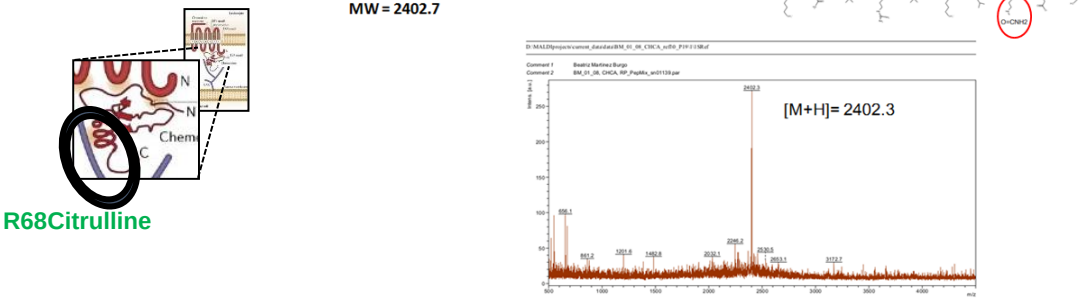


Figure 3-7. Synthesis of R68A CXCL8 C-terminal peptide acetylated-KENWVQRVVEKFLKAAENS-amide.
a) CXCL8 tertiary structure and selected peptide (circled). Peptide charge is +1 (4 positive charges, 3 negative charges). This peptide is hypothesised to have decreased GAG binding, as Arg68, core aa in GAG binding is substituted with Ala. b) Peptide sequence and MALDI of crude peptide. c) RP-HPLC. Below, some positive peptide fractions. Analytical HPLC shows 75% pure product.

3.4.1.5 Chemical synthesis of CXCL8 C-terminal R68Citrulline peptide

acetylated-KENWVQRVVEKFLKCitAENS-amide

a) Truncated Chemokine Region b) Peptide Sequence and MALDI of crude peptide



b) HPLC of crude peptide, and MALDI and analytical HPLC of pure fractions

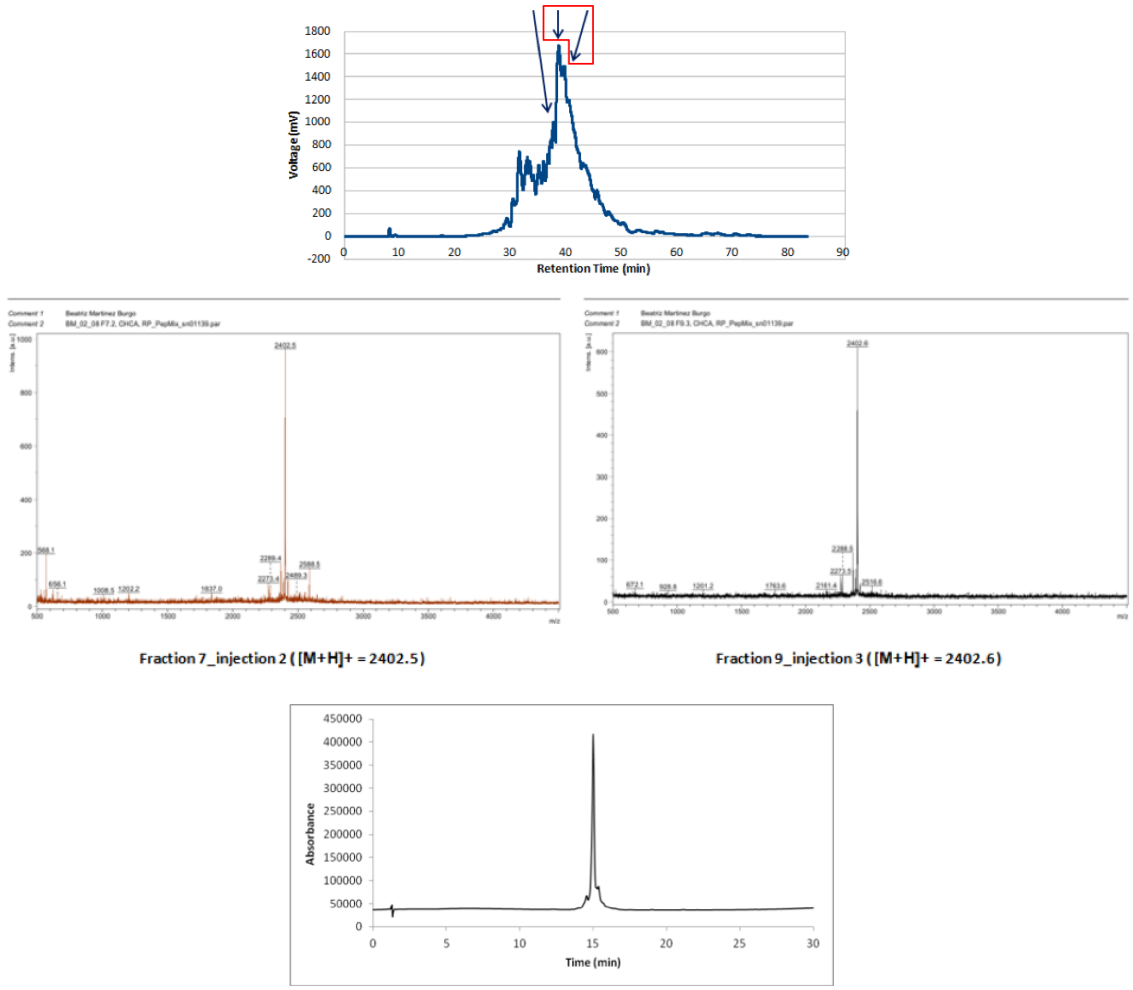


Figure 3-8. Synthesis of R68Citrulline CXCL8 C-terminal peptide acetylated-KENWVQRVVEKFLKRACitNS-amide.
a) CXCL8 tertiary structure and selected peptide (circled). Peptide charge is +1 (4 positive charge, 3 negative charges). Peptide is hypothesised to have decreased GAG binding, as core residue Arg68 is substituted with Citrulline. b) Peptide sequence and MALDI of crude peptide. c) RP-HPLC. Below, some positive peptide fractions. Analytical HPLC shows 85% pure product.

Final yields of peptide synthesis are summarised in Table 5.

PEPTIDE	CHEMOKINE REGION	MASS OF CRUDE PEPTIDE	MASS OF PURE PEPTIDE	YIELD [a]	PURITY [b]
WT	C-terminal	240mg	145mg	60.4%	approx. 90%
E70K	E70K C-terminal	239.9mg	25mg	10.4%	approx. 85%
R68A	R68A C-terminal	231.53mg	55mg	23.8%	approx. 75%
R68Cit	R68Cit C-terminal	240.27mg	56mg	23.3%	approx. 85%
Scrambled	Scrambled (C-t)	-	5mg	-	approx. 95%

Table 5. Summary of synthesised chemokine peptides, with corresponding yield and purity.

Mass of crude peptide refers to theoretical mass at 100% yield. a. Yield is based on 0.1mmol resin (0.1mmol peptide) = 100% peptide = x mg peptide. b. Purity is obtained from analytical HPLC.

According to previous reports on chemokine modulation, studies continued to further characterise three out of five synthesised peptides: the WT peptide, the E70K peptide, based on a mutation which has been previously associated with increased GAG binding within the full-length chemokine, and a scrambled peptide used as control. Biophysical peptide characterisation was used to analyse the peptide structure by CD, and their GAG-binding ability by Surface Plasmon Resonance.

Synthesised peptides R68A and R68Cit were not further biophysically assessed. Both were hypothesised to present lower affinity binding than WT peptide, as previously reported within the whole chemokine (Proost *et al.*, 2008) which suggests that peptide binding might be undetectable by SPR studies of GAG binding.

3.4.2 Biophysical characterisation of peptides for GAG binding

Initial biophysical characterisation of synthesised peptides was performed during my POSAT IDP secondment period at Durham University Chemistry and Biophysics Departments, under joint supervision of Dr Steven Cobb and Dr Ehmke Pohl. Further, studies continued at

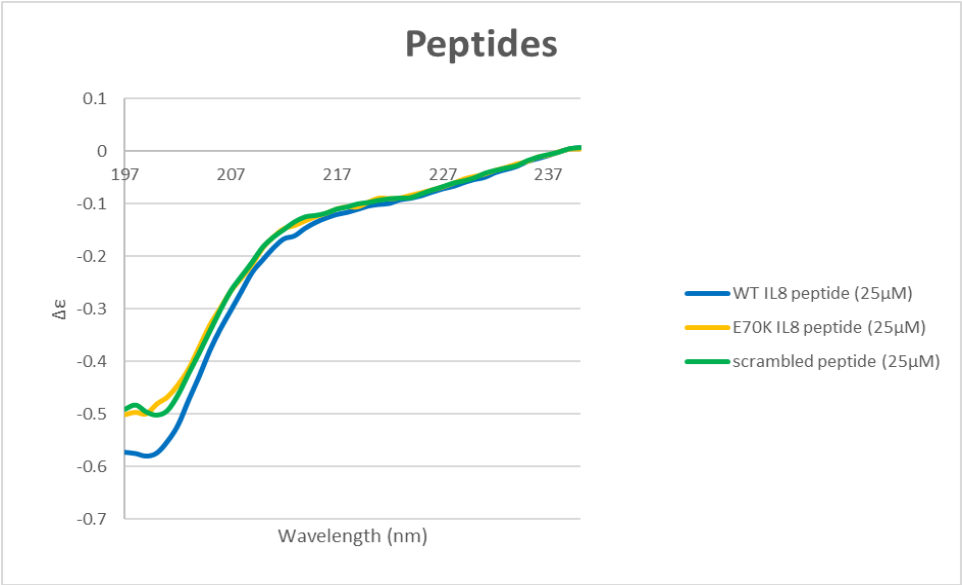
Institute of Cellular and Molecular Biology (ICaMB, Newcastle University) in collaboration with Prof Jeremy Lakey and Dr Helen Waller.

3.4.2.1 Circular Dichroism (CD)

3.4.2.1.1 CD for chemokine peptides in solution

The secondary structure of synthesised peptides was assessed by CD. Chemokines in native form present a folded structure. For instance, CXCL8 has a folded structure which consists on secondary structural motifs, primarily one alpha helix and one triple-stranded beta sheet (Figure 1-5) that fold in its 3D tertiary structure. Hydrogen (H) bonding in a protein contributes to the maintenance of the secondary structure, hence lack of H bonding in the synthesised short-length peptide hypothetically unfolds its structure. Therefore, peptide structure potentially differs significantly from the corresponding structure within the whole chemokine. Chemokine peptides were shown extended, non-helical or irregularly structured (Figure 3-9), differently to the structure of the region to which they correspond within the full-length CXCL8 (an α-helix) (Figure 3-9b).

a)



b)

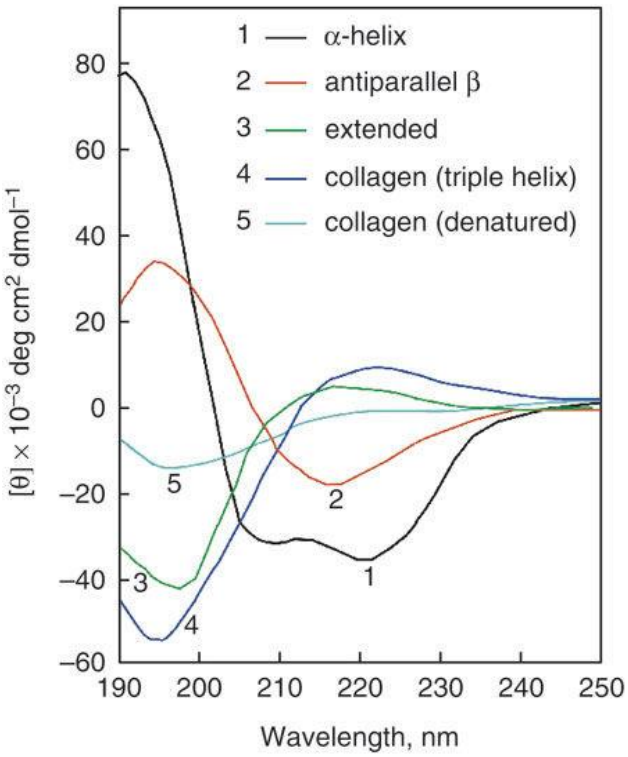


Figure 3-9. Circular Dichroism (CD) spectra for peptides.
a) CD spectra for WT peptide, E70K mutant peptide, and scrambled peptide (control peptide). Peptides were used at 25µM in PBS buffer, and were analysed at 20°C, with 220nm light, in the range of (240-197) nm wavelength, using PBS for background substraction. Spectra show the peptides are extended, non-helical or irregularly structured. Data is representative of three independent experiments (n=3). b) Spectra of an α–helix sequence (labelled as "1") compared to spectra of an extended or non-helical sequence (labelled as "3") (adapted from (Greenfield, 2006)).

3.4.2.1.2 CD for chemokine peptides in solution with heparin

Further studies were performed in order to evaluate whether presence of the highly sulphated GAG heparin would induce any conformational change in these short-length positively charged peptides. Synthesised peptides are hypothetically more flexible or plastic than the chemokine as they lack hydrogen bonding. Incubation of C-terminal peptides with heparin showed detectable change in their structure, as opposed to the scrambled peptide (Figure 3-10). Study was analysed at room temperature (20 ºC). It could be of interest to analyse it at 37ºC. Even though visible change is marginal, it indicates that presence of GAG induces a degree of peptide folding. This hypothetically increases the structural correlation with the corresponding region within CXCL8, which suggests how peptides function *in vitro*, and thus how peptides might act *in vivo*, in presence of GAG.

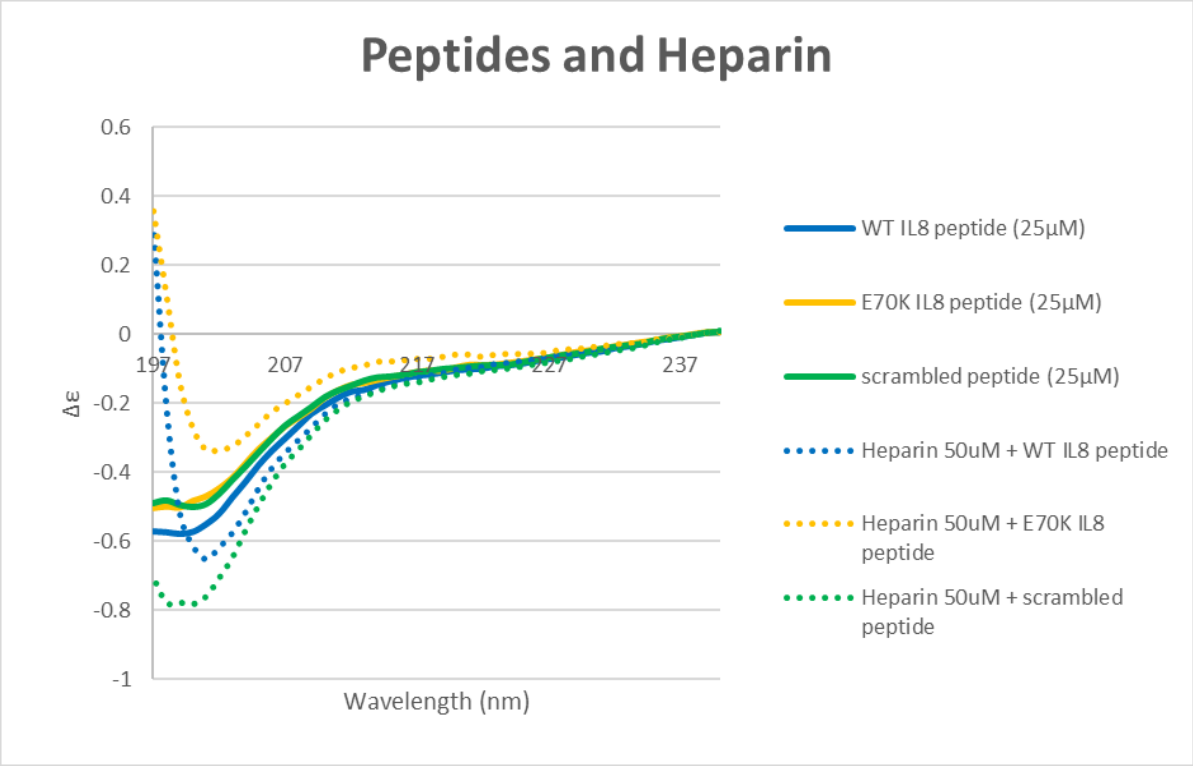


Figure 3-10. Circular Dichroism spectra of peptides, compared to peptides with heparin.
CD spectra for the CXCL8/IL8 WT peptide, the E70K mutant peptide and the scrambled peptide, and CD spectra for each peptide in solution with 50µM heparin (dotted lines). Peptides were used at 25µM in PBS buffer, and were analysed at 20°C, with 220nm light, in the range of (240-197) nm wavelength, using PBS for background substraction. Spectra show peptides are extended or unfolded. The WT C-terminal and the mutant peptide structures marginally change in presence of heparin as opposed to the scrambled peptide. Data is representative of three independent experiments (n=3).

3.4.2.2 Surface Plasmon Resonance (SPR)

Surface Plasmon Resonance (SPR) is widely used to assess GAG-binding capabilities of different molecules such as chemokines. In this study, we assessed CXCL8 and the synthesised CXCL8 C-terminal peptides. The SA chip was used to immobilise biotinylated heparin which was generously provided by Yoan Monneau (Prof Hughes Lortat-Jacob's laboratory, Institute of Structural Biology, Grenoble, France). We confirmed binding of CXCL8 to heparin using concentrations in the nanomolar to micromolar range (Figure 3-11). CXCL8-heparin binding was earlier shown by SPR to be of low affinity (KD=8.3µM) (Gerlza *et al.*, 2014). Low shifts or positive bulk effects are often seen at 5-10 response units (RU). Binding response units of chemokine peptides were higher than 10RU which shows significance of heparin binding.

A) B)

Figure 3-11. Surface Plasmon Resonance of heparin-CXCL8.
A) SPR sensorgram shows CXCL8 concentration-dependent binding in the range of (50-1000) nM. Chemokine was flowed at 30 µL/min over chip. B) Binding reported for each CXCL8 concentration. Data was analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. **P <0.01, ***P<0.001. Data is representative of three independent experiments over a single heparin-coated SA chip.

I then studied the binding of CXCL8 C-terminal peptides to heparin. Binding was demonstrated for heparin-CXCL8 C-terminal peptides at 5µL/min (Figure 3-12). Therefore, we concluded that synthesised peptides can bind GAG, particularly E70K mutant peptide, which showed significant binding as opposed to WT peptide and scrambled peptides, both showing similar levels of binding. This suggests that E70K substitution (peptide net charge +4) enhances significantly the peptide GAG-binding ability. Peptides showed lower affinity of binding than full-length chemokine as significant peptide binding was detected at a concentration $2x10^4$-fold higher (E70K peptide) or $4x10^4$-fold higher (WT peptide, scrambled peptide) than the whole chemokine Figure 3-12.

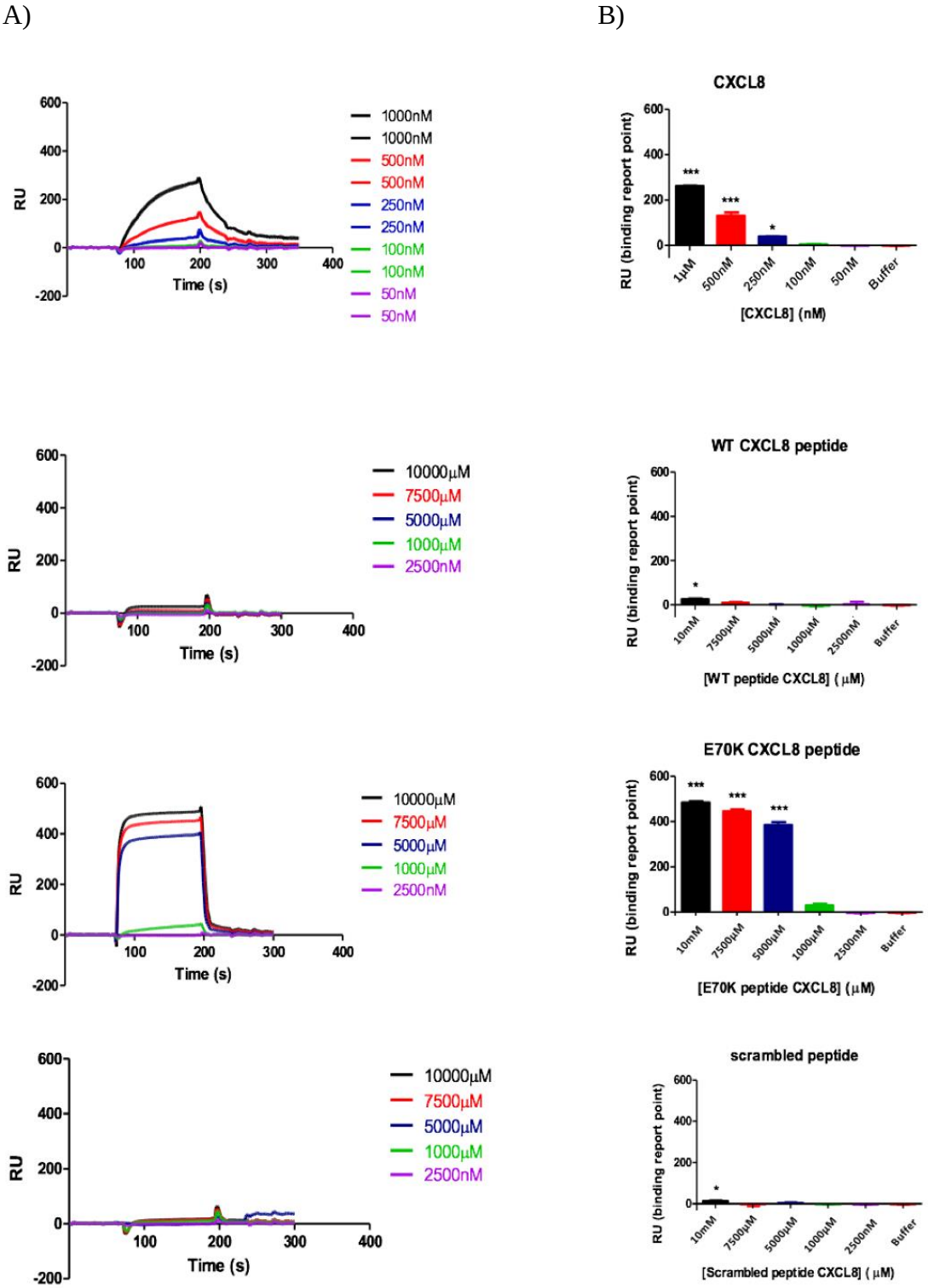


Figure 3-12. Surface Plasmon Resonance of heparin-CXCL8 peptide.
A) SPR sensorgram shows heparin-CXCL8 binding in the range of (50-1000) nM CXCL8, and heparin-CXCL8 peptide binding in the range of (2.5-10000) μM peptide. Chemokine or peptide were flowed at 5 μL/min over the chip. B) Binding shown for each chemokine or peptide concentration. Appendix I shows sensorgram of binding of WT peptide, and scrambled peptide with magnified y-axis. Data were analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. *$p<0.05$, ***$P<0.001$. Data is representative of three independent experiments over a single heparin-coated SA chip.

E70K mutant peptide was the only peptide that showed significant binding to GAG at different concentrations. The SPR off-rate of all peptides was observed in SPR sensorgram to be much quicker than that of the chemokine, perhaps as predicted since they are non-helical structures. WT peptide required 2-fold higher concentration than E70K peptide to show GAG binding. Equivalent GAG binding to WT peptide was observed for the scrambled peptide. E70K peptide showed significant specific binding in the range of 5 to 10 millimolar. Hence, further investigations were performed to define whether E70K peptide has the potential to compete with or modulate chemokine binding on the heparin coated chip.

The peptide dissociation phase from the heparin chip was faster than the chemokine one. Hence analysis of the peptide potential to out-compete CXCL8, by injection of chemokine followed by peptide, was hypothesised to be difficult to read or non-detectable by SPR. Alternatively, a mixture of E70K peptide and CXCL8 was initially studied. Initial assay showed higher heparin binding of the mixture than chemokine or peptide alone (Figure 3-13). However, the mode of binding of E70K peptide should be evaluated in a dose-dependent manner, perhaps at different concentration, and using WT and scrambled control peptides. The E70K peptide might act as a competitive small molecule in presence of CXCL8. This remains a question that may be explored if further knowledge and development of the chemokine peptide modulatory role is to be realised.

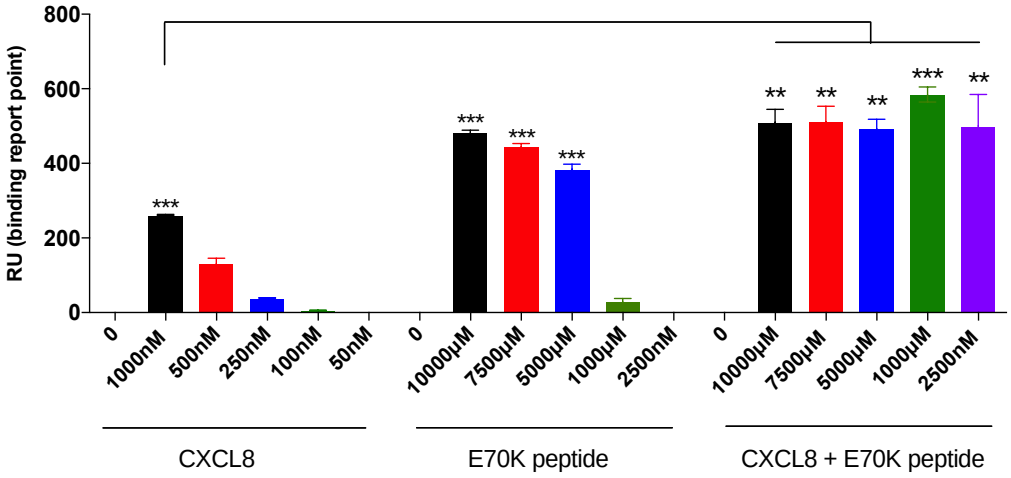


Figure 3-13. Surface Plasmon Resonance of E70K peptide and CXCL8 binding to heparin. Graph shows SPR binding of (1000-50) nM CXCL8 to heparin; (10000-2.5) µM E70K peptide to heparin; and E70K peptide at the same concentration range combined with 1000nM CXCL8 to heparin. Both chemokine and chemokine peptide were flowed over the chip at 5 µL/min. Data was analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. **P <0.01, ***P<0.001. Statistical evaluation of the initial assay showed increased binding of combined chemokine and E70K peptide compared with chemokine alone. Data is representative of three independent experiments over a heparin-coated SA chip.

Taken together, synthesised peptides showed significant heparin binding. The mode of binding appears to be of low affinity as fast SPR off-rate was observed at the end of injection of each chemokine peptide, even though E70K peptide binding showed significance at different concentrations. Thus, initial data suggests that E70K peptide may play a modulatory role on cellular GAG binding, and consequently, could affect CXCL8-mediated leukocyte recruitment.

3.5 Discussion

The truncated version of CXCL8 C-terminal region, core GAG-binding region, the variants E70K, R68A and R68Citrulline, and a scrambled peptide were synthesised at approx. 85-95% purity and biophysically characterised by circular dichroism and surface plasmon resonance.

Among synthesised CXCL8 C-terminal peptides, R68A and R68Cit peptides were not further evaluated by biochemical assays, both were hypothesised to have lower GAG binding. R68A mutation was earlier studied within the full-length chemokine, showing reduction in the ability of CXCL8 to bind to heparin (Dyer *et al.*, 2014). Citrullination of chemokines has been also previously reported, for instance in CXCL8, which showed lower leukocyte recruitment into the peritoneum (Proost *et al.*, 2008), but was more effective at neutrophil mobilisation from the bone marrow into the bloodstream (Loos *et al.*, 2009). For other chemokines studied - CXCL5, CXCL10, CXCL11, and CXCL12 - citrullination generally decreased receptor binding or activation (Loos *et al.*, 2008; Struyf *et al.*, 2009; Mortier *et al.*, 2010).

CXCL8, and CXCL8 peptide GAG-binding was assessed by Surface Plasmon Resonance. Chemokine, or chemokine peptide were flowed at 5μL/min over GAG heparin on a biochip. Peptide binding was only evident at concentrations $2x10^4$-fold to $4x10^4$-fold higher than CXCL8. E70K mutant peptide showed significantly higher binding than WT peptide or scrambled peptide. This suggests that E70K peptide, with higher positive charge (net charge +4, as opposed to WT net charge +2), might have a competitive role in GAG binding, thus modulating CXCL8-GAG heparin binding. Further SPR decay assays are required to confirm

mode of action of each peptide, particularly E70K peptide in presence of CXCL8 if better definition of mode of GAG binding is to be realised. In addition, it would be of interest to evaluate whether the CXCL8 peptides have ability to interact with CXCL8. Previously reported studies using chemokine peptides have demonstrated peptide ability to bind GAG and compete with whole CXCL chemokine by biophysical IFT studies and *in vivo* (Vanheule *et al.*, 2015; Vanheule *et al.*, 2017).

Synthesised peptides were non-helical, extended or irregularly structured as shown by Circular Dichroism. This was expected as synthesised peptides are short-length sequences with electrostatic bonding and no hydrogen bonding. Hence, data confirmed higher flexibility or plasticity of the non-helical peptide structures compared to the full length CXCL8. In addition, however, peptides in solution with heparin showed a degree of structural change by CD analysis, as opposed to scrambled peptide. Thus, although peptides have low affinity for GAG binding, they showed a significant role in GAG interaction.

Further investigations might contribute to better understand the biochemical role of CXCL8 C-terminal peptides. Strategies to enhance peptide specificity could involve, for instance, substitution with more positively charged amino acids in the sequence, synthesis of a longer sequence or the full-length chemokine variant. Also, chemokine and peptide oligomerisation and folding of the unfolded states contribute to the plasticity of GAG binding and it is crucial to know how this influences the specificity of GAG binding, and whether peptides can oligomerise with CXCL8.

Taken together, the present chemokine peptide approach improves understanding of the regulation of GAG-CXCL8 binding. It could be helpful towards development of synergistic combined strategies aimed to modulate neutrophil trafficking and infiltration into damaged tissue. In the following section, investigations focus on studying the biological role of synthesised peptides, using different *in vitro* static and dynamic models of leukocyte recruitment.

4. Regulation of CXCL8 Function: The Role of GAG Binding

4.1 Introduction

As previously discussed, chemokine function can be regulated by several means including GAG binding. In the past, investigations within our group developed non-GAG binding mutant chemokines by substitution of basic amino-acid residues with alanine residues to understand their modulatory role. These mutant chemokines bind their cognate receptors normally and competitively inhibit binding of their wild type counterparts. Occupation of chemokine receptors by non-GAG binding chemokine variants, prevents migration along a gradient and therefore inhibits chemotaxis, as previously shown with CCL7 (Ali *et al.*, 2010) and CXCL12 (O'Boyle *et al.*, 2009). Studies have shown that CXCL8 mutants with reduced GAG-binding abilities induced less recruitment of neutrophils than wild type CXCL8 (Kuschert *et al.*, 1998).

Studies have also developed mutants with increased GAG binding. A variant of CXCL8 with no ability to bind GPCRs but with increased GAG binding affinity has been shown to inhibit transendothelial migration of neutrophils by displacing CXCL8 from the surface of endothelial cells (Gschwandtner *et al.*, 2017). This effect could displace multiple chemokines, overcoming issues of redundancy, however, high concentrations of chemokine may be required to occupy binding sites on all GAGs (Bedke *et al.*, 2010; Gerlza *et al.*, 2015). Nevertheless, this approach represents another potential method of regulating chemokine function.

In addition to whole chemokine mutants, chemokine peptides have been developed to block chemokine-GAG binding. Small peptide fragments of CXCL9 C-terminal region have been shown to compete with CXCL8, CXCL11 and CCL2 for binding to GAG (K_D=61.21nM for LMWH; K_D=4.76nM for HS) (Vanheule *et al.*, 2015; Vanheule *et al.*, 2017). A linear octapeptide of interferon gamma (IFN-γ), MC-2, derived from the GAG-binding region also inhibited cytokine GAG binding (IC_{50}=0.18μM), which was suggested to be related to the number of positive charges (Fernandez-Botran *et al.*, 2002; Fernandez-Botran *et al.*, 2004; Cripps *et al.*, 2005). In addition, a single dose of MC-2 (50mg/kg) added to the resuscitation

regimen in a rat model of experimental hemorrhagic shock was associated with diminished liver injury and improved intestinal barrier function (Matheson *et al.*, 2016).

Furthermore, earlier reported CXCL8 peptides targeting the CXCL8 GPCR-binding region based on *in silico* analysis, bind the chemokine receptor with a K_D of 252μM (Jiang *et al.*, 2015). Small molecule chemokine receptor antagonists are a valid strategy, as the CXCR1/2 non-competitive allosteric inhibitor reparixin (K_D= 1nM) has been proven to prevent graft dysfunction in early phase clinical trials. The CXCR1/2-antagonist SB225002 has been shown to significantly inhibit chemokine-mediated calcium mobilisation (IC_{50}= 10nM for CXCL1, IC_{50}= 8nM for CXCL8) (White *et al.*, 1998; Lane *et al.*, 2001), and also to inhibit HIV replication in lymphocytes and macrophages. It has been proven effective in preclinical trials of Chronic Obstructive Pulmonary Disease (Hay and Sarau, 2001). Moreover, the CXCR2-antagonist SB265610 was shown to interfere with CXCL1-mediated calcium mobilisation in lung injury (IC_{50}=3.4nM) by CXCR2 binding (K_D=2.51nM) (Widdowson *et al.*, 2004).

In this chapter, the ability of C-terminal chemokine peptides (WT peptide: KENWVQRVVEKFLKRAENS; E70K peptide: KENWVQRVVEKFLKRAKNS; and scrambled peptide: KVREKNEKWFVEQRVALNS, shown in Figure 3-1) to bind GAG and their potential to modulate chemokine-GAG binding was assessed by *in vitro* migration assays and flow-based models.

In addition, the role of CXCL8 C-terminal peptides was compared to the inhibitory effect of the LMWH tinzaparin. LMWHs have been reported to be involved in the inhibition of chemokine-GAG binding. Chemokine regulation during the inflammatory response was also studied using the CXCR1/2 chemokine receptor antagonists reparixin, SB225002 or SB265610, to compare levels of neutrophil recruitment associated with modulation of GPCR binding.

4.2 Specific aims

- Characterise cells to be selected for neutrophil recruitment

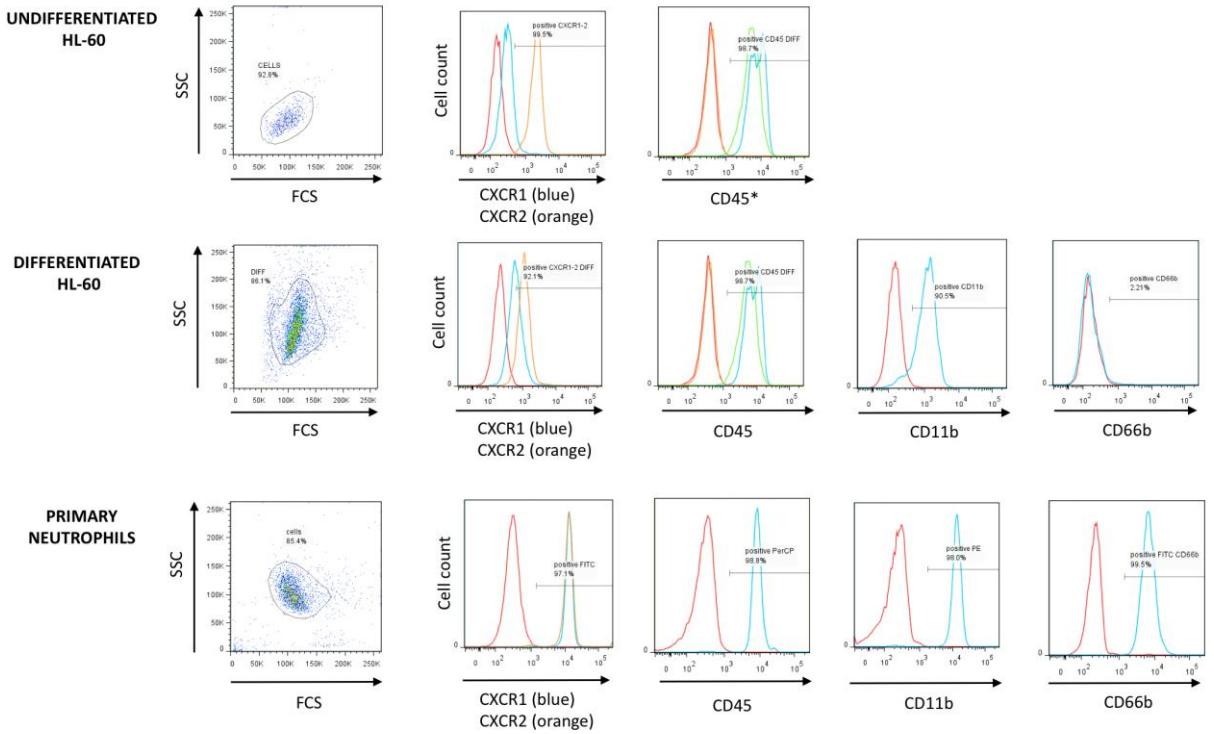
- Investigate the biological function of CXCL8 C-terminal peptides during CXCL8-mediated neutrophil recruitment

4.3 Results

4.3.1 Cell surface molecule expression on HL-60 and primary blood neutrophils

Cell surface expression of neutrophil markers was evaluated. HL-60 cell line both undifferentiated, and DMSO-treated for 6 days to induce differentiation were used. Primary neutrophils (PMN) obtained from blood donated by healthy volunteers were also characterised. Cells were tested for their expression of CD45, CD11b and CD66b, as antigens displayed on neutrophils, and for chemokine receptors CXCR1 and CXCR2 (Lakschevitz *et al.*, 2016). Undifferentiated cells showed significant levels of CD45 only. In contrast, differentiated HL-60 express high levels of CD45, CD11b, CXCR1 and CXCR2, and no CD66b. Primary blood neutrophils expressed all analysed surface molecules, CD45, CD11b, CD66b, CXCR1 and CXCR2 in higher levels than the cell line (Figure 4-1). Thus, further biological investigations primarily used primary neutrophils.

A)



B)

MFI					
	CXCR1	**CXCR2**	**CD45**	**CD11b**	**CD66b**
Undifferentiated HL-60	200	580	11500	-	-
Differentiated HL-60	281	512	11450	1177	203
Primary Neutrophils (PMN)	12400	12400	7879	12600	5915

C)

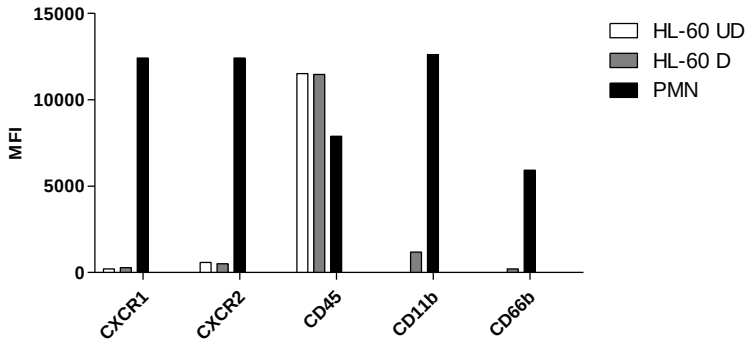


Figure 4-1. Cell surface expression of neutrophil antigens.
A) Neutrophil cell line HL-60 and Primary Neutrophils were analysed for cell surface expression of CD45, chemokine receptors CXCR1 and CXCR2, and adhesion molecules CD11b and CD66b by flow cytometry, in relation to the respective isotype controls. *In histogram for CD45, note that blue corresponds to undifferentiated cell line (UD), whereas green refers to undifferentiated cell line (D). B) Median fluorescence intensity (MFI) representative values of A. C) Bar chart of B. Data is representative of two independent experiments for differentiated HL-60 cell line and for PMN (n=2).

4.3.2 Chemokine receptor expression at RNA level in HL-60 and PMN

To further investigate the chemokine receptor expression in differentiated HL-60 and PMN, mRNA expression was measured by qRT-PCR using HPRT as housekeeping gene. Expression of CXCR1, CXCR2, CXCR4 and CXCR7 were measured (Figure 4-2).

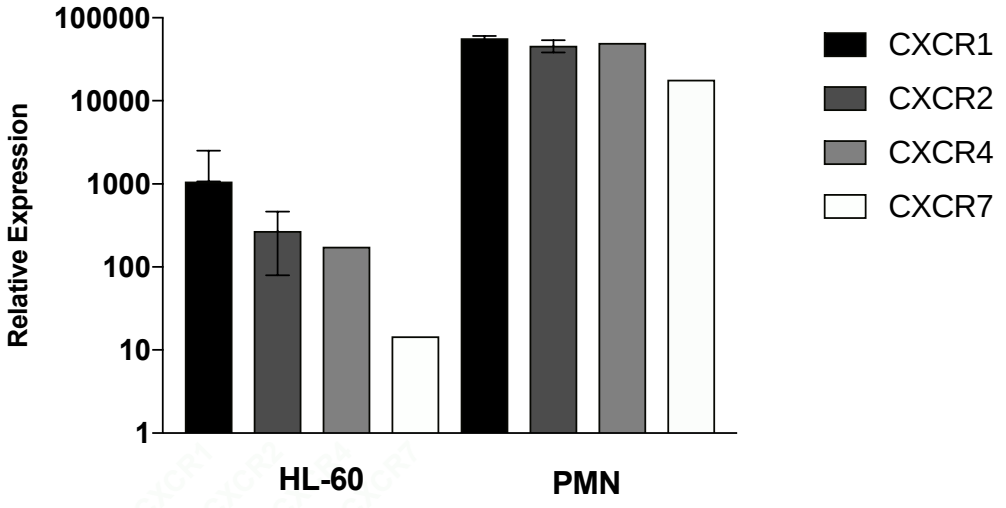


Figure 4-2. Neutrophil CXCR receptor mRNA expression.

Differentiated HL-60 cell line and primary neutrophils (PMN) mRNA was assessed for the expression of chemokine receptors CXCR1, CXCR2, CXCR4 and CXCR7, normalised to HPRT. HL-60 was treated with 1.25% DMSO RPMI 1640 media for 6 days for differentiation. PMN were extracted from blood of healthy donors. Relative expression to undifferentiated HL60 expression levels. Data is representative of two independent experiments, though CXCR4 and CXCR7 were only analysed once, and each experiment was performed in triplicate.

CXCR1 and CXCR2 were shown to be highly expressed, both in HL-60 cell line and PMN. Data is consistent with flow cytometry as both mRNA and surface expression levels were higher for PMN than for the cell line. CXCR4 and CXCR7 expression in neutrophils was confirmed, as shown (Zhang *et al.*, 2009).

4.3.3 Neutrophil diffusion gradient chemotaxis directed by CXCL8 and CXCL1

The aim of this study was to assess chemokine regulation of chemotaxis. We first studied the neutrophil migration directed by the neutrophil chemoattractants CXCL8 and CXCL1. Concentrations of 10-26nM were used for the optimisation of neutrophil diffusion gradient

chemotaxis assays, with 20nM to 26nM giving highest migration. Primary neutrophils showed maximum chemotactic response at 20nM CXCL8, after which neutrophil migration appeared to decrease (Figure 4-3), as earlier observed (Proost *et al.*, 2008) possibly due to overstimulation leading to receptor desensitisation, internalisation, and recycling or degradation. Chemotaxis data confirmed neutrophils isolated from healthy volunteers were a better model for our studies, showing a greater chemotactic response than HL-60 cell line. Hence, subsequent work was performed using primary neutrophils.

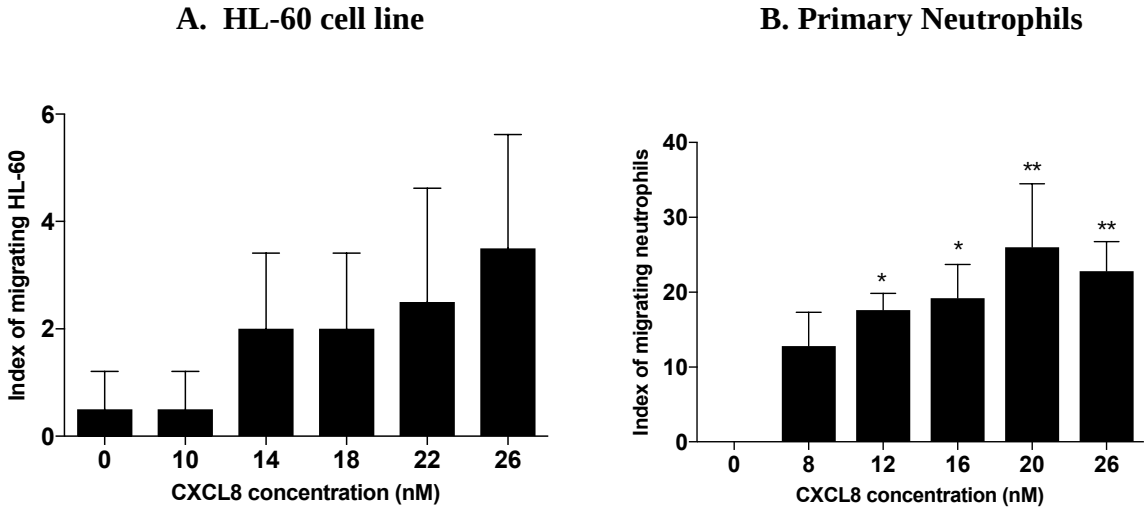


Figure 4-3. CXCL8 dose-response in diffusion gradient neutrophil chemotaxis
CXCL8 was added to the transwell bottom chamber at different concentrations. Then, 200,000 neutrophils (A. HL-60 cell line, or B. primary neutrophils) were added on to the insert, and cells were incubated at 37°C for 90 minutes. Cells were counted using a haemocytometer. Index of migrating cells is relative to the negative control, 0nM CXCL8. Index= (Sample/ Negative). Data were analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. *$P < 0.05$, **$P < 0.01$. Data are representative of two independent experiments (n=2), each performed in triplicate.

CXCL1-mediated diffusion gradient chemotaxis induced no significant migratory response on primary blood neutrophils. 10-20nM of chemokine was used (Figure 4-4 and Figure 4-5). CXCL1 used in chemotaxis assays was no-BSA-conjugated protein, as opposed to CXCL8. Further evaluation of the protein quality by silver gel stain or mass spectrometry would confirm whether protein concentration is preserved.

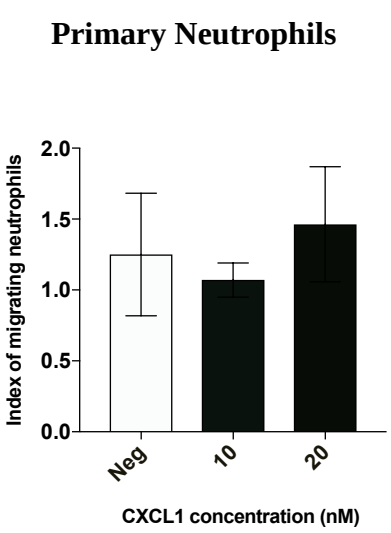
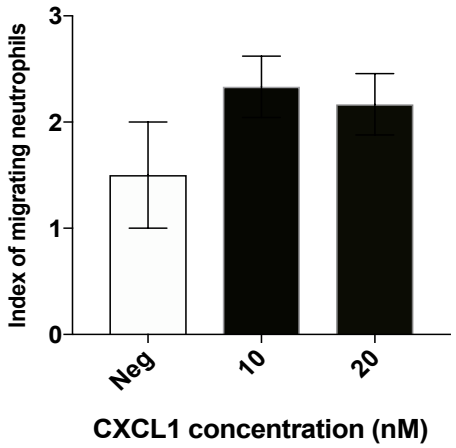


Figure 4-4. CXCL1 dose-response in diffusion gradient neutrophil chemotaxis.
CXCL1 was added to the transwell bottom chamber at different concentrations. 200,000 primary neutrophils were added on to the insert, and cells were incubated at 37°C for 90 minutes. Cells were counted using a haemocytometer. Index of migrating cells is relative to the negative control, 0nM CXCL1. Index= (Sample/Negative). Data were analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. Data showed no significance. It is representative of two independent experiments (n=2), each performed in triplicate.

4.3.4 Neutrophil transendothelial chemotaxis directed by CXCL8 or CXCL1

Transendothelial chemotaxis (TEC) was also investigated, to evaluate the effect of endothelial GAGs on chemokine gradient formation and neutrophil migration. Overall, CXCL8 induced significant neutrophil transendothelial chemotaxis at both 10nM and 20nM, with highest response at 20nM (Figure 4-5). CXCL1 showed no statistically significant neutrophil migration in presence of an endothelial layer, which correlated with solute diffusion gradient chemotaxis.

A. CXCL1



B. CXCL8

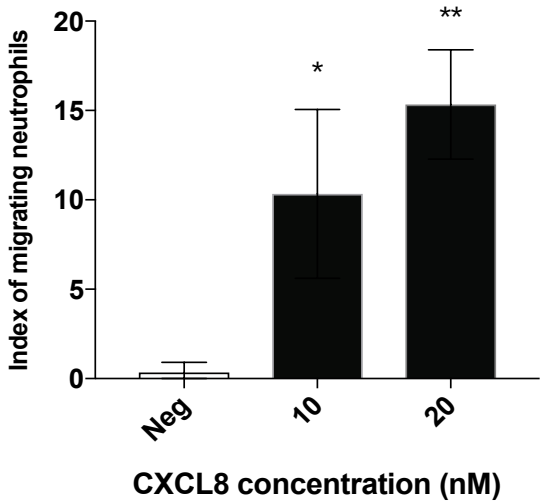


Figure 4-5. Neutrophil transendothelial migration towards CXCL1 or CXCL8.
CXCL1 (A) or CXCL8 (B) was added to the transwell bottom chamber at different concentrations once endothelial layer was formed on the insert after three days and assessed with a microscope. Then, 200,000 neutrophils were added on the transwell upper chamber, and cells were incubated at 37°C for 90 minutes. Cell count was done using a haemocytometer. Index of migrating cells is relative to the negative control, 0nM chemokine. Index= (Sample/Negative). Data were analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. *$P < 0.05$, **$P < 0.01$. Data are representative of three independent experiments (n=3), each performed in triplicate.

CXCL1 was not further evaluated in this study, since CXCL1 showed no statistically significant neutrophil migration. Hence, the focus was on CXCL8 function and on evaluation

of the potential role of CXCL8-derived peptides at modulating chemokine biological activity. 10nM CXCL8 were next used for migration assays aimed to assess the biological function of synthesised peptides.

4.3.5 Role of CXCL8 C-terminal peptides in neutrophil recruitment

The CXCL8 C-terminal peptide, core GAG-binding region within CXCL8, and peptide variants (Figure 3-1) were studied to examine the potential role at displacing GAG-bound CXCL8 and affecting chemokine gradient formation during neutrophil recruitment.

4.3.5.1 C-terminal peptides do not interfere with diffusion gradient chemotaxis

Firstly, we evaluated whether the peptides interfered with chemokine receptor binding. Neither WT peptide (KENWVQRVVEKFLKRAENS), E70K peptide (KENWVQRVVEKFLKRAKNS), nor scrambled peptide (KVREKNEKWFVEQRVALNS) showed significant inhibition on CXCL8-mediated neutrophil diffusion gradient chemotaxis. Thus, we assume the peptides do not affect CXCR1/2 receptor binding (Figure 4-6). Cell viability in presence of peptides was assessed by cell exclusion trypan blue and showed no significant cell death (>95% viability).

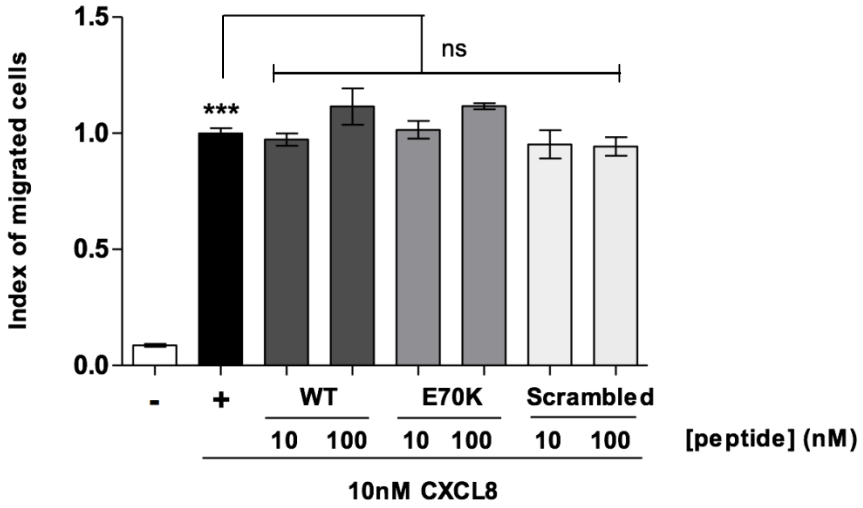


Figure 4-6. Diffusion gradient chemotaxis in response to CXCL8 combined with each peptide. 10nM CXCL8 were used (positive control). Synthesised CXCL8 C-terminal peptides (10, 100) nM showed no interference with neutrophil migration in absence of endothelial GAG surface, which suggests no binding with CXCR1/2 receptors. WT peptide (KENWVQRVVEKFLKRAENS); E70K peptide (KENWVQRVVEKFLKRAKNS); or scrambled peptide (KVREKNEKWFVEQRVALNS) were studied. Data were analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test shows significant migration in response to CXCL8 compared to negative control. ***$P < 0.001$. ns: no significant. Representative data of three independent experiments (n=3), each performed in triplicate.

4.3.5.2 C-terminal peptides do not inhibit neutrophil calcium signaling

In order to confirm that CXCL8 C-terminal peptides have no effect on CXCL8-GPCR binding and signaling, we evaluated whether peptides have a modulatory effect on CXCL8-mediated neutrophil intracellular calcium (Ca^{2+}) signaling. Consistent with diffusion gradient chemotaxis, and using the same concentrations, no significant effect of WT peptide, E70K peptide or scrambled peptide was shown on CXCL8-mediated calcium signaling (Figure 4-7).

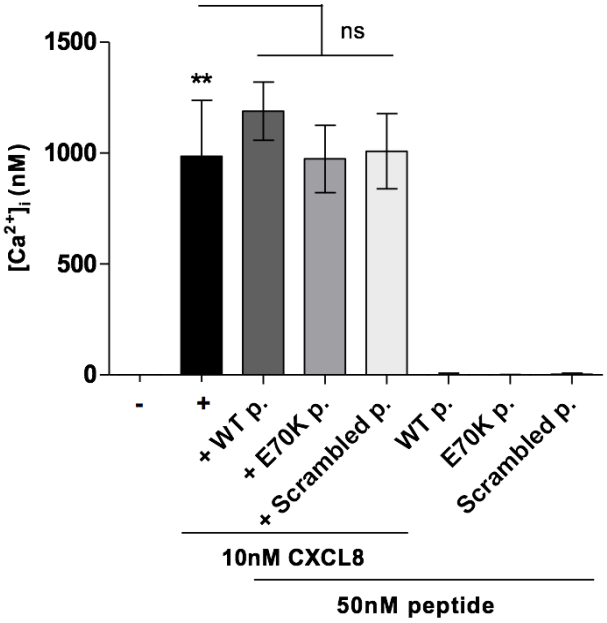


Figure 4-7. Neutrophil calcium flux in response to CXCL8 combined with each peptide.
Intracellular calcium ($[Ca^{2+}]_i$) was measured in response to CXCL8, or CXCL8 combined with each peptide (WT: KENWVQRVVEKFLKRAENS; E70K: KENWVQRVVEKFLKRAKNS; or scrambled peptide: KVREKNEKWFVEQRVALNS). Primary blood neutrophils were labelled with Indo-1, AM. Then, cells were analysed in response to HBSS only (negative control), 10nM CXCL8 (positive control) or CXCL8 combined with peptide (CXCL8 WT peptide, CXCL8 E70K peptide or scrambled peptide) at 50nM, within range of (10-100) nM previously analysed. Data was analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. **$P < 0.01$ shows significant adhesion in response to CXCL8 compared to negative control. ns: no significant. Data is representative of three independent experiments (n=3).

4.3.5.3 Effect of C-terminal peptides on neutrophil transendothelial migration

Transendothelial migration directed by CXCL8 in presence of each peptide was next studied. CXCL8 WT or scrambled peptide showed no significant modulation of CXCL8-mediated neutrophil migration. In contrast, E70K peptide showed significant modulation of CXCL8-mediated transendothelial migration (Figure 4-8).

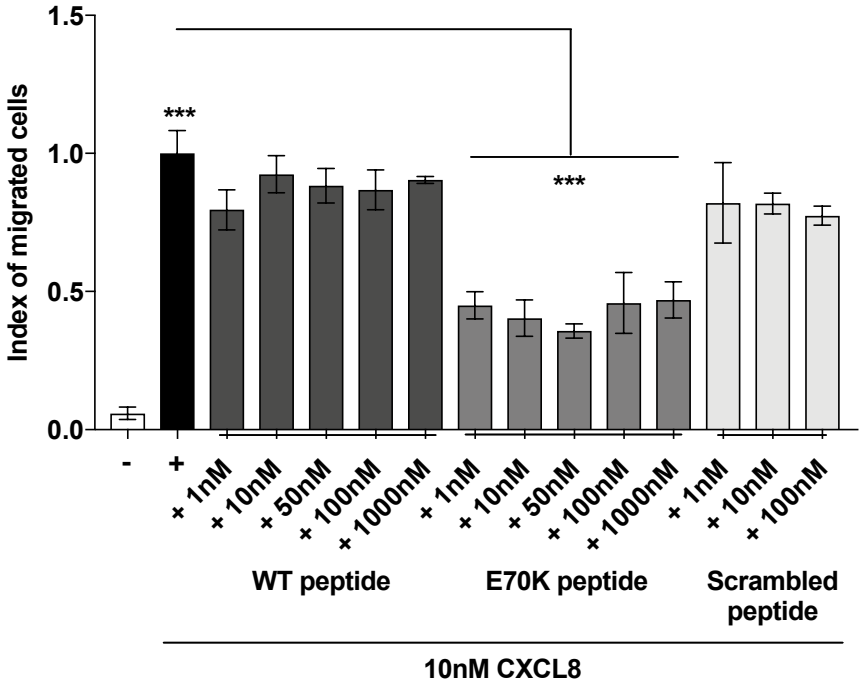


Figure 4-8. Transendothelial migration in response to CXCL8 combined with each peptide. Neutrophil transendothelial migration was assessed as previously described. Response to CXCL8 (10nM), or CXCL8 combined with peptide (at (1-1000) nM) (WT: KENWVQRVVEKFLKRAENS; E70K: KENWVQRVVEKFLKRAKNS; or scrambled: KVREKNEKWFVEQRVALNS) was measured. Cells count was done using counting beads by flow cytometry. Data were analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. ***$P < 0.001$ on black column indicates significant migration in response to CXCL8 compared to negative control. Data is representative of two independent experiments (n=2) from different primary neutrophil preparations, each performed in triplicate.

4.3.6 Leukocyte flow-based adhesion assays

This section of my POSAT project aimed to investigate the inflammatory response associated with ischaemia-reperfusion injury following transplantation. Specifically, I investigated the potential function of chemokine peptides to inhibit cell migration under flow conditions.

Optimisation studies were performed using the THP-I monocyte cell line, due to availability and ease of culture. Figure 4-9 shows main steps followed with VenaFlux platform to study leukocyte adhesion under flow, by using THP-I cell line over a Vena8 Endothelial+ biochip. Once the assay was optimised neutrophils were studied.

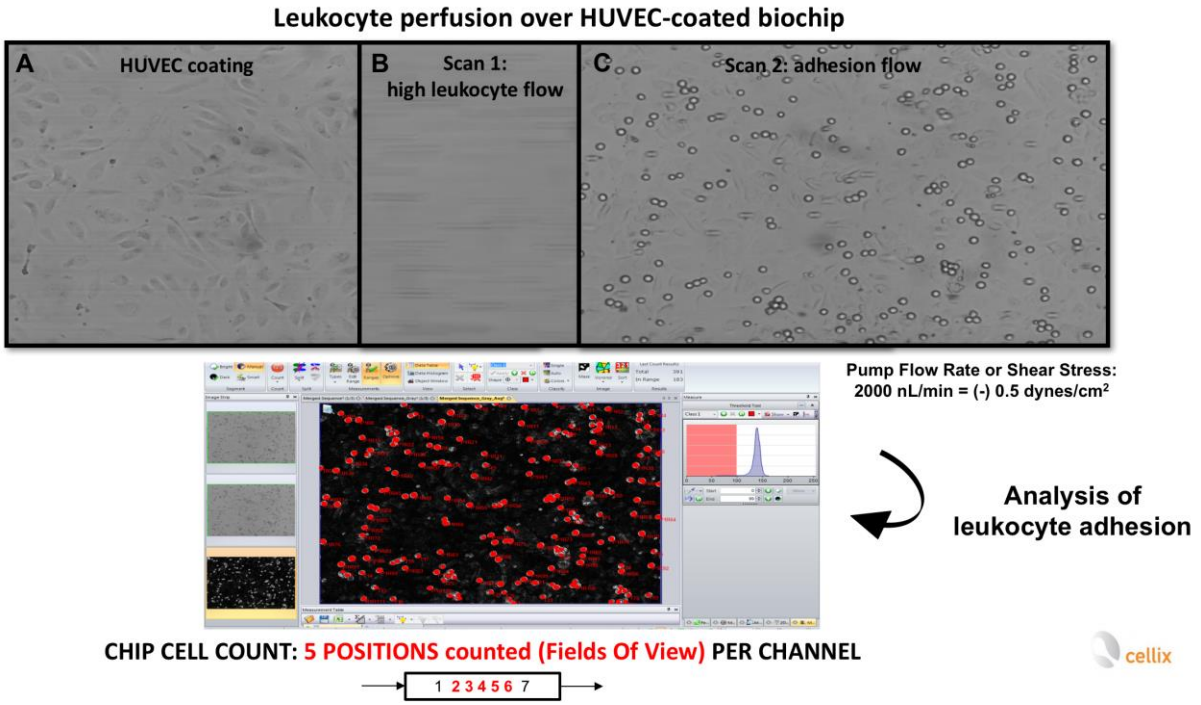
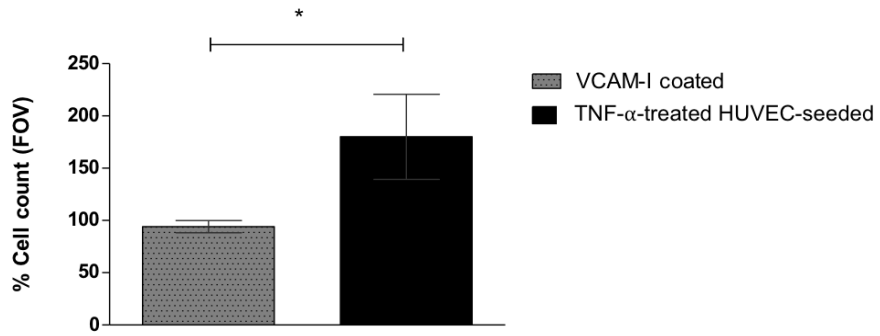


Figure 4-9. Schematic representation of leukocyte perfusion and adhesion over TNF-α-treated primary HUVECs.
A) First, HUVEC endothelial cells were seeded over the fibronectin-coated biochip. B) Next, THP-I cells were loaded onto the endothelial layered chip and initially perfused at high flow rate, -10 dynes/cm^2 for 10 seconds, to allow leukocyte circulation over the chip (negative flow, towards pump). C) Leukocyte adhesion was then analysed (here THP-I) at more physiological flow rate or shear stress, -0.5 dynes/cm^2 for 3 minutes. THP-I cells were fluorescently labelled using 1µM $(DIOC_6)_3$. Count of adherent cells (depicted as red dots) was performed using Image-Pro Premier Analysis software. 5 positions (Fields of View or FOV) were counted per channel. Note that the 5 positions are systematically labelled for each biochip channel, numbered 2-6 (see positions in red).

In order to select the most appropriate biochip, leukocyte adhesion was studied using Vena8 Fluoro+ biochip (400x100cm) or Vena8 Endothelial+ biochip (800x120cm). The Fluoro+ biochip can be coated with adhesion molecules (ICAM-1, VCAM-1), but not endothelial cells due to its dimensions. Instead, the Endothelial+ biochip is used to generate an endothelial layer, for instance with primary Human Umbilical Vein Endothelial Cells (HUVECs). Study using both biochips showed significant cell adhesion. However, Vena8 Endothelial+ biochip which has larger dimensions allows endothelial seeding and showed higher THP-I cell adhesion, thus it was more suitable for our leukocyte flow-based studies (Figure 4-10).

A) Biochip Optimisation (Vena8 Fluoro+ versus Vena8 Endothelial+)



B) TNF-α Optimisation (Vena8 Endothelial+ biochip)

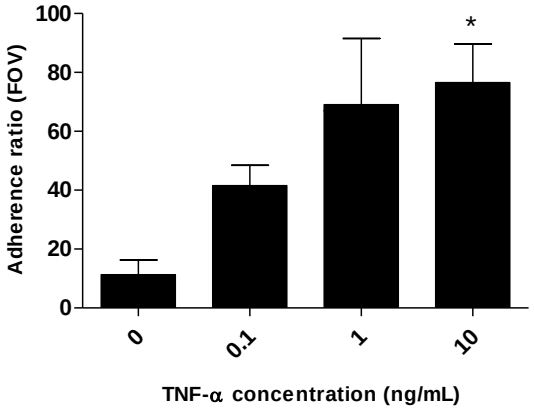


Figure 4-10 Biochip optimisation, followed by TNF-α optimisation for flow-based adhesion.
A) THP-I adhesion was analysed using VCAM-1 as substrate (on Vena8 Fluoro+ biochip), and compared to adhesion over activated HUVECs (on Vena8 Endothelial+ biochip) which showed more significant cell adhesion. B) Optimisation of TNF-α concentration for endothelial cell activation. 10ng/mL TNF-α were initially used (as shown in A). Then 1ng/mL TNF-α were used for peptide studies. Data was normalised to negative control (No TNF-α). Adhesion (cell count or adherence) was analysed by ANOVA, followed by Bonferroni post-hoc test. *$p<0.05$. Data is representative of three independent experiments (n=3), each performed in triplicate.

4.3.6.1 Neutrophil flow-based adhesion in response to CXCL8

4.3.6.1.1 HL60 neutrophil-like cell line flow-based adhesion

Flow-based adhesion assays initially used the HL60 cell line, undifferentiated (UD) or differentiated (D, or DMSO-treated cells), and later were performed for primary blood

neutrophils. HL-60 adhesion assays under shear stress showed significant adhesion to TNF-α-treated HUVECs, positive control, compared to the negative control (HUVEC layer over fibronectin substrate).

A range of CXCL8 concentrations (1-200 nM) applied to TNF-α treated HUVECs induced significant neutrophil adhesion compared to the negative control (Figure 4-11, Figure 4-12). No significant effect was observed in response to TNF-α plus chemokine compared to TNF-α only. Chemokine is expressed in response to TNF-α, and this endogenous production may be higher than exogenous chemokine. Hence, an alternative strategy might be the use of other adhesion-inducing molecules or cytokines e.g. IL-1β, IFN-γ; lower TNF-α concentration, or to use different chemokine concentrations. It might also be of interest to assess neutrophil adhesion in response to TNF-α combined with anti-inflammatory cytokines such as IL-4 or IL-10, to compare their effect in cytokine or chemokine-mediated adhesion (Marie *et al.*, 1996; Frangogiannis, 2004).

The potential of heparin, to displace endogenously-produced chemokine from the endothelial GAG surface, was assessed using heparin applied to the HUVEC layer for 1 hour before analysis of neutrophil adhesion. Presence of heparin showed no significant reduction of adhesion of HL-60 cell line or primary neutrophils. Study was confirmed by initial incubation of endothelium with 200nM CXCL8 for 1hour followed by heparin treatment, which had not significant effect compared to CXCL8, or to heparin only (Figure 4-13).

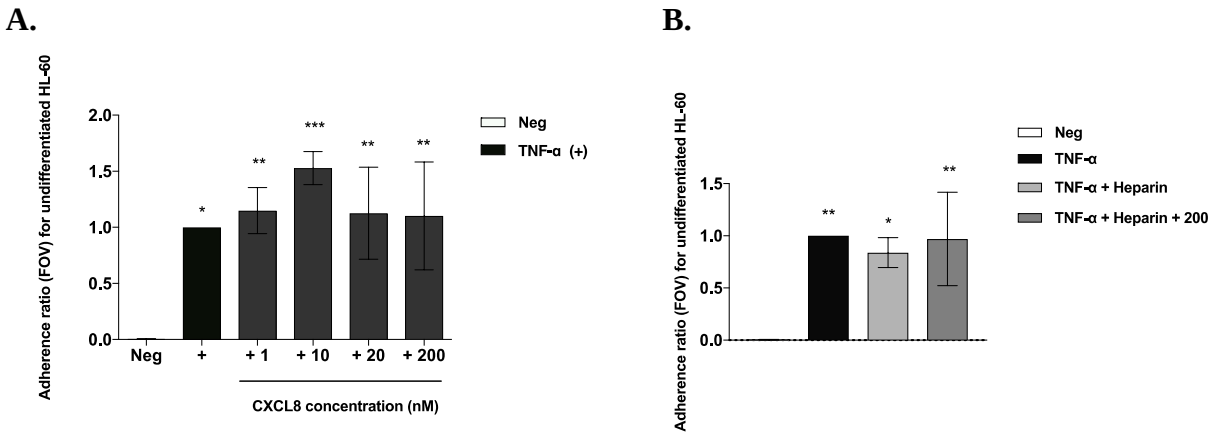


Figure 4-11. UD-HL60 flow-based adhesion mediated by CXCL8.
A) CXCL8 dose-response study. B) Analysis of role of heparin on chemokine displacement (200nM). Negative control is untreated HUVECs (100µg/mL fibronectin as substrate for HUVECs on biochip). Positive control is TNF-α-stimulated HUVECs (10ng/mL TNF-α over HUVECs O/N at 37°C). HUVEC layer was treated with CXCL8 (nM), or with heparin (100µg/mL) for 1 hour. Adherence ratio is obtained from average of 5 fields of view (FOV), with subsequent normalisation to positive control. Data were analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Data is representative of three independent experiments (n=3), each performed in triplicate.

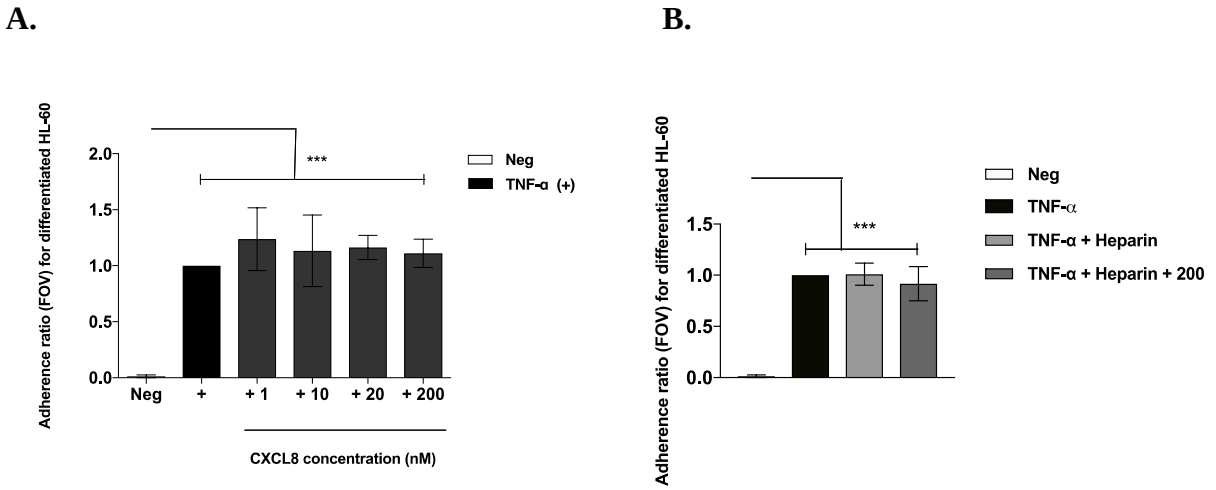


Figure 4-12. D-HL60 (DMSO-treated) flow-based adhesion mediated by CXCL8.
A) CXCL8 dose-response study. B) Analysis of role of heparin on chemokine displacement (200nM). Negative control is untreated HUVECs (100µg/mL fibronectin). Positive control is TNF-α-stimulated HUVECs (10ng/mL TNF-α). HUVEC layer was treated with CXCL8 (nM), or with heparin (100µg/mL) for 1 hour. Adherence ratio is obtained from average of 5 Fields of view (FOV), with subsequent normalisation to positive control. Data were analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. ***$P < 0.001$. Data is representative of three independent experiments (n=3), each performed in triplicate.

4.3.6.1.2 Primary neutrophil flow-based adhesion

Primary neutrophils showed significant adhesion in response to CXCL8 under shear stress compared to negative control (no TNF-α) at a range of 1 to 200nM CXCL8, although no significant difference was observed using different chemokine concentrations (Figure 4-13). No significant effect was observed in response to TNF-α plus chemokine compared to TNF-α only, which may be associated with cytokine-induced expression of endogenous chemokine.

In order to examine the potential role of heparin on displacing chemokine-GAG binding, endothelial layer was treated with heparin for 1 hour. Primary neutrophils showed no significantly different adhesion in presence of heparin. Also, no significant difference was observed in cell adhesion after endothelial treatment with chemokine followed by heparin.

A. **B.**

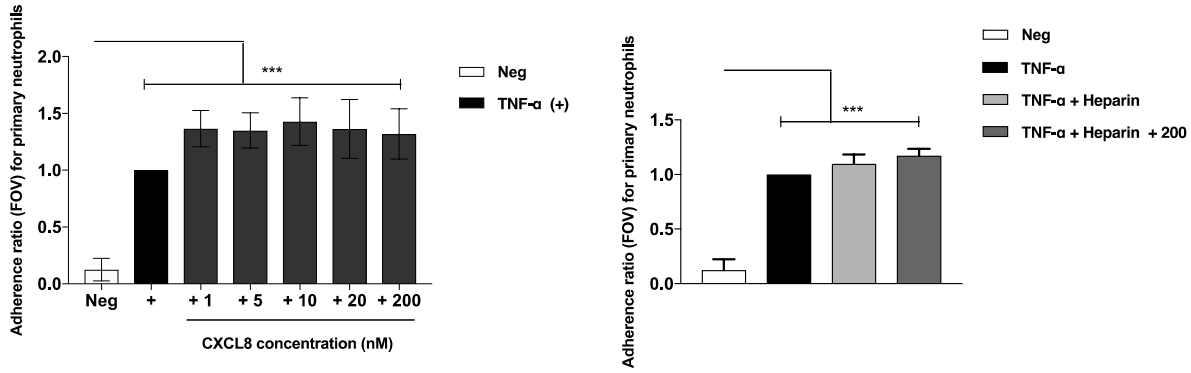


Figure 4-13. Neutrophil flow-based adhesion assay directed by CXCL8.
A) CXCL8 dose-response study. B) Analysis of role of heparin on chemokine displacement (200nM). Negative control is untreated HUVECs (100µg/mL fibronectin). Positive control is TNF-α-stimulated HUVECs (10ng/mL TNF-α). HUVECs were treated with CXCL8 (nM), or with heparin (100µg/mL) for 1 hour. Adherence ratio is obtained from average of 5 fields of view (FOV), and subsequent normalisation to positive control. Data were analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. ***$P < 0.001$. Data is representative of three independent experiments (n=3), each performed in triplicate.

4.3.6.1.3 Modulation of neutrophil flow-based adhesion by C-terminal peptides

The potential of C-terminal peptides to affect flow-based neutrophil adhesion was assessed in presence of 20nM CXCL8, concentration which earlier showed induction of neutrophil adhesion and most significant neutrophil chemotaxis.

All peptides induced a significant reduction in TNF-α and CXCL8 mediated adhesion, with no significant difference between WT peptide, E70K peptide and scrambled peptide, ($p<0.01$), which might be associated with the physiological shear stress (Figure 4-14). Data suggests no significant role of the charge difference among the peptides (WT has net charge +2; E70K has net charge +4) or their sequence difference (WT vs scrambled). Their negative effect in adhesion may be due to their positive charge and ability to bind GAG and modulate neutrophil adhesion. The different role of E70K peptide in transendothelial migration, where only E70K peptide showed potential gradient disruption, might be associated with its higher positive charge to bind polyanionic GAG.

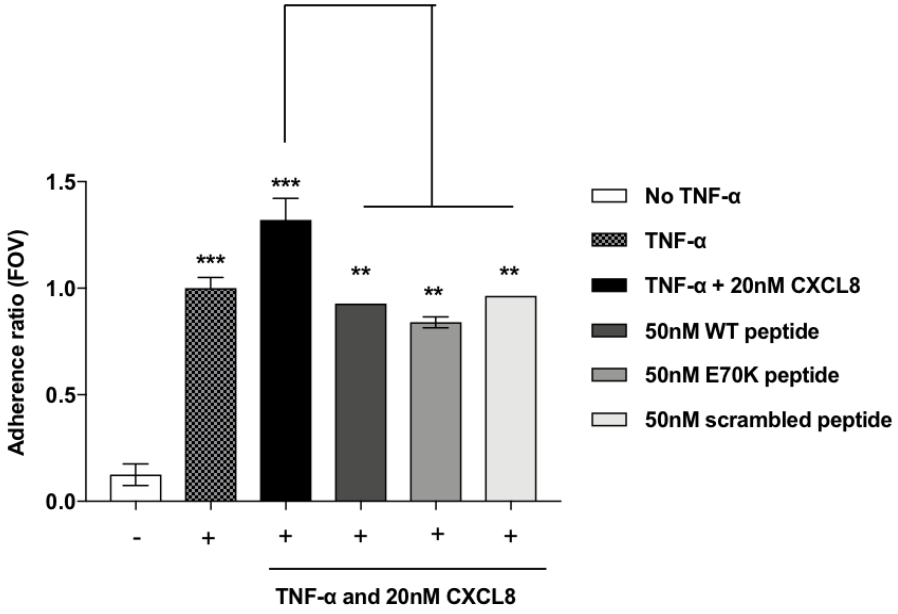


Figure 4-14. Neutrophil flow-based adhesion in response to each peptide.
Negative control is untreated HUVECs (fibronectin only). Positive control is TNF-α-stimulated HUVECs (1ng/mL TNF-α used to treat HUVECs O/N at 37°C) treated with 20nM CXCL8 for 1 hour. Adherence ratio was obtained from the average of 5 fields of view (FOV) per channel of biochip. CXCL8 WT peptide (KENWVQRVVEKFLKRAENS); E70K peptide (KENWVQRVVEKFLKRAKNS); or scrambled peptide (KVREKNEKWFVEQRVALNS) were studied. Data was analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. **P <0.01, ***P < 0.001. Representative data of three independent experiments (n=3), each performed in triplicate.

4.3.6.1.4 Neutrophil flow-based adhesion: the role of modulators of GAG binding or GPCR binding

The effect of the synthesised peptides was compared to the low molecular weight heparin, tinzaparin, in order to assess the LMWH's ability to displace chemokines from surface GAG, thus modulating neutrophil adhesion. LMWH tinzaparin was added to the HUVEC seeded biochip for 1 hour before the neutrophil adhesion assay.

In addition, the effect of peptides on GAG binding was correlated to the ability of small molecule CXCR1/2 chemokine receptor antagonists to interfere with neutrophil chemokine-GPCR binding, their effect was assessed after 1-hour incubation of primary neutrophils. Antagonists studied were reparixin (CXCR1/2 antagonist), SB225002 (CXCR1/2 antagonist), and SB265610 (CXCR2 antagonist). Cell viability in presence of the LMWH, or the antagonists was assessed by cell exclusion trypan blue. No significant cell death was observed (>80% viability).

CXCR1&2 chemokine receptor antagonists showed significant inhibition of flow-based neutrophil adhesion ($p<0.001$). An inhibitory effect was also observed via GAG binding using tinzaparin ($p<0.001$) (Figure 4-15). CXCL8 peptides, studied as part of the same experiment as LMWH and CXCR1/2 antagonists, modulated the cytokine or chemokine function but less than LMWH (WT peptide and scrambled peptide, E70K peptide: $p<0.01$). Thus, peptides may be a tool to better understand CXCL8 function during neutrophil-mediated inflammation or damage associated with ischaemia-reperfusion injury following transplantation.

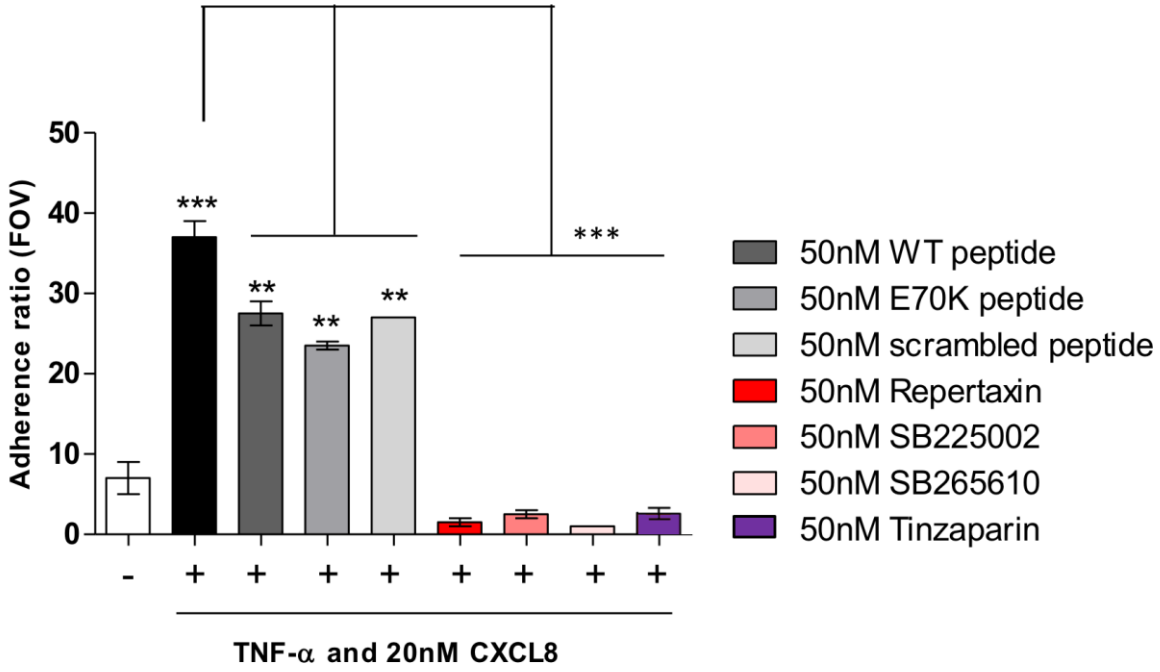


Figure 4-15. Neutrophil flow-based adhesion in response to different modulators.
Negative control is untreated HUVECs (fibronectin only). Positive control is TNF-α-stimulated HUVECs with 20nM CXCL8 (100µg/mL fibronectin, 1ng/mL TNF-α). CXCL8 (20nM) and CXCL8 peptide (50nM) were added over TNF-α-stimulated HUVECs. HUVECs were treated with LMWH tinzaparin at 50nM for 1hour before performing the assay. Neutrophils were treated with each CXCR1&2 antagonist at 50 nM for 1hour before the assay. Adherence ratio was obtained from average of 5 fields of view (FOV) per channel of chip. CXCL8 WT peptide (KENWVQRVVEKFLKRAENS); E70K peptide (KENWVQRVVEKFLKRAKNS); and scrambled peptide (KVREKNEKWFVEQRVALNS) (peptide data, shown in Figure 4-14, and LMWH and CXCR1/2 antagonists data were part of the same experiment, performed three independent days). Data was analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. **$P < 0.01$, ***$P < 0.001$. Representative data of three independent experiments (n=3), each performed in triplicate.

4.4 Discussion

Cellular characterisation showed CXCR1 and CXCR2 chemokine receptors are highly expressed at RNA level in both primary neutrophils and differentiated HL-60, although levels were higher in primary neutrophils. CXCR4 was expressed at similar level as CXCR1/2 in primary cells, and CXCR7 at a lower level. mRNA data was consistent with cell surface expression, as primary neutrophils expressed CXCR1, CXCR2, CD45, CD11b and CD66b. Thus, further *in vitro* static and dynamic studies were performed modelling primary neutrophil responses.

CXCL8 was confirmed to have a significant role in diffusion gradient neutrophil migration and in transendothelial neutrophil migration. CXCL1 showed, though not statistically, significant mediated migration which was not expected as it is a neutrophil chemoattractant. Further evaluation of the protein quality should be performed using e.g. silver gel stain or mass spectrometry, as it would help to confirm chemokine concentration. A range of 10 to 20nM CXCL8 was selected, due to its higher significance, for further functional studies of CXCL8 and of the truncated CXCL8 C-terminal region.

In the previous section, GAG binding was observed for all synthesised chemokine peptides. Higher binding was shown for the E70K peptide (KENWVQRVVEKFLKRAKNS) than for the WT peptide (KENWVQRVVEKFLKRAENS) or a scrambled of the WT peptide (KVREKNEKWFVEQRVALNS), which suggested the significance of positively-charged residues (E70K has overall charge +4, as opposed to charge +2 in WT or scrambled peptides) for specificity of GAG binding. In this section, peptide functional assays showed no interference with neutrophil GPCR binding, as peptides demonstrated no significant inhibition of CXCL8-mediated neutrophil calcium signaling. This was consistent with diffusion gradient neutrophil migration, where peptides did not show significant modulation of CXCL8-mediated migration. It might be of interest to confirm the lack of GPCR interference of peptides at higher concentrations by calcium signaling assay, even though it was shown by solute-diffusion chemotaxis. Studies in the presence of endothelial surface showed that synthesised chemokine peptides modulate neutrophil flow-based adhesion over TNF-α treated HUVECs although there was no major difference among CXCL8 WT peptide, E70K peptide, or scrambled peptide, ($p<0.01$). This shows that the intrinsic GAG binding ability of the peptides, short positively-charged sequences, decreases chemokine GAG-binding, hence integrin-mediated neutrophil adhesion. Transendothelial migration studies showed that only E70K peptide could induce a significant reduction in CXCL8 gradient-mediated neutrophil transendothelial migration ($p<0.001$) using 5 times the chemokine concentration, as opposed to WT peptide or scrambled peptide. This might be due to required additional positive charge in WT peptide or scrambled peptide to bind GAG, and so to interfere with the chemokine gradient formation during transendothelial chemotaxis. Thus, E70K-GAG binding ability is suggested to be associated with its positive charge or specificity to polyanionic GAG. Further biochemical and dynamic studies may help to maximally exploit the ability of chemokine peptides to modulate chemokine function.

Flow-based neutrophil adhesion following treatment of endothelial cells with unfractioned heparin was done to determine its role at displacing GAG-bound chemokine from the cell surface. No significant decrease in neutrophil adhesion was observed. Further analysis however, performed with low molecular weight heparin tinzaparin showed significant chemokine displacement and inhibition of flow-based chemokine-mediated neutrophil adhesion ($p<0.001$). Chemokine peptides might be helpful to avoid issue of LMWH accumulation e.g. during renal damage and facilitate resolution of inflammation by clearance.

Studies of CXCR1/2 chemokine receptor antagonism using reparixin, SB225002 or SB265610 induced significant inhibition of GPCR-chemokine binding as shown by significantly reduced neutrophil flow-based adhesion ($p<0.001$).

The function of synthesised peptides is therefore dependent on a significant binding to GAG. Also, it depends on the cell surface and ECM GAG expression which could be upregulated during inflammation, or altered as part of constant ECM rearrangements (Adage *et al.*, 2012). An alternative approach may involve additional substitution with positively charged amino acids within the GAG-binding region of CXCL8; or peptide folding to potentially increase peptide stability and GAG specificity, if a further impaired chemokine function during neutrophil-mediated injury is to be realised. A recent report showed that CXCL9 C-terminal (74-103) inhibits neutrophil migration *in vivo* through specific interference with GAG interactions (Vanheule *et al.*, 2017). Shortening CXCL9 (74-103) from the full-length chemokine C-terminal increases its specificity for particular GAGs, hence it is essential to know the specificity of GAG-binding peptides for development of leading biomarker candidates competing with chemokines for GAG to avoid potential side effects.

It is also worth noting that chemokine peptides are usually associated with intrinsic properties, such as a low toxicity, immunogenicity, rapid clearance and achievable significant interaction, which contributes to their increasing appreciation as potential candidates for novel drugs (Ezerzer *et al.*, 2009; Pamies and Hartung, 2017).

The application of microfluidics and tissue-on-chip technology in this study, aimed to mimic the inflammatory response. It was shown successful at reproducing leukocyte perfusion using physiological flow rate, as previously reported by pioneering investigations (Huh *et al.*, 2013; Jang *et al.*, 2013; Bhatia and Ingber, 2014). Studies using tissue/organ-on-chip approach

should be further exploited to maximise understanding of the organ physiology (lung, kidney, etc.) to improve patient's health, and to develop leading biomarker candidates.

This section illustrates the application of chemokine peptides as a molecular tool in immunobiological research, and as building blocks for synthetic biology. Taken together, this biochemical approach confirms previous findings on significance of GAG-CXCL8 binding. It also demonstrates that the chemokine peptides interfere with mediated neutrophil recruitment. It may have potential applications, in convergence with established therapeutic approaches and preservative technologies, to improve organ function and decrease neutrophil-mediated damage during ischaemia reperfusion injury, or during the inflammatory response associated with acute or chronic organ injury.

5. Regulation of Chemokine Function: Oxidative Stress and The Role of Post-translational Modifications

5.1 Introduction

Post-translational modifications (PTMs) can be involved in regulation of chemokine function. Examples of PTMs are proteolysis (Moelants *et al.*, 2013; Riva *et al.*, 2014), glycosylation (Stone *et al.*, 2017), nitration (Barker *et al.*, 2014; Thompson *et al.*, 2017) and citrullination (Loos *et al.*, 2008; Proost *et al.*, 2008; Loos *et al.*, 2009; Struyf *et al.*, 2009; Moelants *et al.*, 2014). Phosphorylation (Stone *et al.*, 2017) and sulphation (Tan *et al.*, 2013) are examples of chemokine receptor PTMs. The consequences of these modifications depend on the chemokine, tissue type or type of modification (Meissner *et al.*, 2015; Thompson *et al.*, 2017).

Reactive Oxygen Species and Reactive Nitrogen Species (ROS/RNS) play crucial physiological roles e.g. during signaling or can be formed as by-products of normal metabolism, but high levels can also be pathological (Pizzino *et al.*, 2017). RNS such as $ONOO^-$ have been described to contribute to organ damage, but also have some protective role associated with iNOS (Urtasun *et al.*, 2008).

Protein nitration can occur due to the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as peroxynitrite ($ONOO^-$). Peroxynitrite typically acts on tyrosine or tryptophan side chains. 3-nitrotyrosine (3NT) was used in this study as a marker of presence of RNS (Weber *et al.*, 2014). During injury, e.g. IRI; or infection, chemokine production can be higher. This milieu also correlates with high levels of ROS/RNS which can alter chemokine function. In addition, several human cancers including prostate, colon or liver produce RNS, as observed by strong expression of nitrotyrosine (Kasic *et al.*, 2011; Molon *et al.*, 2011). Investigations have shown that chemokine nitration impedes tumoural infiltration of antigen-specific T cells (Molon *et al.*, 2011). Hence, it is vital to detect the different forms of chemokines present (Chung and Lan, 2011; Barker *et al.*, 2014).

For instance, CCL2 undergoes nitration in response to macrophage activation, and nitrated CCL2 has reduced monocyte-binding and chemotactic function (Molon *et al.*, 2011; Barker *et al.*, 2017). Similarly, nitration of CCL5 attenuates its chemotactic activity (Sato *et al.*, 1999).

This chapter focuses on the role of nitration on the regulation of chemokine function, particularly of CXCL1 and CXCL8. As previously described, CXCL8 has one tyrosine (Y) and one tryptophan (W), whereas CXCL1 does not have either tryptophan nor tyrosine (Figure 5-1). As tyrosine and tryptophan are both residues susceptible to nitration by peroxynitrite, CXCL1 was used in our studies as negative control of chemokine nitration. Investigations were carried out to give evidence of the *in vivo* effect of cellular stress on protein nitration, chemokine nitration and CXCL8 production in tissues. Studies also evaluated the biological function of post-translational nitration of CXCL1 and CXCL8 on neutrophil adhesion and subsequent neutrophil transendothelial migration.

Figure 5-1. Sequence of CXCL1 and CXCL8, with reported GPCR and GAG-binding regions. Chemokine sequence of CXCL1 (P09341, PDB) and CXCL8 (P10145, PDB). As previously described, depicted are GPCR-binding sites (purple), GAG-binding sites (green), and residues involved in both GPCR- and GAG-binding (yellow).

5.2 Specific aims

Chemokine function is fundamental and plays a complex role during inflammation. Chemokines require tight regulation in order to mediate in the inflammatory response and also promote resolution. Post-translational modification of chemokines has a role in control of chemokine function and may regulate the inflammatory responses. Specific aims are:

- Detect protein nitration and CXCL8 expression *in vivo* in different tissue biopsies

- Produce, purify and validate an anti-nitrotyrosine-CXCL8 antibody from hybridoma cells
- Evaluate the biological function of nitrated CXCL1 and nitrated CXCL8

5.3 Materials and methods

5.3.1 Immunological evaluation of patient biopsies

Human tissue access was approved by Research Ethics Committee, Ref. -11/NE/0352. Informed consent was obtained from all subjects.

Formalin-fixed paraffin-embedded (FFPE) tissue sections from a diverse range of patient biopsies (Table 6) were dewaxed. Sections were deparaffinised by placing in xylene twice for 5 minutes. Then, sections were rehydrated by immersion in 99% ethanol twice, 95% ethanol, 90% ethanol and 75% ethanol for 20 secs each. Then, they were rinsed in tap water. Antigen retrieval was carried out using citrate buffer (pH6) for 2 minutes in pressure cooker. Sections were blocked for 30 minutes with 20% normal goat serum.

The range of patient biopsies studied correspond to normal kidney, damaged kidney, liver Primary Biliary Cholangitis (PBC), inflamed tonsil and normal placenta. Normal kidney, PBC liver, inflamed tonsil and normal placenta sections were kindly provided by Mrs Barbara Innes in collaboration with the Pathology Department (Royal Victoria Infirmary, Newcastle, UK). Normal Human Kidney (NHK) sections NHK0036 and NHK0040, which correspond to unaffected poles of tumour nephrectomies, were kindly provided by Dr Anna Moles and Professor Neil S. Sheerin (Institute of Cellular Medicine). NEVP kidney tissue sections were kindly provided by Dr Avinash Sewpaul (Institute of Cellular Medicine).

HUMAN TISSUE	DIAGNOSIS	
KIDNEY	Normal	Normal Human Kidney NHK0036, NHK0040 obtained from unaffected poles of tumour nephrectomies.
		Normal Human Kidney obtained from unaffected area of injured tissue
	Damaged	Normothermic *ex-vivo* perfused (NEVP) (case 137)
LIVER	Primary Biliary Cholangitis (PBC) 21368/05 1F	
	PBC R25359/03 1B	
	PBC 97/19355	
	PBC P33113/08 1A	
	PBC PR40197/14 2B	
TONSIL	Inflamed (mildly inflamed or abnormal)	
PLACENTA	Normal	

Table 6. Tissue cases and corresponding diagnosis.

5.3.1.1 Immunohistochemistry

In order to detect protein localisation and tissue morphology, immunohistochemistry (IHC) was used. After section dehydration, 0.3% hydrogen peroxide was used for 10 minutes to block any endogenous peroxidase activity when using HRP, followed by PBS wash for 5 minutes. Sections were stained sequentially with primary antibody for 1 hour at 4°C and secondary antibody for 2 hours at room temperature. Blocking was done with avidin-biotin when using primary biotinylated antibody. Primary mouse anti-nitrotyrosine monoclonal (1/100, ab61392) was detected using biotinylated anti-mouse secondary antibody from Vectastain Elite ABC HRP kit (PK-6102). Primary rabbit anti-CXCL8 polyclonal was selected using ab106350 (Abcam), AHC0881 (Thermo Scientific) or ab114500 (Abcam) at 1:50. Anti-CXCL8 was detected using anti-rabbit IgG secondary antibody. Slides were then treated from anti-rabbit Vectastain Elite ABC HRP kit, after being washed with TBS three times for 5 minutes. Then developed using Impact DAB substrate solution for 5 minutes. For double stained sections, 3-nitrotyrosine antibody was detected using ImPRESS-AP anti-mouse IgG polymer detection kit (MP-5402, Vector Labs). Sections were then counterstained with haematoxylin for 1 min and 20 secs, transferred to tap water and finally, dehydrated by immersion in 75% ethanol, 90% ethanol, 95% ethanol, and 99% ethanol twice for 5 minutes

each. Slides were mounted using DPX and left to air dry overnight. Analysis was performed using upright microscope (Leica Microsystems).

5.3.1.2 Immunofluorescence

In order to improve detection of protein localisation *in vivo*, immunofluorescence was used. Sections were stained sequentially with primary antibody for 1 hour at 4°C and secondary for 2 hours at room temperature. Primary mouse anti-nitrotyrosine PAb (1/100, ab61392) was detected using a goat anti-mouse DyLight 488 secondary antibody at 1/50 (ImmunoReagents), and primary rabbit anti-CXCL8 PAb (1/50; AHC0881 (Thermo Fisher Scientific)) was detected using a goat anti-rabbit Dylight 550 secondary antibody at 1/100 (ImmunoReagents). Slides were then incubated in 0.1% Sudan Black B in 70% ethanol for 20 minutes at room temperature, washed in PBS, and mounted using Vectashield mounting medium (Vector Labs). Co-localization analysis was performed using both primary antibodies. Analysis was performed using AxioImager microscope with Apotome (Zeiss).

5.3.2 Chemokine nitration

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 10min. Figure 5-2 shows amino-acid residues in CXCL8 previously reported to be susceptible of nitration or nitration/oxidation by reactive species (Nagai *et al.*, 2002; Pacher *et al.*, 2007).

N-terminal

SAKELRCQCIKTYSKPFHPKFIKELRVIESGPHCANTEIIVKLSDGRELCLDPKENWVQRVVEKFLKRAENS

C-terminal

Figure 5-2. CXCL8 sequence showcasing reported amino acids affected by oxidative stress. Residues susceptible of nitration (red) or of nitration/oxidation (blue) by reactive species.

5.3.3 Western Blot (WB)

Western Blot was carried out as earlier described in General Materials and Methods.

In order to identify nitrated protein, 0.5 µg of chemokine or nitrated chemokine were loaded per well on the SDS-PAGE gel. Bovine Serum Albumin (BSA), protein rich in tyrosine was used as positive control treated with peroxynitrite. Nitrated protein was detected using rabbit

anti-nitrotyrosine primary antibody (06-284, Millipore) at dilution 1:1000 for 2 hours, followed by anti-rabbit-HRP (Sigma) at 1:5000 for 2 hours.

Furthermore, in order to assess IgG type of clones produced in ascitic fluid to detect nitrotyrosine-CXCL8, once they showed specificity for their SPPS-synthesised peptide by Dot Blot, they were loaded on an SDS-PAGE gel. Protein was then transferred on to a membrane and probed with anti-mouse IgG-HRP at 1:5000 for 1 hour at room temperature to detect total IgG. Also, each clone was incubated with anti-mouse IgG1, IgG2a, IgG2b or IgG3 antibodies for 2 hours at RT, then incubated with goat anti-mouse HRP for 1hour at room temperature before developing.

5.3.4 Dot Blot

Blotting was performed as earlier detailed in General Materials and Methods. CXCL8 was studied using anti-CXCL8 polyclonal antibody (AHC0881) at 1:1000. Nitration was studied using anti-nitrotyrosine polyclonal antibody (9691, CST) at 1:1000.

5.3.5 Antibody purification

Purification of antibodies from clones, aimed to detect specifically nitrotyrosine CXCL8, was performed using HiTrap Protein G Sepharose column.

5.4 Results

5.4.1 *In vivo* detection of CXCL8 and 3NT in kidney biopsies

5.4.1.1 Optimisation of nitration staining in normal kidney tissue

In order to evaluate the effect of cellular stress on protein nitration, biopsies corresponding to normal kidney tissue were stained for 3NT. First, optimisation to select treatment for antigen retrieval used citrate, EDTA, trypsin or no treatment. Result showed both citrate and EDTA were suitable for antigen retrieval (Figure 5-3). Citrate was then used to study co-localisation with CXCL8, which also required citrate.

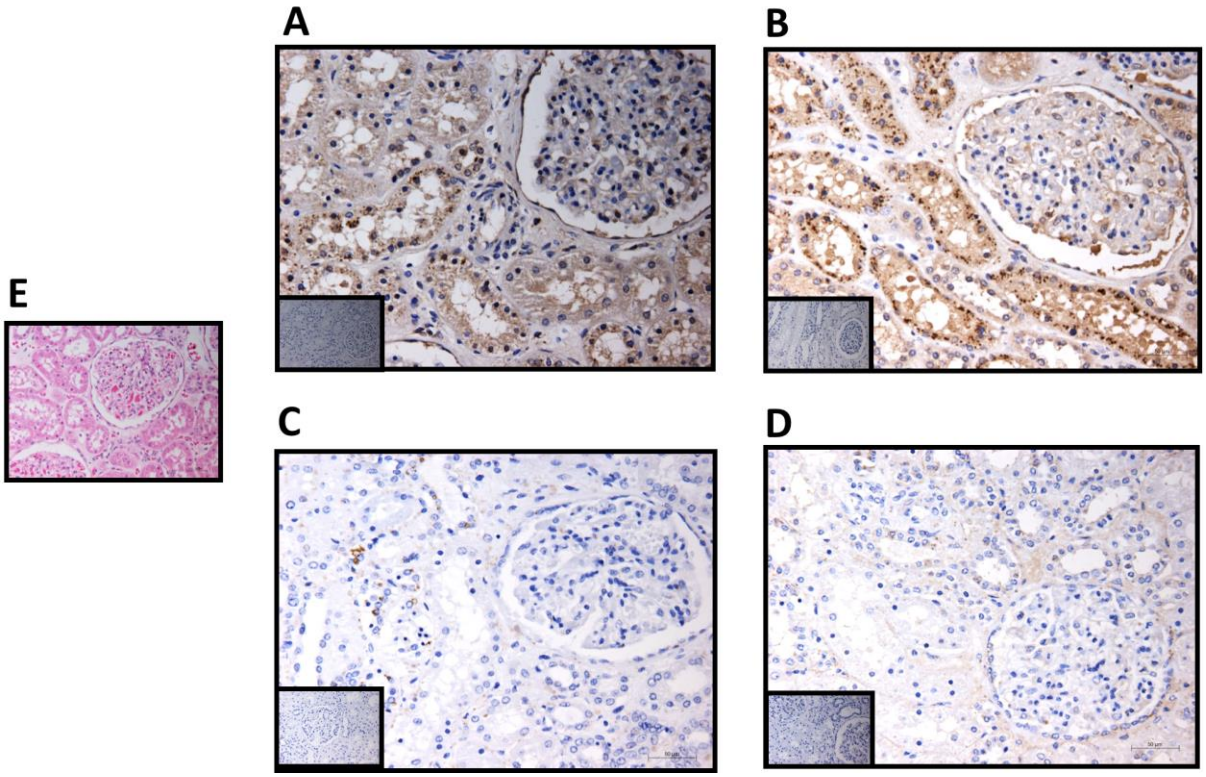


Figure 5-3. Immunohistochemistry optimisation for 3NT on paraffin-embedded sections of normal kidney biopsies.
Optimisation for antigen retrieval used citrate (A), EDTA (B), trypsin (C) or no treatment (D). Primary mouse 3-nitrotyrosine antibody (ab61392) was used at 1:100, and developed using Vectastain Elite ABC HRP kit (peroxidase, mouse IgG, brown). Boxes (in bottom left): No Primary Antibody (NPA). E: Haematoxylin and Eosin (H&E) staining for tissue visualisation. 20x magnification.

5.4.1.2 Optimisation of CXCL8 staining in normal placenta and inflamed tonsil

In order to evaluate the effect of cellular inflammation on chemokine production, biopsies from normal placenta were analysed for CXCL8 expression (Elliott *et al.*, 1998) (Figure 5-4). Optimisation of CXCL8 staining was performed and antigen retrieval using citrate was the most suitable method (data not shown), as earlier shown for 3-nitrotyrosine (Figure 5-3). Sections were positive for CXCL8 particularly on the cell surface (Figure 5-4A).

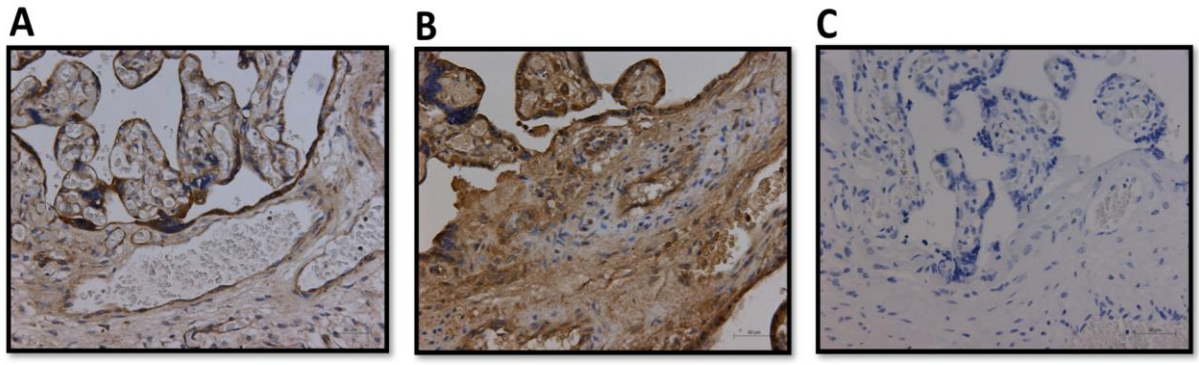


Figure 5-4. Immunohistochemistry optimisation for CXCL8 on paraffin-embedded sections of normal placenta biopsies.
Antigen retrieval was performed using sodium citrate buffer. CXCL8 primary antibody (A. ab106350, B. AHC0881 or C. ab114500) was used at 1:50, and developed using Vectastain Elite ABC HRP kit (peroxidase, rabbit IgG, brown). Antibody in A was selected for further assays. 20x magnification.

Next, other tissue types were also evaluated to optimise the detection of CXCL8. Inflamed tonsil also showed positive signal (Uhlen *et al.*, 2010), similar to normal placenta, (Figure 5-5). Clearer CXCL8 signal however, was shown in normal placenta (Figure 5-4). It would be of interest to compare signal with mouse control tissue, where CXCL8 is not expressed.

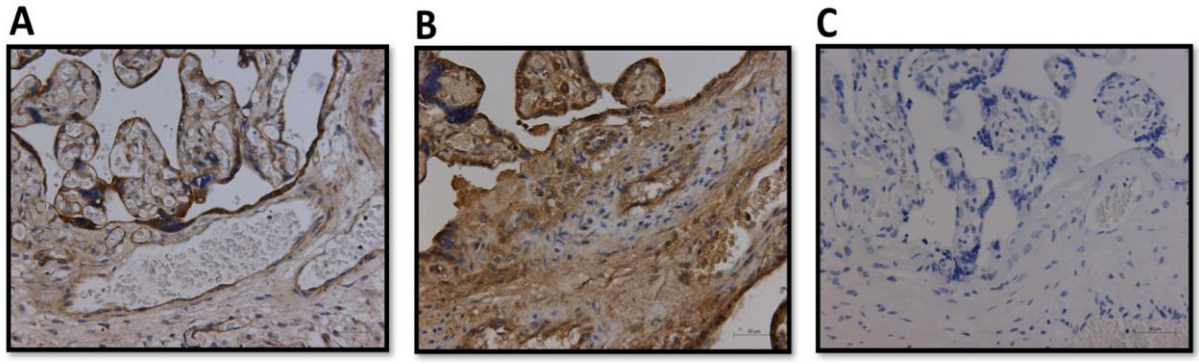


Figure 5-5. Immunohistochemistry optimisation for CXCL8 on paraffin-embedded sections of inflamed tonsil.
Antigen retrieval was performed using sodium citrate buffer. Each of the CXCL8 primary antibodies (A. ab106350, B. AHC0881 or C. ab114500) was used at 1:50, and developed using Vectastain Elite ABC HRP kit (peroxidase, rabbit IgG, brown). 20x magnification.

5.4.1.3 Detection by immunofluorescence of CXCL8 and 3NT in kidney tissue

5.4.1.3.1 Detection of CXCL8 and 3NT in different kidney tissues

In order to examine the effects of oxidative stress in chemokine expression and protein nitration *in vivo*, we used biopsy sections obtained from ECD ischaemic kidney after a time of preservation by NEVP immediately before transplantation. This was compared to unaffected poles of tumour nephrectomies. As earlier described, it is known that CXCL8 is present in normal kidney, particularly in the tubular area (Uhlen *et al.*, 2010). Similar levels of CXCL8 were observed in NEVP compared with normal kidney (Figure 5-6). 3NT appeared to be expressed at different levels and constitutively, particularly in the tubules. Data could be associated with previous report in which $ONOO^-$-induced damage was not different between Acute Tubular Necrosis (ATN) following ischaemia and normal kidney, whereas damage was increased in diabetic nephropathy (Thuraisingham *et al.*, 2000). Interestingly, NEVP kidney, with signs of tissue damage, appeared to have different 3NT levels compared to normal kidney from unaffected poles of nephrectomies, mainly within the tubular area (Figure 5-6). This could be explained by the physiological role of post-translational modification of proteins in the kidney.

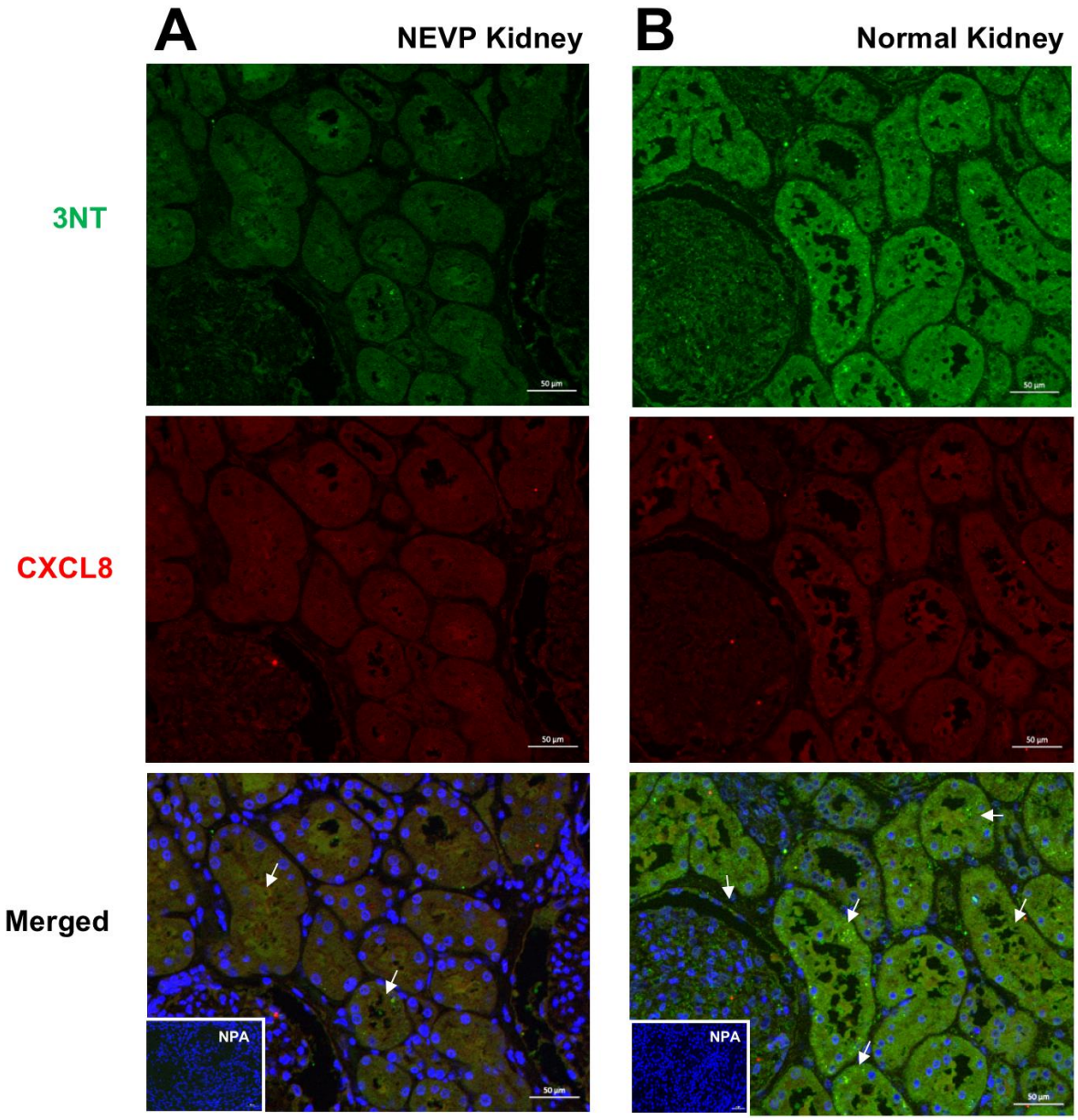


Figure 5-6. Nitration and CXCL8 observed in damaged kidney or in normal kidney.
Kidney biopsies from patients with different diagnosis were stained for CXCL8, 3NT and double-stained. Analysed biopsies are (A) Normothermic *ex vivo* perfused (NEVP) kidney. (B) Normal kidney with slight signs of inflammation. Arrows - protein nitration, primarily shown in tubules. Insert - No Primary Antibody. 20x magnification. Data is representative of two independent experiments (n=2).

5.4.2 Evaluation of chemokine nitration for CXCL8 and CXCL1

5.4.2.1 Western Blot analysis of the effect of peroxynitrate on chemokine function

In order to detect and evaluate the role of peroxynitrite on the chemokine function, we performed a qualitative assay to detect nitration of CXCL8 after peroxynitrite treatment. CXCL1, in contrast, has no tyrosine nor tryptophan so it was a negative control.

We detected nitrated CXCL8 (n-CXCL8), at approximately 10KDa. BSA, rich in tyrosine, was nitrated and used as positive control (n-BSA). In addition, signal was observed in n-CXCL8 lane at a higher molecular weight (>10 KDa). CXCL8 used in this assay had BSA as a carrier, which might explain the high molecular weight band in CXCL8 lane, also present in n-BSA. In contrast, no CXCL1 or n-CXCL1 was detected by 3-nitrotyrosine antibody as it lacks tyrosine (Figure 5-7) and has no carrier BSA. A band seen in CXCL8 lane, might be due to non-specific signal. Hence chemokines with no BSA (carrier-free, CF), were used in subsequent experiments to analyse the nitration.

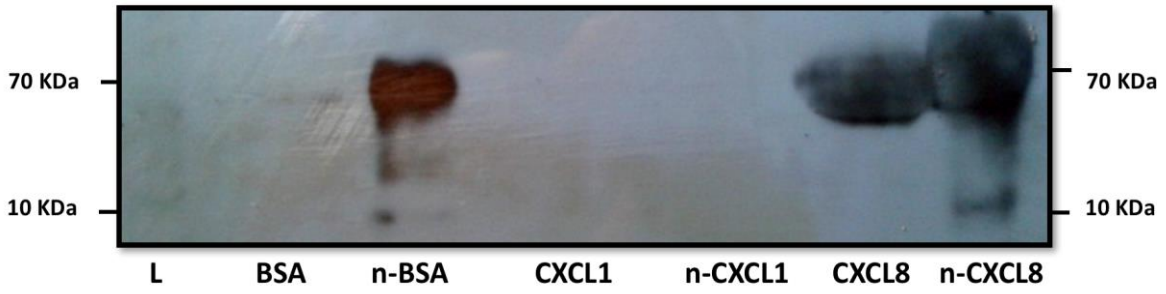


Figure 5-7. Initial visualisation of nitrated-tyrosine on CXCL8.

Chemokines CXCL1 (no BSA-conjugated) and CXCL8 (BSA-conjugated) (approx. 10KDa) were used. Peroxynitrite (ONOO-) was used to induce chemokine nitration. 0.5µg chemokine was treated with a final concentration of 1mM peroxynitrite in PBS, for 10 minutes at 37°C. 12% SDS-PAGE gel was used to run the protein for 90 minutes. Rabbit 3-nitrotyrosine primary antibody (1:1000, 2 hour) and anti-rabbit-HRP secondary antibody (1:5000, 2 hour) were used to detect nitrated tyrosine. BSA is rich in tyrosine, so nitrated BSA (n-BSA) was used as positive control. BSA, CXCL8 and CXCL1 were used as negative controls. Note that signal on n-CXCL8 higher than 10KDa may be due to BSA present as a carrier protein. Nitrated chemokine: n-CXCL. L: Ladder. Data obtained from one experiment.

5.4.2.2 Dot Blot analysis of the effect of peroxynitrate on chemokine function

In order to confirm the possibility of detection of n-CXCL8, we assessed samples by Dot Blot. Polyclonal antibodies against CXCL8 and nitrotyrosine were used.

Figure 5-8 shows detection of both CXCL8 and n-CXCL8 using anti-CXCL8 polyclonal antibody, whereas no signal for nitrated CCL2, used as a negative control. This suggests the specificity of the antibody. The lower blot in Figure 5-8 shows detection of nitrated chemokine using anti-nitrotyrosine polyclonal antibody. Nitrated CXCL8 is clearly detected, and nitrated CCL2, even though at a low level, was positive. CXCL8 was used here as a negative control. Importantly, further evaluation of the potential protein degradation by nitration should be performed using e.g. silver gel stain or mass spectrometry, as it would help to confirm both protein concentration and protein nitration.

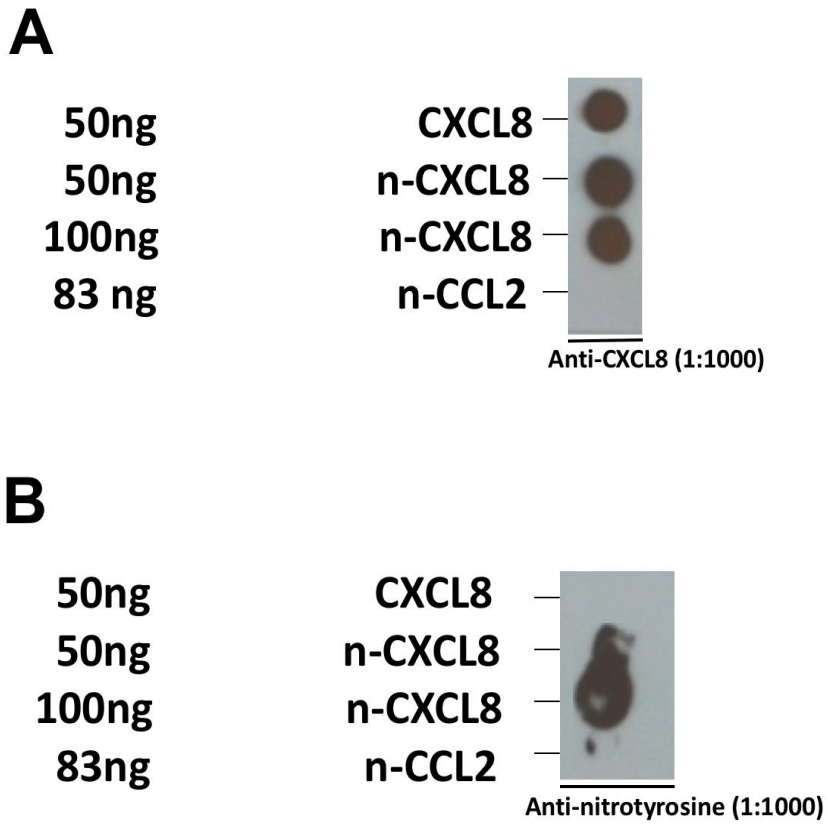


Figure 5-8. Dot Blot detection of CXCL8 and nitrotyrosine using polyclonal antibodies.
A) Detection of CXCL8 (50ng), and n-CXCL8 (50ng, 100ng) by anti-CXCL8 PAb at 1:1000. No signal for n-CCL2 (83 ng), negative control, shows specificity. B) Detection of nitrated chemokine using anti-nitrotyrosine PAb (dilution 1:1000) for n-CXCL8 (50ng, 100ng), and with a low signal for n-CCL2. CXCL8 was negative control. Representative data of two independent experiments (n=2).

5.4.2.3 Analysis of nitrotyrosine-CXCL8

In order to generate a monoclonal antibody to specifically detect nitrated CXCL8, two different sequences within CXCL8 covering the region of Tyr13 were designed (Table 7). The antibody was produced in collaboration with Abmart Inc. (Shanghai). The validation of the ascites samples for antibody was carried out by Dot Blot at Abmart Inc. We then performed Dot Blot, immunostaining and IgG immunoblotting evaluation studies.

5.4.2.3.1 Ascitic clone production and validation for nitrotyrosine-CXCL8

CXCL8 peptides for production of clones for nitrotyrosine-CXCL8 are shown in Table 7.

	CONTROL	**NO_2-PEPTIDE**
PEPTIDE REGION 1	QCIKTYSKP	QCIKTY**(NO2)**SKP
PEPTIDE REGION 2	IKTYSKPFHPC	IKTY**(NO_2)**SKPFHPC

Table 7. Selected CXCL8 regions for production of clones against nitrotyrosine-CXCL8.

Two CXCL8 peptide regions in nitrated form (NO_2-PEPTIDE), synthesised by Fmoc SPPS, were selected for mouse immunisation in order to produce clone of monoclonal antibody against nitrotyrosine-CXCL8. Nitrated peptides are later named peptide 1 and peptide 2, respectively.

Five clones (C1, C2, C4, C17 and C26) were obtained from ascitic fluid of mice injected with CXCL8 peptide region 1 or region 2. Clones for detection of nitrotyrosine-CXCL8 initially tested at Abmart Inc by Dot Blot are shown in Figure 5-9. Data shows all clones detect their specific target peptide at 1:1000 dilution at different detection limits.

Project ID	Target protein	Product Name	The Epitope Identification/Peptide sequence	product type	Detection limit(ng)	Data*
20377-1	IL8	20377-1-1/C1-S_15020	Ac-QCIKTY(NO2)SKP-NH2	Ascites	0.01ng	
20377-1	IL8	20377-1-1/C2-S_15020	Ac-QCIKTY(NO2)SKP-NH2	Ascites	0.25ng	
20377-1	IL8	20377-1-2/C17-D_1502	Ac-IKTY(NO2)SKPFHPC-N	Ascites	5ng	
20377-1	IL8	20377-1-2/C26-S_1502	Ac-IKTY(NO2)SKPFHPC-N	Ascites	1ng	
20377-1	IL8	20377-1-2/C4-D_15021	Ac-IKTY(NO2)SKPFHPC-N	Ascites	1ng	

*Peptide amount of antigen in every line is 25ng,5ng,1ng,0.25ng,0.05ng and 0.01ng

Figure 5-9. Produced clones and validation data from Abmart Inc.
Report shows the two designed CXCL8 peptide sequences as 1 and 2 (as earlier indicated, in Table 7) and five clones, with product name: C1, C2, C4, C17 and C26, raised to detect nitrotyrosine-CXCL8.

Validation studies performed in house used peptide alone bound to a PVDF membrane, instead of conjugated BSA-SMCC peptide (Abmart Inc. protocol), which meant that more peptide was required to be shown by the corresponding clone. C1 and C4 were positive for their specific peptides (Figure 5-10). Clones C2, C17 and C26 showed background or no signal (data not shown) so were not studied any further.

To validate specificity, clones C1 and C4 were first tested for detection of nitrated CXCL8 compared to CXCL8 by Dot Blot. Both clones C1 and C4 detected nitrated CXCL8, although with a faint signal. One of the samples of n-CXCL8 was also heated at 96°C for 15 minutes to induce degree of unfolding in the chemokine structure, in order to facilitate resemblance to the peptides generated to raise the clones, which are unfolded. Heated n-CXCL8 showed similar signal to n-CXCL8. Clones showed no signal for CXCL8, used as negative control. Also, no signal was observed for n-CCL2, which suggested some specificity (Figure 5-10).

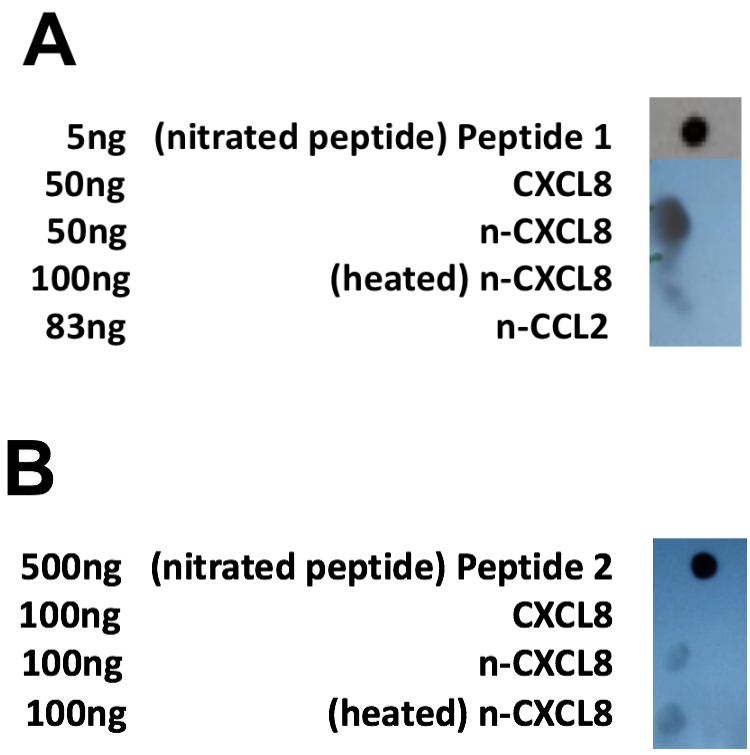


Figure 5-10. Validation of clones for specific detection of nitrotyrosine-CXCL8 by Dot Blot. Clones C1 (A) and C4 (B) were assessed at 1:1000 (1mg/mL stock) by Dot Blot. They specifically detected peptide 1 or peptide 2, respectively, used as positive controls. For clone C1, 50ng and 100ng n-CXCL8 were used. 100ng n-CXCL8 were used for clone C4, as Dot Blot titre was higher according to Abmart Inc. validation data (Figure 5-9). Heated n-CXCL8 refers to sample heated at 95°C. CXCL8 and n-CCL2 were used as negative control. Representative data from three independent experiments (n=3).

5.4.2.3.2 Validation of clones C1 and C4 for detection of nitrotyrosine-CXCL8 by immunohistochemistry

In order to investigate clones C1 and C4 for their ability to detect nitrotyrosine-CXCL8 *in vivo*, immunohistochemistry was performed, using liver biopsies from patients diagnosed with Primary Biliary Cholangitis (PBC) and different kidney biopsies. Initial optimisation using paraffin-embedded liver tissue samples was performed with different antigen retrieval methods: Citrate, Trypsin, EDTA, or no treatment, and two dilutions of antibody (1:50 and 1:200) (data not shown). Optimisation showed EDTA antigen retrieval and antibody dilution 1:200 to be most appropriate for both clones (Figure 5-11). Signal was apparently non-specific for each C1 and C4 on PBC tissue compared to negative control. PBC staining was confirmed by study of C4 and CXCL8 on another available liver PBC tissue case, which

showed that CXCL8 was not significantly expressed, and C4 appeared to show no specificity (Figure 5-11).

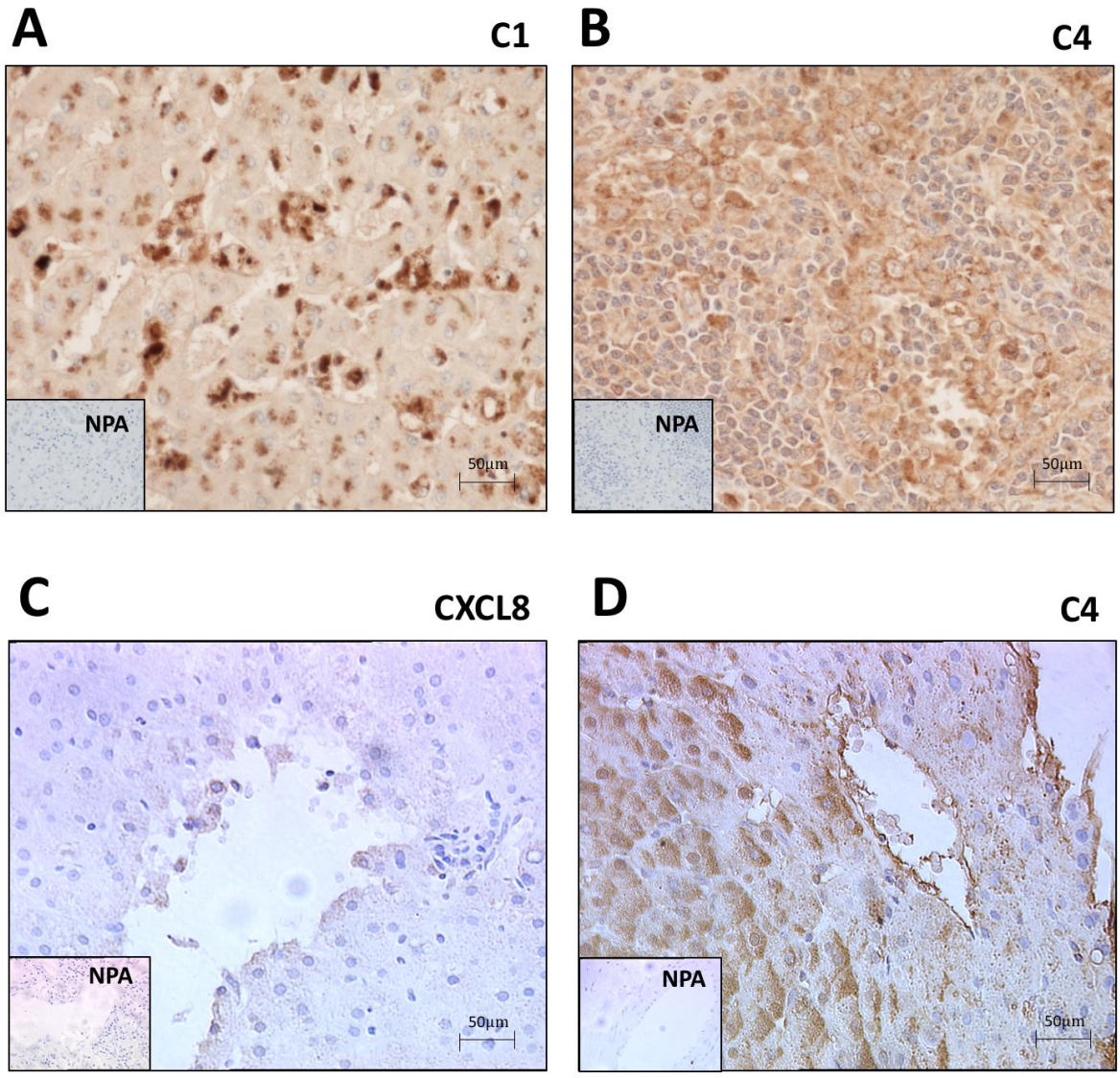


Figure 5-11. Immunohistochemistry of liver PBC sections for nitrotyrosine-CXCL8 using clones C1 or C4.
Liver PBC sections correspond to two different PBC cases (case 1: A & B, case 2: C & D). EDTA was selected for antigen retrieval after optimisation. Clones C1 (A) and C4 (B, D), and anti-CXCL8 PAb (C) were used at 1:200. C1 and C4 showed non-specific signal and background. C) CXCL8 was studied using polyclonal anti-CXCL8 antibody, showing no significant signal, and otherwise lower than for clones. Immunostaining was performed by Mrs Barbara Innes and Mrs Nicola Townshend, in collaboration as the secondment at Durham University was at the same time. 40x magnification.

Similar pattern of strong signal from C4, with apparent no specificity, was observed in renal tissue biopsy from patient diagnosed with Acute Tubular Necrosis (ATN) (Figure 5-12).

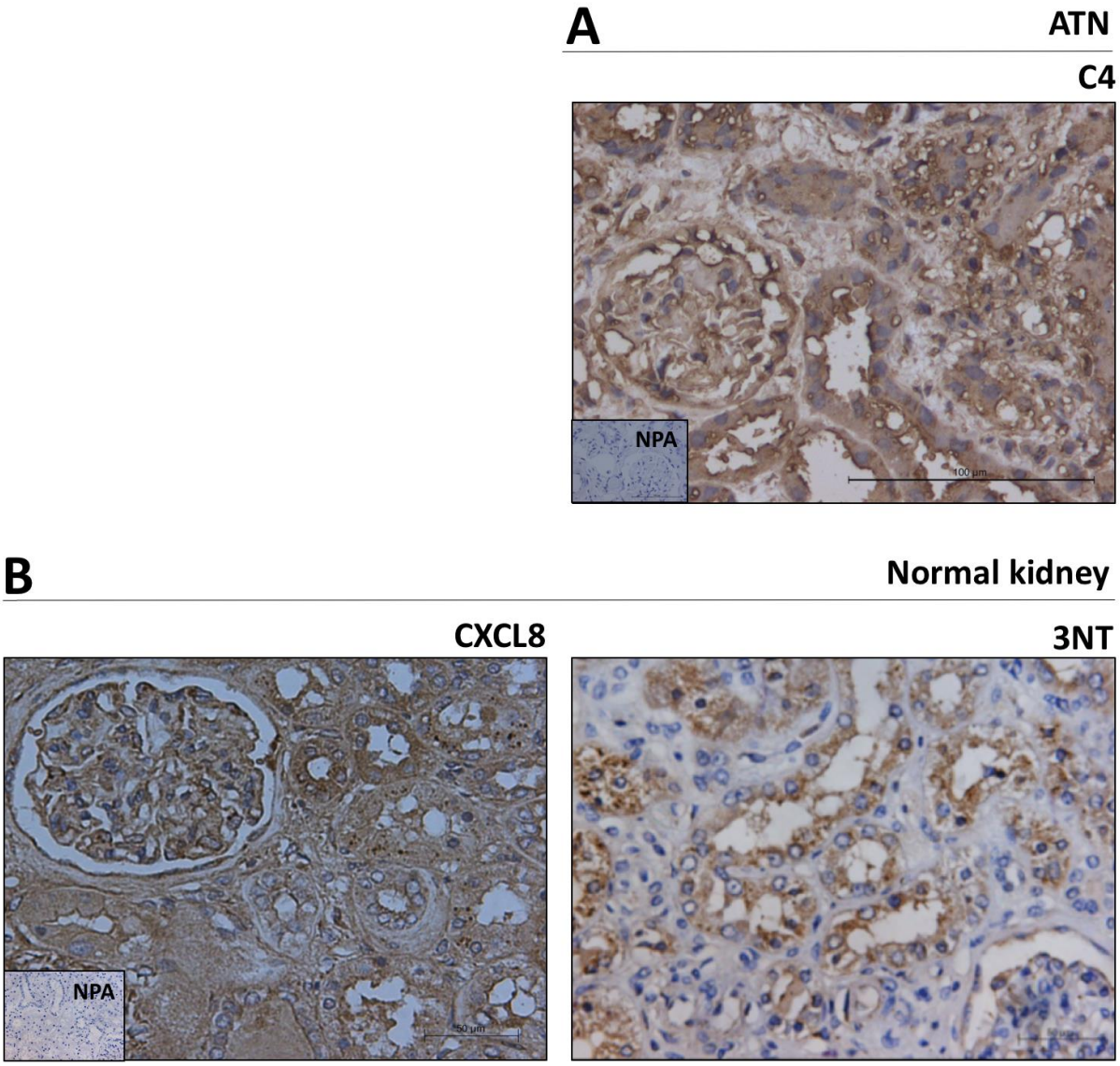


Figure 5-12. Immunohistochemistry of a renal biopsy with ATN for nitrotyrosine-CXCL8.
A) Antigen was retrieved by EDTA using clone C4 at 1:200. C4 signal in ATN showed non-specific staining. 40x magnification. B) In a normal kidney tissue, antigen retrieval was performed using citrate buffer for 3NT and for CXCL8. CXCL8 signal is observed in the glomerular tuft, proximal tubular cells and in some cells in the insterstitium. 3NT signal is mainly in tubules. CXCL8 antibody (ab106350) was used at 1:50, and developed with Vectastain Elite ABC kit (peroxidase, rabbit IgG, brown). 3NT (ab61392) was used at 1:100, and developed with Vectastain Elite ABC kit (peroxidase, mouse IgG, brown). 20x magnification. Data is representative of two independent experiments (n=2).

Since IHC and Dot Blot data showed no specificity of clones C1 and C4 for nitrotyrosine-CXCL8, IgG immunostaining was next performed to determine their IgG type.

5.4.2.3.3 IgG immunoblotting validation of clones C1 and C4

Clone C1 and C4 were further evaluated for their IgG type by immunoblotting studies performed in collaboration with Dr Jeremy Palmer (Institute of Cellular Medicine). Validation data showed that clones, produced to detect specifically nitrotyrosine-CXCL8 were polyclonal. Clone C1 appeared to be positive for IgG1 and IgG3 (Figure 5-13A), and clone C4 showed to be positive for IgG1, IgG2a, IgG2b and IgG3 (Figure 5-13B). Previous reports suggest that hybridoma enrichment and recloning (e.g. stemcell technologies), or alternatively development of a phage-display library could help to enhance cloning efficiency and achieve required specificity (McKinney *et al.*, 1995; Imai *et al.*, 2011).

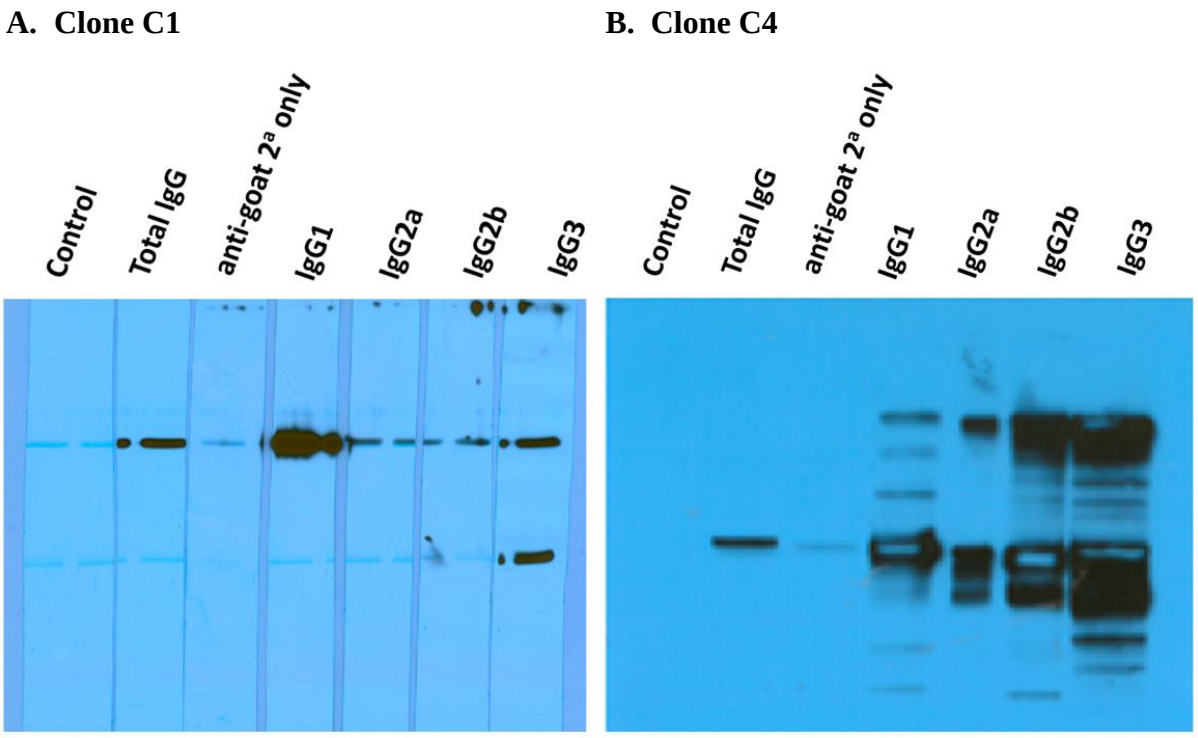


Figure 5-13. IgG immunoblotting of clones C1 and C4 shows polyclonal antibodies.
Each clone C1 or C4 was loaded, and then anti-mouse IgG-HRP was added at 1:5000 for 1 hour at room temperature to detect total IgG. IgG has a molecular weight of about 150kDa. Also, each C1 and C4 were incubated with anti-mouse IgG1, IgG2a, IgG2b or IgG3 antibodies for 2 hours at room temperature, and then incubated with goat anti-mouse for 1hour at room temperature before film development. Immunoblotting was performed in collaboration with Dr Jeremy Palmer (Institute of Cellular Medicine). Data is representative of one experiment.

Previously in this chapter, the detection of post-translational nitration of proteins by Reactive Nitrogen Species during inflammation was assessed *in vivo*. Next section addresses the effect of post-translational nitration of chemokines by Reactive Nitrogen Species *in vitro* by static and flow-based studies.

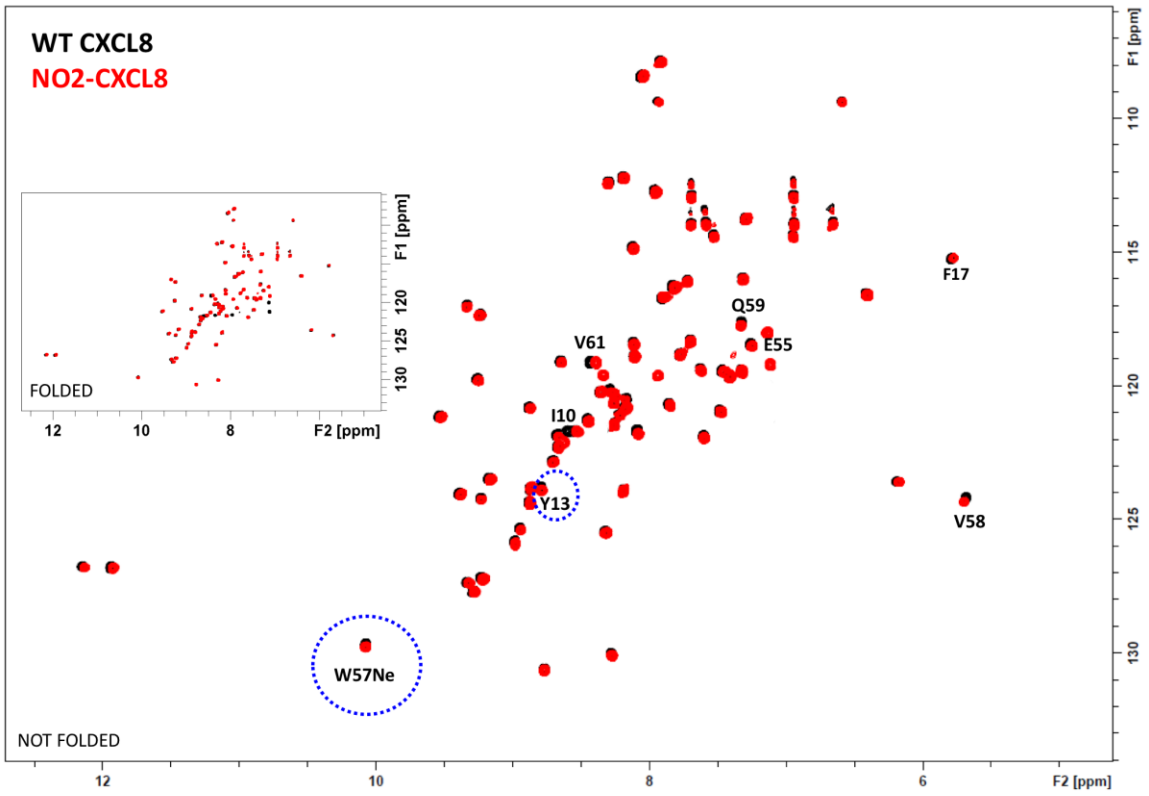
5.4.3 Nuclear magnetic resonance (NMR)

5.4.3.1 Study of the role of chemokine nitration in the chemokine structure

5.4.3.1.1 Structure of CXCL8 or nitrated CXCL8

CXCL8 structure was studied by NMR and compared to nitrated CXCL8 to determine whether nitration has a significant influence on the chemokine structure. Studies were performed in collaboration with Professor Krishna Rajarathnam's group (Texas University Medical Branch, USA). NMR of CXCL8 and nitrated CXCL8 showed that spectra are superimposable indicating essentially no change in structure (Figure 5-14). Spectra of nitrated chemokine shows that tyrosine Y13 and tryptophan W57 can be nitrated (Figure 5-14A). Data on tyrosine nitration is consistent with previously shown western blot data. Also, data correlates with mass spectrum of nitrated CXCL8 which shows that the chemokine can undergo single or double nitration (data not shown). Thus, the nitrated chemokine has net negative charge that affects its local environment. Chemical shift perturbations (CSPs) occur in the vicinity of nitrated residues (I10, F17, E55, V58, Q59, V61) (Figure 5-14B). It is worth noting that a few of the peaks are not superimposed. Those may arise from side-chain arginine. Overall, data shows that any altered function on CXCL8 could be due to nitration, not to global structural changes (Figure 5-14).

a)



b)

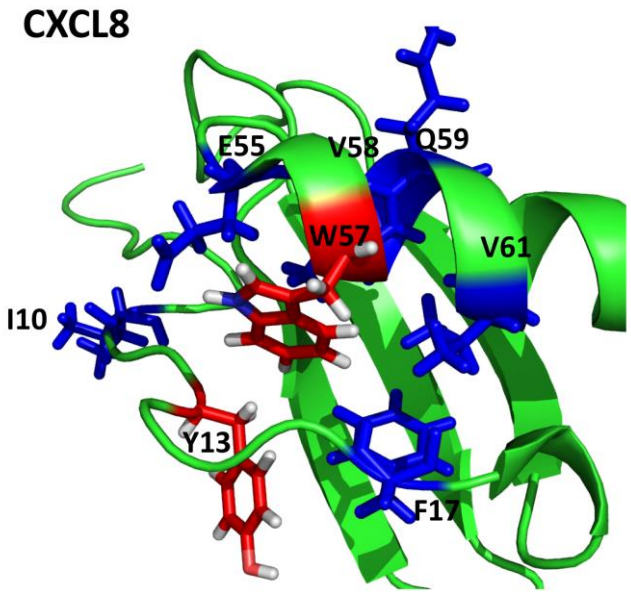


Figure 5-14. NMR of CXCL8 versus nitrated CXCL8.

a) 1H-15N HSQC spectra shows structure of CXCL8 (black) compared to that of nitrated CXCL8 (red). It shows that most of the peaks are superimposed, indicating that nitration does not alter chemokine structure. b) Ribbon presentation of CXCL8 structure. Residues that can be modified are in red (Y13, W57). Residues that show chemical shift perturbation are in blue. NMR CXCL8 experiment was performed by Prof Krishna Rajarathnam's group (Texas University, USA).

5.4.3.1.2 Structure of CXCL1 or nitrated CXCL1

Studies were also performed to analyse the effect of nitration on CXCL1 structure. Data showed that nitration did no significantly change CXCL1 structure (Figure 5-15).

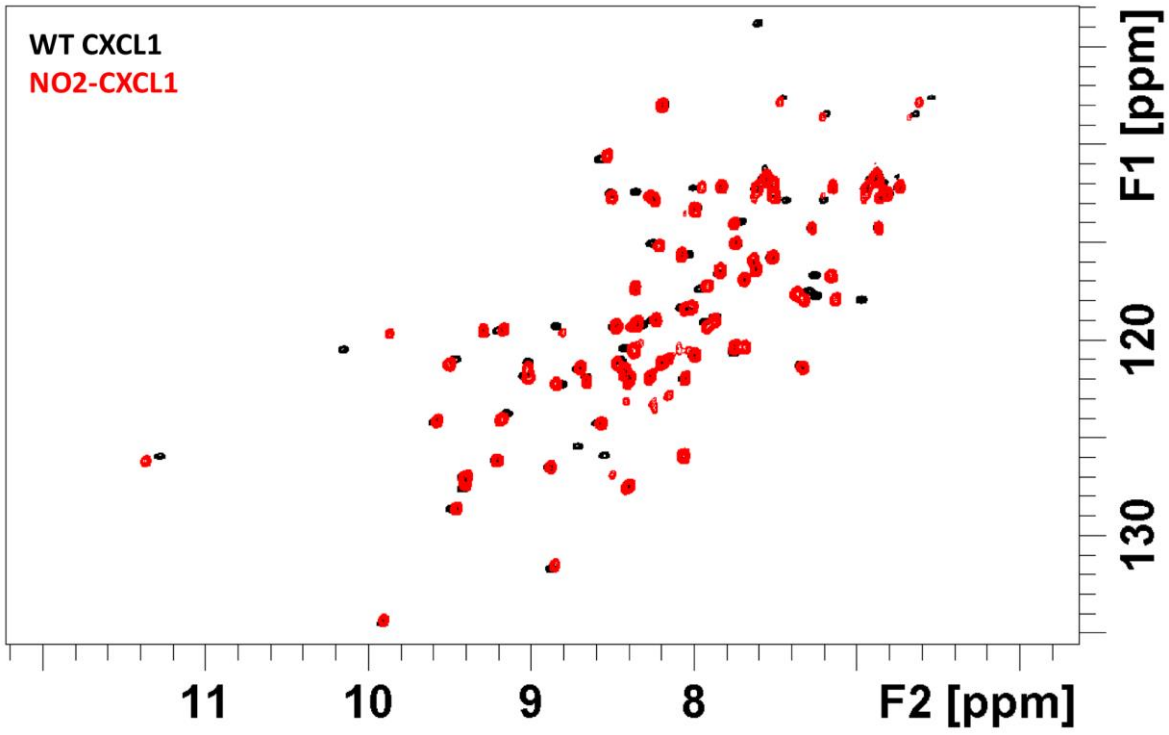


Figure 5-15. NMR of CXCL1 versus nitrated CXCL1.

1H-15N HSQC spectra shows folded structure of CXCL1 (black) compared to that of nitrated CXCL1 (red). It shows that most of the peaks are superimposed, indicating that nitration does not alter chemokine structure, suggesting that any changes in CXCL1 function are due to nitration. NMR CXCL1 study was also performed at Prof Krishna Rajarathnam's laboratory (Texas University, USA).

5.4.4 Neutrophil chemotaxis directed by CXCL8 or nitrated CXCL8

5.4.4.1 Diffusion gradient chemotaxis

In vitro studies to investigate the effect of post-translational nitration on CXCL8 function were performed by analysis of neutrophil migration through diffusion gradient chemotaxis and transendothelial chemotaxis.

Diffusion gradient chemotaxis of primary neutrophils was significantly decreased when CXCL8 underwent nitration by peroxynitrite ($p<0.05$) (Figure 5-16). As earlier indicated, evaluation of potential protein degradation by nitration using silver gel stain or mass spectrometry would help to determine whether protein concentration used is well preserved.

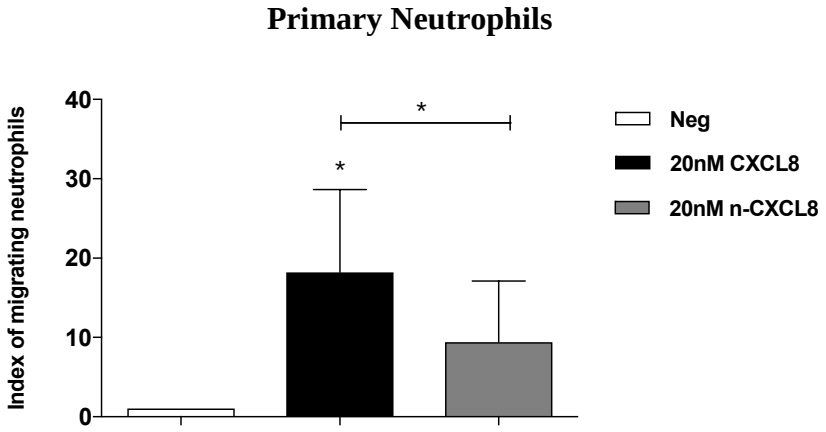


Figure 5-16. Neutrophil diffusion gradient chemotaxis in response to CXCL8 versus n-CXCL8. CXCL8 or n-CXCL8 (20nM) was added to the transwell bottom chamber. Chemokine was nitrated using a final concentration of 1mM peroxynitrite per 1µM chemokine at 37°C for 10minutes. Media only was used as negative control. 250,000 cells were added on the transwell top chamber and incubated at 37°C for 90 minutes. Cell count was done using haemocytometer. Index of migrating cells is relative to negative control, Index= (Sample/Negative). Data was analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. *$P < 0.05$ on black column indicates significant neutrophil migration in response to chemokine compared with negative control. Data is representative of two independent experiments (n=2), each performed in triplicate.

5.4.4.2 Transendothelial chemotaxis

Transendothelial chemotaxis was performed to look at the role of CXCL8 nitration in neutrophil migration through an endothelial layer. CXCL8 post-translational nitration significantly decreased neutrophil transendothelial migration ($p<0.01$). Thus, data confirmed chemokine nitration as a negative regulator of neutrophil migration, as earlier described for other chemokines such as CCL2 or CCL5 (Figure 5-17).

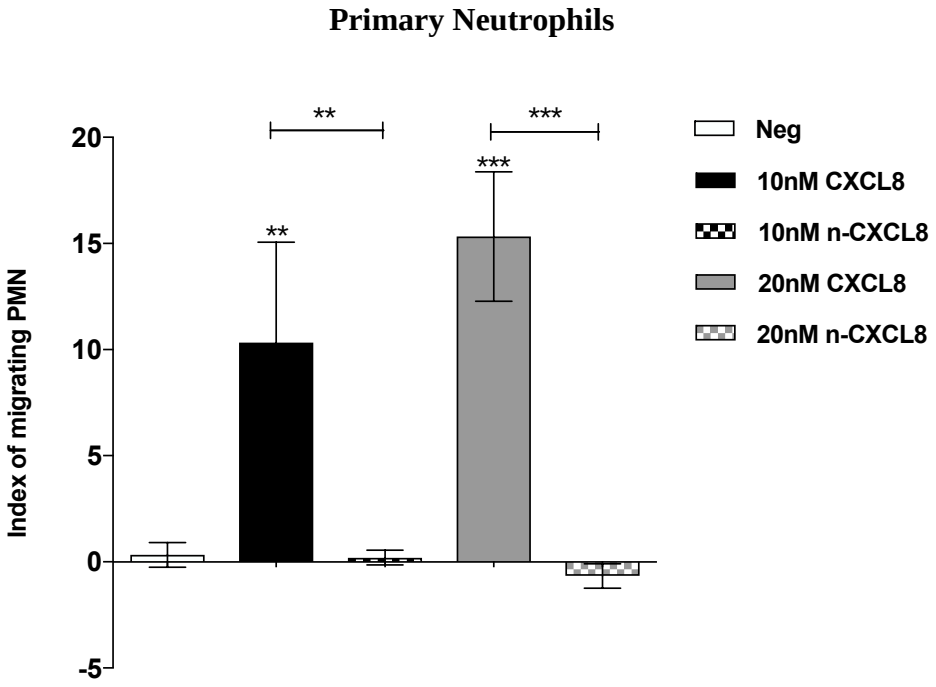


Figure 5-17. Neutrophil transendothelial chemotaxis in response to CXCL8 versus n-CXCL8. HMEC-1 layer was generated by incubation of HMEC-1 cells on the insert 3-4 days before the assay. CXCL8 or n-CXCL8 was added to the transwell bottom chamber. Media only was used as negative control. 250,000 cells were added on the top chamber and incubated at 37°C for 90 minutes. Cell count was done using haemocytometer. Index of migrating cells is relative to negative control, Index= (Sample/Negative). Data was analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. **$P < 0.01$, ***$P < 0.001$. Data is representative of two independent experiments (n=2), each performed in triplicate.

5.4.5 Neutrophil flow-based adhesion directed by CXCL8 or nitrated CXCL8

An *in vitro* flow-based adhesion assay was performed to assess the ability of nitrated CXCL8 to regulate neutrophil adhesion compared to the effect of CXCL8. Flow-based adhesion of primary neutrophils directed by nitrated CXCL8 was significantly diminished ($p<0.001$) compared to CXCL8 response (Figure 5-18). This is in agreement with previously reported data where nitrated CXCL8 decreased neutrophil transendothelial migration ($p < 0.001$).

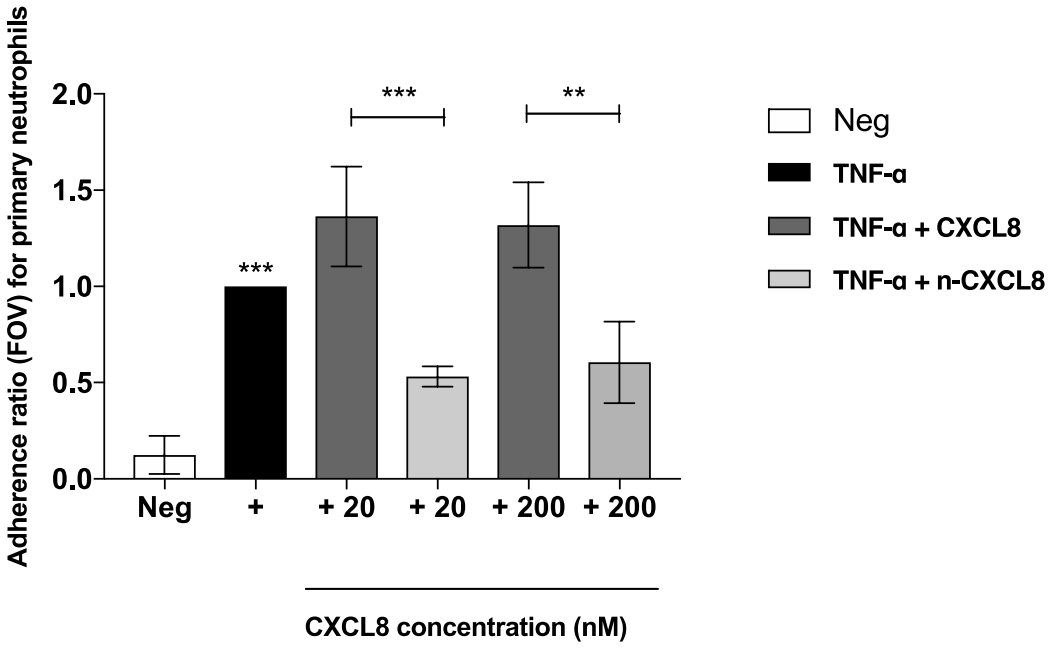


Figure 5-18. Neutrophil flow-based adhesion in response to CXCL8 versus n-CXCL8.
Negative control is untreated HUVECs (100µg/mL fibronectin only). Positive control is TNF-α-treated HUVECs (10ng/mL TNF-α treatment over HUVECs). CXCL8 treatments (20nM, 200nM) were done over TNF-α-stimulated HUVECs. Adherence ratio is obtained from average of five standard fields of view (FOV) per biochip channel, and subsequent normalisation to positive control. Data was analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. **P <0.01, ***P < 0.001. Representative data of three independent experiments (n=3), each performed in triplicate.

5.5 Discussion

Investigations in this chapter focused on post-translational nitration of CXCL1 and CXCL8, and on chemokine production during the inflammatory response. Chemokines CXCL1 and CXCL8, two prototypical neutrophil chemoattractants, were studied as they are expressed in various cell types, particularly CXCL8 during diverse inflammatory situations such as during renal transplantation (Chiao *et al.*, 1997; Tabary *et al.*, 1998; Guan *et al.*, 2016). The study was designed to enhance understanding of the role of chemokine nitration on the regulation of chemokine function during inflammation.

Firstly, *in vivo* evaluation of a range of biopsies corresponding to different tissue types showcased the physiological role of post-translational nitration of proteins in the kidney. Studies suggested an association between protein nitration and stress-induced inflammation or grade of injury. This association was not further studied due to constrains of time and a

highly tailored access to tissue biobank. Hence, it might be clarified by further evaluation of diabetic nephropathy models (Thuraisingham *et al.*, 2000), or different time point of ischaemic injury, or acute damage (Chiao *et al.*, 1997; Tabary *et al.*, 1998; Guan *et al.*, 2016).

The role of inflammatory chemokines in renal injury is highly dependent on the type of chemokine. Data in this study showed that CXCL8, neutrophil chemoattractant known to be expressed in renal physiological conditions, is in fact expressed at comparable level in normal and injured kidney.

Functional studies of post-translational nitration of chemokines by NMR showed that CXCL8 nitration has no significant effect on the chemokine structure but creates a net negative charge in the chemokine. CXCL8 can be nitrated in Y13, and potentially in W57, and this has an effect on the chemokine local environment and reduces neutrophil adhesion and migration. CXCL1 nitration did not affect its function and showed no statistically significant decreased neutrophil migration compared to native chemokine. CXCL8 nitration acts therefore as a negative regulator of chemokine function. Data correlate with previously reported studies on nitration of CCL2, which showed that chemokine nitration significantly dampens CCL2-mediated leukocyte recruitment *in vitro* and *in vivo* (Molon *et al.*, 2011; Barker *et al.*, 2017). According to chemokine migration data on nitrated CXCL8, chemokine nitration might have profound consequences in organ function. Better understanding of chemokine nitration should be maximally exploited to define its role during the acute and chronic inflammatory response that may lead to organ fibrosis, and thus to define ways to ameliorate the damage.

As earlier reported, nitration of other proteins has also suggested to act as a negative regulator e.g. for phosphatase A2 (PPA2) (Deng *et al.*, 2016). Report demonstrated that blockage of PPA2 nitration, using a high-performance peptide-based drug, impedes Endothelial-Mesenchymal Transition (End-MT) that can contribute to renal fibrosis, as demonstrated by a mouse model of unilateral ureteral obstruction (UUO). The role of protein nitration could therefore, respond to a "switch-off" mechanism in the protein regulation during inflammation aimed to turn on a regulatory process that can lead to homeostasis (Thompson *et al.*, 2017).

Stress-induced protein changes e.g. in chemokines are increasingly studied, vital to understand the inflammatory response e.g. during transplantation. This is because chemokines, key players in inflammation, do not provide the whole picture, for instance to

determine organ function during different stages of transplantation or how organ rejection may occur. Investigations have shown that post-translational modified proteins may be more biologically significant than absolute protein levels (Barker *et al.*, 2014). Post-translational modifications of chemokines may change chemokine structure, or chemokine function or even impede detection of the chemokine, limiting the potential of some immunochemical or proteomic biomarkers (Molon *et al.*, 2011). Hence, development of functional assays, such as antibodies to detect a specific chemokine modification, mass spectrometry or nano-HPLC to investigate chemokines in patient samples, could help to maximally exploit knowledge of the chemokine regulation and its therapeutic implications.

Clones were produced to specifically detect nitrotyrosine-CXCL8. Clones C1 and C4 showed to successfully detect their specific peptide. Validation for detection of nitrated CXCL8 using clones C1 or C4 showed no clear specificity by Dot Blot. IHC studies of liver PBC and ATN tissue sections were consistent with blot data, as neither clone C1 nor C4 showed specific signal but significant background. Clones probed they are polyclonal by IgG immunoblotting. Hence, studies aimed to detect nitrotyrosine-CXCL8 within our group are ongoing.

Chemokine nitration has previously been identified *in vivo* for CCL2 by IHC and ELISA (Molon *et al.*, 2011), but chemokine nitration studies still remain challenging. One explanation for the difficulty of detection, observed for CCL2, could be that the antibody may be specific for nitration only at one position and so unable to detect more heavily nitrated CCL2 (Molon *et al.*, 2011). *In vivo* detection of nitrated protein in sites rich in chemokines is shown here, and also in numerous other studies of transplantation and other inflammatory situations (MacMillan-Crow *et al.*, 1996; Molon *et al.*, 2011; Barker *et al.*, 2014). Thus, co- and post-translational modifications of chemokines, as well as chemokine function, should be explored in order to improve understanding of their role and potential clinical value.

6. Final Discussion

6.1 Summary of aims and outcomes

- To synthesise, purify and biophysically characterise CXCL8 C-terminal peptides

The role of glycosaminoglycans is known to be crucial in the regulation of chemokines. Chapter 3 shows that chemokine peptides are helpful to study chemokine GAG interactions. A truncated version of CXCL8 C-terminal region [CXCL8 (54-72)], the E70K peptide, and a scrambled peptide of the WT were synthesised and purified at 80-95% purity.

Biophysical characterisation by SPR showed that the peptides significantly bind GAG heparin, even though peptides were extended or non-helical, differently to the corresponding region within CXCL8, an α-helical structure. E70K peptide, which has two net positive charges more than WT or scrambled peptides showed significantly higher GAG binding, using $2x10^4$ molar excess over the chemokine. Scrambled peptide showed similar binding ability to the WT peptide. Data suggested significance of the increased positive charge in E70K peptide to bind polyanionic GAG.

Detection of peptide GAG-binding by SPR raised question about the potential biological activity of synthesised C-terminal peptides in the modulation of chemokine function. Thus their potential application as a tool to study the regulation of chemokine-mediated leukocyte recruitment during different inflammatory situations, such as during IRI after transplantation.

- To evaluate the biological function of CXCL8 peptides in endothelial GAG-binding and the potential role in modulation of CXCL8 function

Cell surface markers of primary neutrophils during the inflammatory response are known. Characterisation of HL-60 cell line and primary neutrophils showed significant CXCR1/2 and also CD45, CD11b and CD66b expression at surface level for primary neutrophils, which

correlated with high RNA levels of CXCR1/2/4/7 in primary neutrophils, as opposed to limited RNA expression and cell surface CXCR1/2 and CD45 levels in differentiated HL-60. Functional chemokine assays for neutrophil chemotaxis and adhesion were consistent with CXCR1/2 expression at RNA and cell surface levels. *In vitro* primary neutrophil migration towards CXCL1 or CXCL8, and adhesion towards CXCL8 was studied. Response mediated by CXCL8 was, as expected, significant in both adhesion and migration assays.

Chapter 4 shows chemokine peptides to be viable approach to study chemokine function. CXCL8 C-terminal peptides showed to modulate CXCL8 chemokine activity via endothelial GAG binding, with no effect on the chemokine binding with neutrophil GPCR CXCR1/2. Firstly, no interference with GPCR-mediated neutrophil signaling was shown by calcium flux studies in response to CXCL8 combined with each CXCL8 C-terminal peptide. This was consistent with diffusion gradient neutrophil migration assays. Furthermore, flow-based adhesion assays in response to CXCL8 or CXCL8 peptide demonstrated a significant modulatory role of all peptides in adhesion. Particularly, E70K peptide, showed significant modulation of both neutrophil flow-based adhesion and also transendothelial migration using a minimum of 2.5-fold higher peptide concentration than CXCL8. WT peptide, and similarly a scrambled peptide, showed no significant role in transendothelial migration. Thus, E70K peptide role may be associated with its higher positive net charge (+4), or significance to interfere with CXCL8-GAG binding, and formation of gradient with polyanionic GAG.

- To investigate the role of post-translational nitration in the regulation of chemokine function

Protein nitration is reported in *in vivo* human models of stress-induced damage at the beginning of chapter 5. Data emphasizes the role of oxidative stress and generated reactive species in modifying protein function by inducing protein nitration as a regulatory process. It is shown to act as a negative regulator by decreasing chemokine function, affecting leukocyte recruitment during inflammation to switch on homeostatic processes, as earlier reported (Thompson *et al.*, 2017). The modification can also be associated with an immune function of neutrophils, monocytes or other leukocytes to regulate their pro-inflammatory response on the affected area such as during transplantation, as a negative-feedback loop mechanism.

The clinical value of targeting a particular chemokine such as CXCL8, shown in this study expressed at similar levels in normal and damaged tissue, is as yet undetermined. However, it has been recognized that post-translational modification of chemokines and the overall chemokine biological functions, not only the chemokine presence, play vital role to understand the complexity of chemokine regulation in health and disease.

Post-translational nitration of CXCL8 was demonstrated to dampen chemokine function. CXCL1, which lacks Tyr or Trp (Figure 5-1), was used as a negative control of protein nitration, and we confirmed signal of n-CXCL8 using a 3-NT antibody. Further evaluation of potential protein degradation by nitration using silver gel stain or mass spectrometry would help to determine protein concentration and whether is preserved after nitration. Interestingly, CXCL1 or CXCL8 nitration did not significantly modify chemokine structure, although it showed change in side-chain arginine. Nitrated CXCL8 NMR showed that Y13 and W57 residues can be nitrated, which induces chemical shift perturbations in the vicinity. CXCL8 nitration showed significant inhibition of neutrophil recruitment towards the inflamed tissue in *in vitro* models of neutrophil flow-based adhesion and migration over endothelial cells. Hence, changes occurring during CXCL8 nitration are not global structural changes or interaction alterations, and can act as a negative regulator of chemokine-mediated inflammation.

The showcased approaches, chemokine peptide synthesis and chemokine post-translational nitration, have a role in the modulation of chemokine-GAG binding. They are fundamental mechanisms of chemokine regulation at protein level. The present study helps to better understand how chemokines can mediate the leukocyte recruitment in heath and disease. It may allow discovery of leading biomarker candidates in inflammation by analysis of chemokine truncation and detection of chemokine nitration levels in human samples, thus could contribute to advance our knowledge of the therapeutic relevance of chemokine function.

6.2 Implications

Chemokines are key players during inflammation, regulating both initiation and resolution. Better understanding of the chemokine regulation is essential in order to prevent pathological inflammation. As aforementioned, considering that many conditions undergo inflammation, chemokines are logical target as biomarker candidate and for drug development. However, to date, only two chemokine receptor antagonists, for CCR5 and for CXCR4, are in the market FDA approved (Fatkenheuer *et al.*, 2005; Uy *et al.*, 2008; Cooper *et al.*, 2017). Hence, further investigation is required in order to maximally understand the fundamentals of chemokine function regulation. This study focused on better understanding the role of GAG binding at modulating chemokine activity, and the role of post-translational nitration on chemokine function in *in vitro* and *in vivo* models.

Figure 6-1 summaries the results of this study. The inflammatory response initiates when different stress-derived or damage responses induce upregulation of inflammatory mediators (e.g. cytokines, chemokines, growth factors, etc) (Figure 6-1A). Cell surface GAGs bind chemokines at high concentration towards the site of damage or injury. Chemokine gradient leads to leukocyte recruitment via leukocyte GPCR binding, integrin activation and leukocyte adhesion and final transmigration towards the injured site. Prolonged leukocyte GPCR activation commonly induces receptor desensitization (homologous or heterologous) (Steele *et al.*, 2002; Richardson *et al.*, 2003) and internalisation. Thus, the receptor is then recycled or degraded via ubiquitin-proteasome or via autophagic pathway for resolution of inflammation. Chemokine regulation, by truncation (B) and nitration (C) was of focus here.

Figure 6-1B represents the role of the truncated CXCL8 C-terminal region [54-72], particularly with regards to the E70K peptide. Reported E70K peptide data showed interference with the chemokine-GAG interaction, as also observed for the WT peptide and scrambled peptide, reducing neutrophil-endothelial adhesion. E70K peptide also reduced neutrophil transendothelial chemotaxis, potentially affecting the chemokine gradient formation, with consequently reduced the neutrophil-mediated inflammatory response. Our cross-disciplinary approach in biochemistry, biophysics and biology offers better understanding of the chemokine function, particularly its regulation by GAG binding. It mimics the natural chemokine truncation using synthetic chemokine peptides. Studies of

chemokine peptides or chemokine receptor peptides contribute to better understand how chemokines function and how chemokine receptors fine-tune their activity, by homo/heterodimerisation or oligomerisation (Kobayashi *et al.*, 2017). Further development of the chemokine peptide approach may be beneficial to prevent CXCL8-mediated neutrophil-derived damage during severe reperfusion injury, e.g. following ischaemic injury during transplantation or during organ failure.

During inflammation, generation of oxidative stress (ROS/RNS), depicted in Figure 6-1C can have significant impact on the regulation of protein function, for instance on chemokines. Meanwhile stress can increase chemokine production, it can also increase chemokine nitration with profound consequences on the chemokine functionality. It affects the chemokine-mediated leukocyte recruitment and development of the inflammatory response. Therefore, chemokine nitration may act as a negative regulator to dampen chemokine-mediated inflammation, suggesting a switch-off mechanism to control the immune response. Different chemokines undergo distinct chemokine post-translational modifications; hence generalization is not practical in this regard. Moreover, many currently available antibodies cannot detect the nitrated form of a chemokine e.g. CCL2. Thus, case-by-case analysis of specific chemokine presence and function, its tissue type and the timeframe should be maximally exploited (Wisastra *et al.*, 2011; Quan *et al.*, 2015).

In vivo detection of chemokine function and post-translational modifications is crucial, e.g. during transplantation or other situations. Dysregulation of inflammation has been broadly reported to be pathological and associated with alterations in chemokine production and chemokine modifications. Dysregulation can contribute to infectious diseases, such as bacterial pyelonephritis where decreased CXCR1 levels were associated with susceptibility to disease (Frendeus *et al.*, 2000); or to inflammatory conditions such as lung disease, rheumatoid arthritis or cystic fibrosis. Investigations in rheumatoid arthritis have shown alteration of the glycosylation patterns of IL-6 (Raghav *et al.*, 2006; Dewald *et al.*, 2016). During respiratory syncytial viral infection, age-related delay in the pulmonary cytokine response (IL-6, IL-10, IFN- γ), and imbalance in their production have shown to dramatically influence the immunological response and can contribute to viral immune evasion (Tripp *et al.*, 2000; Boukhvalova *et al.*, 2007). In chronic granulomatous inflammation, dysfunction of granulocytes and associated dysregulation of chemokine production and oxidative burst (ROS/RNS) is caused by the dysfunction of phagocytic NADPH oxidase. It is therefore,

critical to understand how chemokine levels and their modifications can contribute to failure of the inflammatory response and the resolution (Kawai *et al.*, 2013; Eirin *et al.*, 2014).

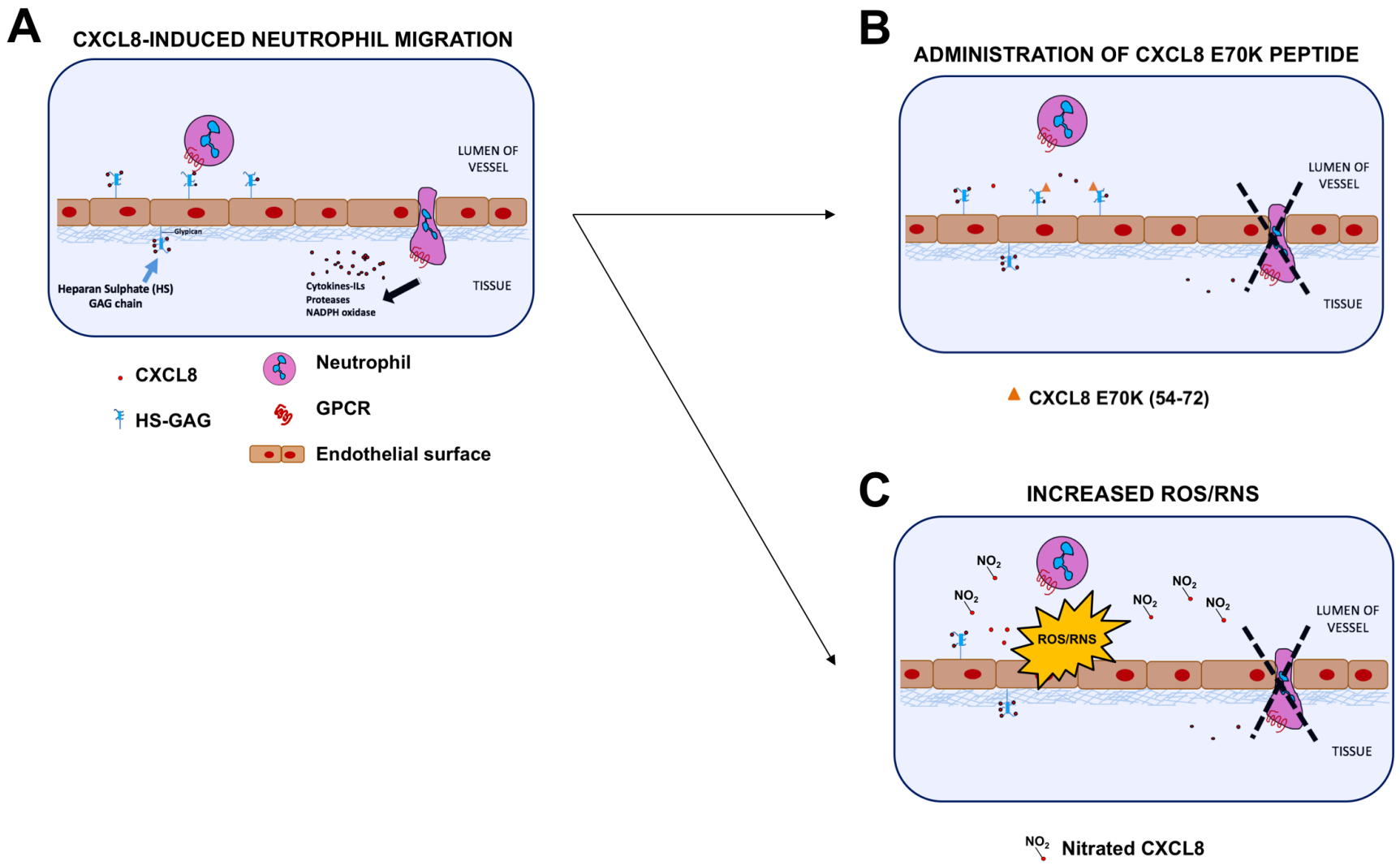


Figure 6-1. Proposed model of neutrophil recruitment in response to: CXCL8; the CXCL8 E70K C-terminal peptide; or nitrated CXCL8.
A) Neutrophil migration directed by CXCL8. B) Modulatory activity of CXCL8 E70K (54-72). C) Inhibition by nitrated CXCL8 (NO_2-CXCL8).

6.3 Future directions

Chemokine investigations in this study have focused on CXCL1 and CXCL8 function. Future work on other inflammatory chemokine may help to understand how functional modulation of a particular chemotactic cytokine can influence the expression and/or function of others, such CXCL9, CXCL10 or CXCL11, and their cognate receptors. This way, design of precise chemotactic immunotherapies to limit different immunopathologies may be facilitated and, for instance, it might contribute to prevent or reverse vascular disease development, or renal morbidity in hypertensive patients (Rudemiller and Crowley, 2017).

Chemokine peptides were synthesised in order to better understand the chemokine GAG binding. This approach is a tool that enhances understanding on modulation of chemokine function by GAG binding. It would be of interest to determine whether chemokine peptide studied in one SPR surface could compete with chemokine GAG binding, or with GAG-bound chemokine oligomers. It could also be of interest to define the role of the peptides in the modulation of other chemokines (CXCL1, CXCL2, CXCL9). Furthermore, with regards to E70K peptide, it could be helpful to determine the particular role of the single mutation within full-length CXCL8 to determine its GAG binding specificity and its role in chemokine modulation. Produced R68A and R68Citrulline C-terminal peptides, purified but not further analysed due to constrains of research secondment, might be of further biochemical interest. Peptide studies on chemokine-GPCR binding residues were not further developed due to requirement for a more complex biosynthetic chemistry likely to involve further *in silico* analysis, longer coupling, or folding.

Data shown in relation to *in vivo* detection of CXCL8 and chemokine nitration contributes to better understand the chemokine function in different transplant or inflammatory situations. However, further investigations should follow, with the aim to detect chemokine production and modifications during particular inflammatory conditions e.g. other time points during early ischaemic injury. Importantly, in this regard, it is of interest to specifically detect nitrated chemokine in human samples. The initial design of an antibody to detect nitrotyrosine-CXCL8 was not successful to detect CXCL8 nitration on patient samples as our validation studies confirmed produced clones to be non-specific for detection of

nitrotyrosine-CXCL8. Therefore, currently alternative investigations are ongoing within the group towards design of monoclonal antibody against nitrotyrosine-CXCL8. An alternative approach considering phage-display library technology might provide specific clone, and it might allow detection of nitration of a particular chemokine in patient samples. Thus, if validation succeeds, the antibody will be used to detect nitrated chemokine. Particularly, chemokines in plasma are hypothesised to be mainly in monomer form, which might facilitate detection, whereas in tissue they tend to dimerise, and undergo further oligomerisation by GAG presence. Initial focus is on nitrated CXCL8 in serum, plasma and urine samples derived post transplantation. In order to characterise the amount of nitrated chemokine in patient samples, it will be critical to optimise protocols for sample concentration, particularly in plasma due to low concentration of chemokine, and to perform further quantitative analysis for instance by nano-HPLC and mass spectrometry.

In addition, in order to further understand how tyrosine residue is important in function, chemokine mutants for both CXCL1 and CXCL8 were designed and synthesised in collaboration with Prof Krishna Rajarathnam's group (Texas University Medical Branch, USA) (Figure 6-2). As CXCL1 does not have tyrosine, this amino acid was introduced, and in CXCL8 tyrosine was replaced with phenylalanine, hypothesised to affect chemokine activity. Also, nitrated versions of both CXCL1 and CXCL8, in wild type and mutant chemokine, were produced. Furthermore, it would also be of interest to determine whether chemokine regulation by post-translation nitration can be prevented on GAG-bound chemokine.

CHEMOKINE	***SEQUENCE***
CXCL1	**MARAALSAAPSNPRLLRVALLLLLLVAAGRRAAGASVATELRCQCLQTLQGIHP KNIQSV NVKSPGPHCAQTEVIATLKNGRKACLNPASPIVKKIIEKMLNSDKSN (C-terminal)**
CXCL1 L15Y	**MARAALSAAPSNPRYLRVALLLLLLVAAGRRAAGASVATELRCQCLQTLQGIHP KNIQSV NVKSPGPHCAQTEVIATLKNGRKACLNPASPIVKKIIEKMLNSDKSN (C-terminal)**
CXCL8	**SAKELRCQCIKTYSKPFHPKFIKELRVIESGPHCANTEIIVKLSDGRELCLDPKEN WVQRVVEKFLKRAENS (C-terminal)**
CXCL8 Y13F	**SAKELRCQCIKTFSKPFHPKFIKELRVIESGPHCANTEIIVKLSDGRELCLDPKEN WVQRVVEKFLKRAENS (C-terminal)**

Figure 6-2. CXCL1 and CXCL8 sequences and corresponding single amino acid mutants. CXCL1 leucine 15 (L15) residue plays a role in receptor binding as part of the chemokine N-terminal. Here, CXCL1 was mutated by substitution of L15 with tyrosine (L15Y CXCL1), to determine its role on chemokine reorganisation and/or potential impact on the chemokine activity. CXCL8 tyrosine 13 has a reported role in receptor binding. Here, CXCL8 was mutated by substitution of Y13 with phenylalanine (Y13F CXCL8) to better identify its significance in the chemokine function.

Studies were designed to investigate the differential ability of produced chemokine versions to bind GAG and GPCR, and their role to induce neutrophil migration by diffusion gradient and transendothelial chemotaxis. As aforementioned, it was confirmed that nitration of CXCL8 resulted in a significant decrease in migration ($P<0.05$) (Figure 5-16, Figure 5-17). Previous work has shown the role of CXCL8 Tyr13, and potentially other amino acids such as His and Arg, in receptor binding (Figure 1-5) (Williams *et al.*, 1996; Gschwandtner *et al.*, 2017). CXCL1 is known to present basic residues involved in receptor binding, such as His or Lys (Figure 5-1) (Sepuru and Rajarathnam, 2016). Initial CXCL1 studies unexpectedly

showed no statistically significant neutrophil migration; otherwise showed lower response than CXCL8. Investigations with chemokine mutants and with nitrated mutant variants are ongoing within the group. Studies aim to better understand chemokine function, post-translational modifications, and how chemokine function can be dysregulated during different conditions, e.g. triggered by NADPH oxidase dysfunction (Kawai *et al.*, 2013; Eirin *et al.*, 2014), to develop knowledge and strategies to maintain a control of the inflammatory response.

6.4 Final conclusion

The role of chemokines CXCL1 and CXCL8 during inflammation has been showcased. Strategic modulation of CXCL8 function using truncated CXCL8 C-terminal [CXCL8 (54-72)] demonstrated that the chemokine peptides have ability to specifically bind GAG and induce a partial inhibition of flow-based neutrophil adhesion. The function of E70K C-terminal peptide, which showed significant GAG-binding and a modulatory role in neutrophil transendothelial migration, is potentially associated with its higher charge which enhances specificity for polyanionic GAG.

During the inflammatory response, chemokine production changes and chemokine levels can be affected by oxidative stress-derived modifications. Regulation of chemokine function by post-translational nitration due to ROS and RNS such as $ONOO^-$ shows to act as a negative regulatory mechanism. These processes can have profound consequences on the chemokine detection, structure and/or function. CXCL8 nitration has no significant effect on its structure. However, it showed a profound effect on the chemokine function by analysis of chemokine-GAG binding, flow-based neutrophil adhesion and neutrophil migration, as observed by diffusion gradient and transendothelial chemotaxis.

Investigations on chemokine function and post-translational modifications such as nitration improve our understanding of the chemokine role during homeostasis and pathophysiological conditions. Studies highlight the tight chemokine regulation required during the inflammatory response and resolution. There is, therefore a complex balance between oxidative stress, cell

death mechanisms (autophagy, apoptosis or necrosis), senescence, and proliferation, regeneration and tolerance, which raises questions for new and exciting avenues of discovery.

7. References

Abecassis, M., Bartlett, S.T., Collins, A.J., Davis, C.L., Delmonico, F.L., Friedewald, J.J., Hays, R., Howard, A., Jones, E., Leichtman, A.B., Merion, R.M., Metzger, R.A., Pradel, F., Schweitzer, E.J., Velez, R.L. and Gaston, R.S. (2008) 'Kidney transplantation as primary therapy for end-stage renal disease: A National Kidney Foundation/Kidney Disease Outcomes Quality Initiative (NKF/KDOQI (TM)) conference', *Clinical Journal of the American Society of Nephrology*, 3(2), pp. 471-480.

Abramowicz, D., Oberbauer, R., Heemann, U., Viklicky, O., Peruzzi, L., Mariat, C., Crespo, M., Budde, K., Oniscu, G.C. and Descartes advisory, b. (2018) 'Recent advances in kidney transplantation: a viewpoint from the Descartes advisory board', *Nephrol Dial Transplant*.

Adage, T., del Bene, F., Fiorentini, F., Doornbos, R.P., Zankl, C., Bartley, M.R. and Kungl, A.J. (2015) 'PA401, a novel CXCL8-based biologic therapeutic with increased glycosaminoglycan binding, reduces bronchoalveolar lavage neutrophils and systemic inflammatory markers in a murine model of LPS-induced lung inflammation', *Cytokine*, 76(2), pp. 433-41.

Adage, T., Piccinini, A.M., Falsone, A., Trinker, M., Robinson, J., Gesslbauer, B. and Kungl, A.J. (2012) 'Structure-based design of decoy chemokines as a way to explore the pharmacological potential of glycosaminoglycans', *Br J Pharmacol*, 167(6), pp. 1195-205.

Addison, C.L., Daniel, T.O., Burdick, M.D., Liu, H., Ehlert, J.E., Xue, Y.Y., Buechi, L., Walz, A., Richmond, A. and Strieter, R.M. (2000) 'The CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR+ CXC chemokine-induced angiogenic activity', *J Immunol*, 165(9), pp. 5269-77.

Ades, E.W., Candal, F.J., Swerlick, R.A., George, V.G., Summers, S., Bosse, D.C. and Lawley, T.J. (1992) 'HMEC-1: establishment of an immortalized human microvascular endothelial cell line', *J Invest Dermatol*, 99(6), pp. 683-90.

Al Faruque, H., Kang, J.H., Hwang, S.R., Sung, S., Alam, M.M., Sa, K.H., Nam, E.J., Byun, Y.R. and Kang, Y.M. (2017) 'Stepwise inhibition of T cell recruitment at post-capillary venules by orally active desulfated heparins in inflammatory arthritis', *PloS one*, 12(4), p. e0176110.

Ali, S., Hardy, L.A. and Kirby, J.A. (2003) 'Transplant immunobiology: a crucial role for heparan sulfate glycosaminoglycans?', *Transplantation*, 75(11), pp. 1773-82.

Ali, S., Malik, G., Burns, A., Robertson, H. and Kirby, J.A. (2005) 'Renal transplantation: examination of the regulation of chemokine binding during acute rejection', *Transplantation*, 79(6), pp. 672-9.

Ali, S., O'Boyle, G., Hepplewhite, P., Tyler, J.R., Robertson, H. and Kirby, J.A. (2010) 'Therapy with nonglycosaminoglycan-binding mutant CCL7: a novel strategy to limit allograft inflammation', *Am J Transplant*, 10(1), pp. 47-58.

Alvarez, B., Rubbo, H., Kirk, M., Barnes, S., Freeman, B.A. and Radi, R. (1996) 'Peroxynitrite-dependent tryptophan nitration', *Chemical Research in Toxicology*, 9(2), pp. 390-396.

Amulic, B., Cazalet, C., Hayes, G.L., Metzler, K.D. and Zychlinsky, A. (2012) 'Neutrophil function: from mechanisms to disease', *Annu Rev Immunol*, 30, pp. 459-89.

Anders, H.J., Vielhauer, V. and Schlondorff, D. (2004) 'Current paradigms about chemokines as therapeutic targets', *Nephrol Dial Transplant*, 19(12), pp. 2948-51.

Anderson, D.C., Schmalstieg, F.C., Arnaout, M.A., Kohl, S., Tosi, M.F., Dana, N., Buffone, G.J., Hughes, B.J., Brinkley, B.R., Dickey, W.D. and et al. (1984) 'Abnormalities of polymorphonuclear leukocyte function associated with a heritable deficiency of high molecular weight surface glycoproteins (GP138): common relationship to diminished cell adherence', *J Clin Invest*, 74(2), pp. 536-51.

Andreoni, F., Ogawa, T., Ogawa, M., Madon, J., Uchiyama, S., Schuepbach, R.A. and Zinkernagel, A.S. (2014) 'The IL-8 protease SpyCEP is detrimental for Group A Streptococcus host-cells interaction and biofilm formation', *Frontiers in microbiology*, 5.

Arav, A., Revel, A., Nathan, Y., Bor, A., Gacitua, H., Yavin, S., Gavish, Z., Uri, M. and Elami, A. (2005) 'Oocyte recovery, embryo development and ovarian function after cryopreservation and transplantation of whole sheep ovary', *Hum Reprod*, 20(12), pp. 3554-9.

Arimateia, D.S., da Silva Brito, A., de Azevedo, F.M., de Andrade, G.P.V. and Chavante, S.F. (2015) 'Heparin fails to inhibit the leukocyte recruitment for an extended time following inflammatory stimulus', *Pharmaceutical biology*, 53(1), pp. 72-77.

Arimont, M., Sun, S.L., Leurs, R., Smit, M., de Esch, I.J.P. and de Graaf, C. (2017) 'Structural Analysis of Chemokine Receptor-Ligand Interactions', *J Med Chem*, 60(12), pp. 4735-4779.

Awad, A.S., Rouse, M., Huang, L., Vergis, A.L., Reutershan, J., Cathro, H.P., Linden, J. and Okusa, M.D. (2009) 'Compartmentalization of neutrophils in the kidney and lung following acute ischemic kidney injury', *Kidney Int*, 75(7), pp. 689-98.

Bachelerie, F., Ben-Baruch, A., Burkhardt, A.M., Combadiere, C., Farber, J.M., Graham, G.J., Horuk, R., Sparre-Ulrich, A.H., Locati, M. and Luster, A.D. (2014a) 'International Union of Basic and Clinical Pharmacology. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors', *Pharmacological reviews*, 66(1), pp. 1-79.

Bachelerie, F., Ben-Baruch, A., Burkhardt, A.M., Combadiere, C., Farber, J.M., Graham, G.J., Horuk, R., Sparre-Ulrich, A.H., Locati, M., Luster, A.D., Mantovani, A., Matsushima, K., Murphy, P.M., Nibbs, R., Nomiyama, H., Power, C.A., Proudfoot, A.E., Rosenkilde, M.M., Rot, A., Sozzani, S., Thelen, M., Yoshie, O. and Zlotnik, A. (2014b) 'International Union of Basic and Clinical Pharmacology. [corrected]. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors', *Pharmacol Rev*, 66(1), pp. 1-79.

Bachelerie, F., Graham, G.J., Locati, M., Mantovani, A., Murphy, P.M., Nibbs, R., Rot, A., Sozzani, S. and Thelen, M. (2014c) 'New nomenclature for atypical chemokine receptors', *Nature immunology*, 15(3), pp. 207-208.

Baggiolini, M., Dewald, B. and Moser, B. (1997) 'Human chemokines: an update', *Annual review of immunology*, 15(1), pp. 675-705.

Baldwin, E.T., Weber, I.T., St Charles, R., Xuan, J.C., Appella, E., Yamada, M., Matsushima, K., Edwards, B.F., Clore, G.M., Gronenborn, A.M. and et al. (1991) 'Crystal structure of interleukin 8: symbiosis of NMR and crystallography', *Proc Natl Acad Sci U S A*, 88(2), pp. 502-6.

Bao, Z., Ye, Q., Gong, W., Xiang, Y. and Wan, H. (2010) 'Humanized monoclonal antibody against the chemokine CXCL-8 (IL-8) effectively prevents acute lung injury', *Int Immunopharmacol*, 10(2), pp. 259-63.

Barker, C.E., Ali, S., O'Boyle, G. and Kirby, J.A. (2014) 'Transplantation and inflammation: implications for the modification of chemokine function', *Immunology*, 143(2), pp. 138-45.

Barker, C.E., Thompson, S., O'Boyle, G., Lortat-Jacob, H., Sheerin, N.S., Ali, S. and Kirby, J.A. (2017) 'CCL2 nitration is a negative regulator of chemokine-mediated inflammation', *Sci Rep*, 7, p. 44384.

Batra, S., Cai, S., Balamayooran, G. and Jeyaseelan, S. (2012) 'Intrapulmonary administration of leukotriene B(4) augments neutrophil accumulation and responses in the lung to Klebsiella infection in CXCL1 knockout mice', *J Immunol*, 188(7), pp. 3458-68.

Becker, L.E., Morath, C. and Suesal, C. (2016) 'Immune mechanisms of acute and chronic rejection', *Clin Biochem*, 49(4-5), pp. 320-3.

Beckman, J.S., Ischiropoulos, H., Zhu, L., Van der Woerd, M., Smith, C., Chen, J., Harrison, J., Martin, J.C. and Tsai, M. (1992) 'Kinetics of superoxide dismutase- and iron-catalyzed nitration of phenolics by peroxynitrite', *Archives of Biochemistry and Biophysics*, 298(2), pp. 438-445.

Bedke, J., Nelson, P.J., Kiss, E., Muenchmeier, N., Rek, A., Behnes, C.L., Gretz, N., Kungl, A.J. and Grone, H.J. (2010) 'A novel CXCL8 protein-based antagonist in acute experimental renal allograft damage', *Mol Immunol*, 47(5), pp. 1047-57.

Bennett, L.D., Fox, J.M. and Signoret, N. (2011) 'Mechanisms regulating chemokine receptor activity', *Immunology*, 134(3), pp. 246-56.

Berendsen, T.A., Bruinsma, B.G., Lee, J., D'Andrea, V., Liu, Q., Izamis, M.L., Uygun, K. and Yarmush, M.L. (2012) 'A simplified subnormothermic machine perfusion system restores ischemically damaged liver grafts in a rat model of orthotopic liver transplantation', *Transplant Res*, 1(1), p. 6.

Berendsen, T.A., Bruinsma, B.G., Puts, C.F., Saeidi, N., Usta, O.B., Uygun, B.E., Izamis, M.L., Toner, M., Yarmush, M.L. and Uygun, K. (2014) 'Supercooling enables long-term transplantation survival following 4 days of liver preservation', *Nat Med*, 20(7), pp. 790-3.

Bertini, R., Allegretti, M., Bizzarri, C., Moriconi, A., Locati, M., Zampella, G., Cervellera, M.N., Di Cioccio, V., Cesta, M.C., Galliera, E., Martinez, F.O., Di Bitondo, R., Troiani, G., Sabbatini, V., D'Anniballe, G., Anacardio, R., Cutrin, J.C., Cavalieri, B., Mainiero, F., Strippoli, R., Villa, P., Di Girolamo, M., Martin, F., Gentile, M., Santoni, A., Corda, D., Poli, G., Mantovani, A., Ghezzi, P. and Colotta, F. (2004) 'Noncompetitive allosteric inhibitors of the inflammatory chemokine receptors CXCR1 and CXCR2: prevention of reperfusion injury', *Proc Natl Acad Sci U S A*, 101(32), pp. 11791-6.

Bhatia, S.N. and Ingber, D.E. (2014) 'Microfluidic organs-on-chips', *Nat Biotechnol*, 32(8), pp. 760-72.

Birnie, G.D. (1988) 'The HL60 cell line: a model system for studying human myeloid cell differentiation', *Br J Cancer Suppl*, 9, pp. 41-5.

Biswas, S.K. (2016) 'Does the Interdependence between Oxidative Stress and Inflammation Explain the Antioxidant Paradox?', *Oxid Med Cell Longev*, 2016, p. 5698931.

Blanchet, X., Langer, M., Weber, C., Koenen, R.R. and von Hundelshausen, P. (2012) 'Touch of chemokines', *Front Immunol*, 3, p. 175.

Bolisetty, S. and Agarwal, A. (2009) 'Neutrophils in acute kidney injury: not neutral any more', *Kidney Int*, 75(7), pp. 674-6.

Bonventre, J.V. and Yang, L. (2011) 'Cellular pathophysiology of ischemic acute kidney injury', *J Clin Invest*, 121(11), pp. 4210-21.

Borst, C., Xia, S., Bistrup, C. and Tepel, M. (2015) 'Interleukin-8 transcripts in mononuclear cells determine impaired graft function after kidney transplantation', *PLoS One*, 10(2), p. e0117315.

Boukhvalova, M.S., Yim, K.C., Kuhn, K.H., Hemming, J.P., Prince, G.A., Porter, D.D. and Blanco, J.C. (2007) 'Age-related differences in pulmonary cytokine response to respiratory syncytial virus infection: modulation by anti-inflammatory and antiviral treatment', *J Infect Dis*, 195(4), pp. 511-8.

Breyer, F. and Kliemt, H. (2007) 'The shortage of transplants - An economic analysis of causes and possible solutions', *Jahrbucher Fur Nationalokonomie Und Statistik*, 227(5-6), pp. 466-484.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y. and Zychlinsky, A. (2004) 'Neutrophil extracellular traps kill bacteria', *Science*, 303(5663), pp. 1532-5.

Bromley, S.K., Mempel, T.R. and Luster, A.D. (2008) 'Orchestrating the orchestrators: chemokines in control of T cell traffic', *Nat Immunol*, 9(9), pp. 970-80.

Brown, D.I. and Griendling, K.K. (2015) 'Regulation of signal transduction by reactive oxygen species in the cardiovascular system', *Circ Res*, 116(3), pp. 531-49.

Burg, J.S., Ingram, J.R., Venkatakrishnan, A.J., Jude, K.M., Dukkipati, A., Feinberg, E.N., Angelini, A., Waghray, D., Dror, R.O., Ploegh, H.L. and Garcia, K.C. (2015) 'Structural biology. Structural basis for chemokine recognition and activation of a viral G protein-coupled receptor', *Science*, 347(6226), pp. 1113-7.

Campanella, G.S.V., Grimm, J., Manice, L.A., Colvin, R.A., Medoff, B.D., Wojtkiewicz, G.R., Weissleder, R. and Luster, A.D. (2006) 'Oligomerization of CXCL10 is necessary for endothelial cell presentation and in vivo activity', *The Journal of Immunology*, 177(10), pp. 6991-6998.

Campbell, B.K., Hernandez-Medrano, J., Onions, V., Pincott-Allen, C., Aljaser, F., Fisher, J., McNeilly, A.S., Webb, R. and Picton, H.M. (2014) 'Restoration of ovarian function and natural fertility following

the cryopreservation and autotransplantation of whole adult sheep ovaries', *Hum Reprod*, 29(8), pp. 1749-63.

Campbell, L.M., Maxwell, P.J. and Waugh, D.J. (2013) 'Rationale and Means to Target Pro-Inflammatory Interleukin-8 (CXCL8) Signaling in Cancer', *Pharmaceuticals (Basel)*, 6(8), pp. 929-59.

Cao, P., Aoki, Y., Badri, L., Walker, N.M., Manning, C.M., Lagstein, A., Fearon, E.R. and Lama, V.N. (2017) 'Autocrine lysophosphatidic acid signaling activates beta-catenin and promotes lung allograft fibrosis', *J Clin Invest*, 127(4), pp. 1517-1530.

Carter, N.M., Ali, S. and Kirby, J.A. (2003) 'Endothelial inflammation: the role of differential expression of N-deacetylase/N-sulphotransferase enzymes in alteration of the immunological properties of heparan sulphate', *J Cell Sci*, 116(Pt 17), pp. 3591-600.

Cashen, A.F., Nervi, B. and DiPersio, J. (2007) 'AMD3100: CXCR4 antagonist and rapid stem cell-mobilizing agent', *Future Oncol*, 3(1), pp. 19-27.

Casilli, F., Bianchini, A., Gloaguen, I., Biordi, L., Alesse, E., Festuccia, C., Cavalieri, B., Strippoli, R., Cervellera, M.N., Di Bitondo, R., Ferretti, E., Mainiero, F., Bizzarri, C., Colotta, F. and Bertini, R. (2005) 'Inhibition of interleukin-8 (CXCL8/IL-8) responses by repertaxin, a new inhibitor of the chemokine receptors CXCR1 and CXCR2', *Biochem Pharmacol*, 69(3), pp. 385-94.

Charo, I.F. and Ransohoff, R.M. (2006) 'The many roles of chemokines and chemokine receptors in inflammation', *N Engl J Med*, 354(6), pp. 610-21.

Chiao, H., Kohda, Y., McLeroy, P., Craig, L., Housini, I. and Star, R.A. (1997) 'Alpha-melanocyte-stimulating hormone protects against renal injury after ischemia in mice and rats', *J Clin Invest*, 99(6), pp. 1165-72.

Chishti, A.D., Dark, J.H., Kesteven, P., Powell, H., Snowden, C., Shenton, B.K., Kirby, J.A. and Baudouin, S.V. (2001) 'Expression of chemokine receptors CXCR1 and CXCR2 during cardiopulmonary bypass', *J Thorac Cardiovasc Surg*, 122(6), pp. 1162-6.

Chung, A.C. and Lan, H.Y. (2011) 'Chemokines in renal injury', *J Am Soc Nephrol*, 22(5), pp. 802-9.

Clark-Lewis, I., Dewald, B., Loetscher, M., Moser, B. and Baggiolini, M. (1994) 'Structural requirements for interleukin-8 function identified by design of analogs and CXC chemokine hybrids', *Journal of Biological Chemistry*, 269(23), pp. 16075-16081.

Collins, S.J. (1987) 'The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression', *Blood*, 70(5), pp. 1233-44.

Cooper, T.M., Sison, E.A.R., Baker, S.D., Li, L., Ahmed, A., Trippett, T., Gore, L., Macy, M.E., Narendran, A., August, K., Absalon, M.J., Boklan, J., Pollard, J., Magoon, D. and Brown, P.A. (2017) 'A phase 1 study of the CXCR4 antagonist plerixafor in combination with high-dose cytarabine and etoposide in children with relapsed or refractory acute leukemias or myelodysplastic syndrome: A

Pediatric Oncology Experimental Therapeutics Investigators' Consortium study (POE 10-03)', *Pediatr Blood Cancer*, 64(8).

Cripps, J.G., Crespo, F.A., Romanovskis, P., Spatola, A.F. and Fernandez-Botran, R. (2005) 'Modulation of acute inflammation by targeting glycosaminoglycan-cytokine interactions', *Int Immunopharmacol*, 5(11), pp. 1622-32.

Cugini, D., Azzollini, N., Gagliardini, E., Cassis, P., Bertini, R., Colotta, F., Noris, M., Remuzzi, G. and Benigni, A. (2005) 'Inhibition of the chemokine receptor CXCR2 prevents kidney graft function deterioration due to ischemia/reperfusion', *Kidney Int*, 67(5), pp. 1753-61.

Cypel, M., Yeung, J.C., Machuca, T., Chen, M., Singer, L.G., Yasufuku, K., de Perrot, M., Pierre, A., Waddell, T.K. and Keshavjee, S. (2012) 'Experience with the first 50 ex vivo lung perfusions in clinical transplantation', *J Thorac Cardiovasc Surg*, 144(5), pp. 1200-6.

Daemen, M.A.R.C., van't Veer, C., Denecker, G., Heemskerk, V.H., Wolfs, T.G.A.M., Clauss, M., Vandenabeele, P. and Buurman, W.A. (1999) 'Inhibition of apoptosis induced by ischemia-reperfusion prevents inflammation', *Journal of Clinical Investigation*, 104(5), pp. 541-549.

Das, S.T., Rajagopalan, L., Guerrero-Plata, A., Sai, J., Richmond, A., Garofalo, R.P. and Rajarathnam, K. (2010) 'Monomeric and dimeric CXCL8 are both essential for in vivo neutrophil recruitment', *PLoS One*, 5(7), p. e11754.

De Buck, M., Gouwy, M., Proost, P., Struyf, S. and Van Damme, J. (2013) 'Identification and characterization of MIP-1alpha/CCL3 isoform 2 from bovine serum as a potent monocyte/dendritic cell chemoattractant', *Biochem Pharmacol*, 85(6), pp. 789-97.

de Oliveira, S., Reyes-Aldasoro, C.C., Candel, S., Renshaw, S.A., Mulero, V. and Calado, A. (2013) 'Cxcl8 (IL-8) mediates neutrophil recruitment and behavior in the zebrafish inflammatory response', *J Immunol*, 190(8), pp. 4349-59.

Dedon, P.C. and Tannenbaum, S.R. (2004) 'Reactive nitrogen species in the chemical biology of inflammation', *Archives of Biochemistry and Biophysics*, 423(1), pp. 12-22.

Deng, Y., Guo, Y., Liu, P., Zeng, R., Ning, Y., Pei, G., Li, Y., Chen, M., Guo, S., Li, X., Han, M. and Xu, G. (2016) 'Blocking protein phosphatase 2A signaling prevents endothelial-to-mesenchymal transition and renal fibrosis: a peptide-based drug therapy', *Sci Rep*, 6, p. 19821.

Dewald, J.H., Colomb, F., Bobowski-Gerard, M., Groux-Degroote, S. and Delannoy, P. (2016) 'Role of Cytokine-Induced Glycosylation Changes in Regulating Cell Interactions and Cell Signaling in Inflammatory Diseases and Cancer', *Cells*, 5(4).

Djamali, A., Kaufman, D.B., Ellis, T.M., Zhong, W., Matas, A. and Samaniego, M. (2014) 'Diagnosis and management of antibody-mediated rejection: current status and novel approaches', *Am J Transplant*, 14(2), pp. 255-71.

Doster, A., Schwarzig, U., Zygmunt, M., Rom, J., Schuetz, F. and Fluhr, H. (2016) 'Unfractionated Heparin Selectively Modulates the Expression of CXCL8, CCL2 and CCL5 in Endometrial Carcinoma Cells', *Anticancer research*, 36(4), pp. 1535-1544.

Dyer, D.P., Salanga, C.L., Volkman, B.F., Kawamura, T. and Handel, T.M. (2016) 'The dependence of chemokine-glycosaminoglycan interactions on chemokine oligomerization', *Glycobiology*, 26(3), pp. 312-26.

Dyer, D.P., Thomson, J.M., Hermant, A., Jowitt, T.A., Handel, T.M., Proudfoot, A.E.I., Day, A.J. and Milner, C.M. (2014) 'TSG-6 inhibits neutrophil migration via direct interaction with the chemokine CXCL8', *The Journal of Immunology*, 192(5), pp. 2177-2185.

Eckhard, U., Huesgen, P.F., Schilling, O., Bellac, C.L., Butler, G.S., Cox, J.H., Dufour, A., Goebeler, V., Kappelhoff, R., Keller, U.A.D., Klein, T., Lange, P.F., Marino, G., Morrison, C.J., Prudova, A., Rodriguez, D., Starr, A.E., Wang, Y. and Overall, C.M. (2016) 'Active site specificity profiling of the matrix metalloproteinase family: Proteomic identification of 4300 cleavage sites by nine MMPs explored with structural and synthetic peptide cleavage analyses', *Matrix Biol*, 49, pp. 37-60.

Eckle, T., Faigle, M., Grenz, A., Laucher, S., Thompson, L.F. and Eltzschig, H.K. (2008) 'A2B adenosine receptor dampens hypoxia-induced vascular leak', *Blood*, 111(4), pp. 2024-35.

Eirin, A., Zhang, X., Zhu, X.Y., Tang, H., Jordan, K.L., Grande, J.P., Dietz, A.B., Lerman, A., Textor, S.C. and Lerman, L.O. (2014) 'Renal vein cytokine release as an index of renal parenchymal inflammation in chronic experimental renal artery stenosis', *Nephrol Dial Transplant*, 29(2), pp. 274-82.

El-Zoghby, Z.M., Stegall, M.D., Lager, D.J., Kremers, W.K., Amer, H., Gloor, J.M. and Cosio, F.G. (2009) 'Identifying specific causes of kidney allograft loss', *Am J Transplant*, 9(3), pp. 527-35.

Elliott, C.L., Kelly, R.W., Critchley, H.O., Riley, S.C. and Calder, A.A. (1998) 'Regulation of interleukin 8 production in the term human placenta during labor and by antigestagens', *Am J Obstet Gynecol*, 179(1), pp. 215-20.

Elmoselhi, H., Mansell, H., Soliman, M. and Shoker, A. (2016) 'Circulating chemokine ligand levels before and after successful kidney transplantation', *J Inflamm (Lond)*, 13, p. 32.

Ezerzer, C., Dolgin, M., Skovorodnikova, J. and Harris, N. (2009) 'Chemokine receptor-derived peptides as multi-target drug leads for the treatment of inflammatory diseases', *Peptides*, 30(7), pp. 1296-305.

Fahy, G.M., Wowk, B., Wu, J., Phan, J., Rasch, C., Chang, A. and Zendejas, E. (2004) 'Cryopreservation of organs by vitrification: perspectives and recent advances', *Cryobiology*, 48(2), pp. 157-78.

Falsone, A., Wabitsch, V., Geretti, E., Potzinger, H., Gerlza, T., Robinson, J., Adage, T., Teixeira, M.M. and Kungl, A.J. (2013) 'Designing CXCL8-based decoy proteins with strong anti-inflammatory activity in vivo', *Biosci Rep*, 33(5).

Fatkenheuer, G., Pozniak, A.L., Johnson, M.A., Plettenberg, A., Staszewski, S., Hoepelman, A.I., Saag, M.S., Goebel, F.D., Rockstroh, J.K., Dezube, B.J., Jenkins, T.M., Medhurst, C., Sullivan, J.F., Ridgway, C., Abel, S., James, I.T., Youle, M. and van der Ryst, E. (2005) 'Efficacy of short-term monotherapy with maraviroc, a new CCR5 antagonist, in patients infected with HIV-1', *Nat Med*, 11(11), pp. 1170-2.

Fernandez-Botran, R., Gorantla, V., Sun, X., Ren, X., Perez-Abadia, G., Crespo, F.A., Oliver, R., Orhun, H.I., Quan, E.E., Maldonado, C., Ray, M. and Barker, J.H. (2002) 'Targeting of glycosaminoglycan-cytokine interactions as a novel therapeutic approach in allotransplantation', *Transplantation*, 74(5), pp. 623-9.

Fernandez-Botran, R., Romanovskis, P., Sun, X. and Spatola, A.F. (2004) 'Linear basic peptides for targeting interferon-gamma-glycosaminoglycan interactions: synthesis and inhibitory properties', *J Pept Res*, 63(2), pp. 56-62.

Filomeni, G., De Zio, D. and Cecconi, F. (2015) 'Oxidative stress and autophagy: the clash between damage and metabolic needs', *Cell Death Differ*, 22(3), pp. 377-88.

Finkel, T. and Holbrook, N.J. (2000) 'Oxidants, oxidative stress and the biology of ageing', *Nature*, 408(6809), pp. 239-47.

Frangogiannis, N.G. (2004) 'Chemokines in the ischemic myocardium: from inflammation to fibrosis', *Inflamm Res*, 53(11), pp. 585-95.

Frears, E.R., Zhang, Z., Blake, D.R., O'Connell, J.P. and Winyard, P.G. (1996) 'Inactivation of tissue inhibitor of metalloproteinase-1 by peroxynitrite', *FEBS Lett*, 381(1-2), pp. 21-4.

Frendeus, B., Godaly, G., Hang, L., Karpman, D., Lundstedt, A.C. and Svanborg, C. (2000) 'Interleukin 8 receptor deficiency confers susceptibility to acute experimental pyelonephritis and may have a human counterpart', *J Exp Med*, 192(6), pp. 881-90.

Furuichi, K., Kaneko, S. and Wada, T. (2009) 'Chemokine/chemokine receptor-mediated inflammation regulates pathologic changes from acute kidney injury to chronic kidney disease', *Clin Exp Nephrol*, 13(1), pp. 9-14.

Furuichi, K., Wada, T., Kaneko, S. and Murphy, P.M. (2008) 'Roles of chemokines in renal ischemia/reperfusion injury', *Front Biosci*, 13, pp. 4021-8.

Gainet, J., Chollet-Martin, S., Brion, M., Hakim, J., Gougerot-Pocidalo, M.A. and Elbim, C. (1998) 'Interleukin-8 production by polymorphonuclear neutrophils in patients with rapidly progressive periodontitis: an amplifying loop of polymorphonuclear neutrophil activation', *Lab Invest*, 78(6), pp. 755-62.

Gandhi, N.S. and Mancera, R.L. (2011) 'Molecular dynamics simulations of CXCL-8 and its interactions with a receptor peptide, heparin fragments, and sulfated linked cyclitols', *Journal of chemical information and modeling*, 51(2), pp. 335-358.

Gangavarapu, P., Rajagopalan, L., Kolli, D., Guerrero-Plata, A., Garofalo, R.P. and Rajarathnam, K. (2012) 'The monomer-dimer equilibrium and glycosaminoglycan interactions of chemokine CXCL8 regulate tissue-specific neutrophil recruitment', *J Leukoc Biol*, 91(2), pp. 259-65.

Gelderblom, M., Weymar, A., Bernreuther, C., Velden, J., Arunachalam, P., Steinbach, K., Orthey, E., Arumugam, T.V., Leypoldt, F., Simova, O., Thom, V., Friese, M.A., Prinz, I., Holscher, C., Glatzel, M., Korn, T., Gerloff, C., Tolosa, E. and Magnus, T. (2012) 'Neutralization of the IL-17 axis diminishes neutrophil invasion and protects from ischemic stroke', *Blood*, 120(18), pp. 3793-3802.

Gerlza, T., Hecher, B., Jeremic, D., Fuchs, T., Gschwandtner, M., Falsone, A., Gesslbauer, B. and Kungl, A.J. (2014) 'A combinatorial approach to biophysically characterise chemokine-glycan binding affinities for drug development', *Molecules*, 19(7), pp. 10618-34.

Gerlza, T., Winkler, S., Atlic, A., Zankl, C., Konya, V., Kitic, N., Strutzmann, E., Knebl, K., Adage, T., Heinemann, A., Weis, R. and Kungl, A.J. (2015) 'Designing a mutant CCL2-HSA chimera with high glycosaminoglycan-binding affinity and selectivity', *Protein Eng Des Sel*, 28(8), pp. 231-40.

Gerszten, R.E., Garcia-Zepeda, E.A., Lim, Y.C., Yoshida, M., Ding, H.A., Gimbrone, M.A., Jr., Luster, A.D., Luscinskas, F.W. and Rosenzweig, A. (1999) 'MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions', *Nature*, 398(6729), pp. 718-23.

Ghezzi, P., Jaquet, V., Marcucci, F. and Schmidt, H.H. (2016) 'The oxidative stress theory of disease: levels of evidence and epistemological aspects', *Br J Pharmacol*.

Giaglis, S., Hahn, S. and Hasler, P. (2016) '"The NET Outcome": Are Neutrophil Extracellular Traps of Any Relevance to the Pathophysiology of Autoimmune Disorders in Childhood?', *Front Pediatr*, 4, p. 97.

Giwa, S., Lewis, J.K., Alvarez, L., Langer, R., Roth, A.E., Church, G.M., Markmann, J.F., Sachs, D.H., Chandraker, A., Wertheim, J.A., Rothblatt, M., Boyden, E.S., Eidbo, E., Lee, W.P.A., Pomahac, B., Brandacher, G., Weinstock, D.M., Elliott, G., Nelson, D., Acker, J.P., Uygun, K., Schmalz, B., Weegman, B.P., Tocchio, A., Fahy, G.M., Storey, K.B., Rubinsky, B., Bischof, J., Elliott, J.A.W., Woodruff, T.K., Morris, G.J., Demirci, U., Brockbank, K.G.M., Woods, E.J., Ben, R.N., Baust, J.G., Gao, D., Fuller, B., Rabin, Y., Kravitz, D.C., Taylor, M.J. and Toner, M. (2017) 'The promise of organ and tissue preservation to transform medicine', *Nat Biotechnol*, 35(6), pp. 530-542.

Gluhovschi, G., Gluhovschi, C., Bob, F., Velciov, S., Trandafirescu, V., Petrica, L. and Bozdog, G. (2010) 'Immune compartments of the nephron in relation to the immune system', *Rom J Intern Med*, 48(1), pp. 17-31.

Goger, B., Halden, Y., Rek, A., Mosl, R., Pye, D., Gallagher, J. and Kungl, A.J. (2002) 'Different affinities of glycosaminoglycan oligosaccharides for monomeric and dimeric interleukin-8: a model for chemokine regulation at inflammatory sites', *Biochemistry*, 41(5), pp. 1640-6.

Gonzalez-Molina, M., Ruiz-Esteban, P., Caballero, A., Burgos, D., Cabello, M., Leon, M., Fuentes, L. and Hernandez, D. (2016) 'Immune response and histology of humoral rejection in kidney transplantation', *Nefrologia*, 36(4), pp. 354-67.

Gonzalez-Motos, V., Kropp, K.A. and Viejo-Borbolla, A. (2016) 'Chemokine binding proteins: An immunomodulatory strategy going viral', *Cytokine Growth Factor Rev*, 30, pp. 71-80.

Granger, D.N. and Kvietys, P.R. (2015) 'Reperfusion injury and reactive oxygen species: The evolution of a concept', *Redox Biol*, 6, pp. 524-51.

Greenfield, N.J. (2006) 'Using circular dichroism spectra to estimate protein secondary structure', *Nat Protoc*, 1(6), pp. 2876-90.

Grenz, A., Osswald, H., Eckle, T., Yang, D., Zhang, H., Tran, Z.V., Klingel, K., Ravid, K. and Eltzschig, H.K. (2008) 'The reno-vascular A2B adenosine receptor protects the kidney from ischemia', *PLoS Med*, 5(6), p. e137.

Gross, A., Hashimoto, C., Sticht, H. and Eichler, J. (2015) 'Synthetic Peptides as Protein Mimics', *Front Bioeng Biotechnol*, 3, p. 211.

Groves, D.T. and Jiang, Y. (1995) 'Chemokines, a family of chemotactic cytokines', *Critical Reviews in Oral Biology & Medicine*, 6(2), pp. 109-118.

Gschwandtner, M., Strutzmann, E., Teixeira, M.M., Anders, H.J., Diedrichs-Möhring, M., Gerlza, T., Wildner, G., Russo, R.C., Adage, T. and Kungl, A.J. (2017) 'Glycosaminoglycans are important mediators of neutrophilic inflammation in vivo', *Cytokine*, 91, pp. 65-73.

Guan, X., Hou, Y., Sun, F., Yang, Z. and Li, C. (2016) 'Dysregulated Chemokine Signaling in Cystic Fibrosis Lung Disease: A Potential Therapeutic Target', *Curr Drug Targets*, 17(13), pp. 1535-44.

Guarrera, J.V., Henry, S.D., Samstein, B., Odeh-Ramadan, R., Kinkhabwala, M., Goldstein, M.J., Ratner, L.E., Renz, J.F., Lee, H.T., Brown, R.S., Jr. and Emond, J.C. (2010) 'Hypothermic machine preservation in human liver transplantation: the first clinical series', *Am J Transplant*, 10(2), pp. 372-81.

Ha, H., Debnath, B. and Neamati, N. (2017) 'Role of the CXCL8-CXCR1/2 Axis in Cancer and Inflammatory Diseases', *Theranostics*, 7(6), pp. 1543-1588.

Ham, A., Rabadi, M., Kim, M., Brown, K.M., Ma, Z., D'Agati, V. and Lee, H.T. (2014) 'Peptidyl arginine deiminase-4 activation exacerbates kidney ischemia-reperfusion injury', *Am J Physiol Renal Physiol*, 307(9), pp. F1052-62.

Hammer, M., Mages, J., Dietrich, H., Schmitz, F., Striebel, F., Murray, P.J., Wagner, H. and Lang, R. (2005) 'Control of dual-specificity phosphatase-1 expression in activated macrophages by IL-10', *Eur J Immunol*, 35(10), pp. 2991-3001.

Hammond, M.E., Lapointe, G.R., Feucht, P.H., Hilt, S., Gallegos, C.A., Gordon, C.A., Giedlin, M.A., Mullenbach, G. and Tekamp-Olson, P. (1995) 'IL-8 induces neutrophil chemotaxis predominantly via type I IL-8 receptors', *The Journal of Immunology*, 155(3), pp. 1428-1433.

Hammond, M.E.W., Shyamala, V., Siani, M.A., Gallegos, C.A., Feucht, P.H., Abbott, J., Lapointe, G.R., Moghadam, M., Khoja, H. and Zakel, J. (1996) 'Receptor recognition and specificity of interleukin-8 is determined by residues that cluster near a surface-accessible hydrophobic pocket', *Journal of Biological Chemistry*, 271(14), pp. 8228-8235.

Hancock, W.W., Gao, W., Csizmadia, V., Faia, K.L., Shemmeri, N. and Luster, A.D. (2001) 'Donor-derived IP-10 initiates development of acute allograft rejection', *J Exp Med*, 193(8), pp. 975-80.

Handel, T.M., Johnson, Z., Crown, S.E., Lau, E.K., Sweeney, M. and Proudfoot, A.E. (2005) 'Regulation of protein function by glycosaminoglycans-as exemplified by chemokines', *Annu. Rev. Biochem.*, 74, pp. 385-410.

Hang, L., Frendeus, B., Godaly, G. and Svanborg, C. (2000) 'Interleukin-8 receptor knockout mice have subepithelial neutrophil entrapment and renal scarring following acute pyelonephritis', *J Infect Dis*, 182(6), pp. 1738-48.

Harada, A., Sekido, N., Akahoshi, T., Wada, T., Mukaida, N. and Matsushima, K. (1994) 'Essential involvement of interleukin-8 (IL-8) in acute inflammation', *J Leukoc Biol*, 56(5), pp. 559-64.

Harvard Health (2012). Kidney disease and health. Kidney Transplant. Harvard Medical School. https://www.health.harvard.edu/kidney-disease-and-health/kidney-transplant-. Harvard Health Publishing.

Hay, D.W. and Sarau, H.M. (2001) 'Interleukin-8 receptor antagonists in pulmonary diseases', *Curr Opin Pharmacol*, 1(3), pp. 242-7.

Hoffmann, E., Dittrich-Breiholz, O., Holtmann, H. and Kracht, M. (2002) 'Multiple control of interleukin-8 gene expression', *J Leukoc Biol*, 72(5), pp. 847-55.

Holdsworth, S.R. and Tipping, P.G. (2007) 'Leukocytes in glomerular injury', *Seminars in Immunopathology*, 29(4), pp. 355-374.

Horuk, R. (2009) 'Chemokine receptor antagonists: overcoming developmental hurdles', *Nat Rev Drug Discov*, 8(1), pp. 23-33.

Hughes, C.E. and Nibbs, R.J.B. (2018) 'A guide to chemokines and their receptors', *FEBS J*.

Huh, D., Kim, H.J., Fraser, J.P., Shea, D.E., Khan, M., Bahinski, A., Hamilton, G.A. and Ingber, D.E. (2013) 'Microfabrication of human organs-on-chips', *Nat Protoc*, 8(11), pp. 2135-57.

Huie, R.E. and Padmaja, S. (1993) 'The reaction of no with superoxide', *Free Radic Res Commun*, 18(4), pp. 195-9.

Imai, S., Nagano, K., Yoshida, Y., Okamura, T., Yamashita, T., Abe, Y., Yoshikawa, T., Yoshioka, Y., Kamada, H., Mukai, Y., Nakagawa, S., Tsutsumi, Y. and Tsunoda, S. (2011) 'Development of an antibody proteomics system using a phage antibody library for efficient screening of biomarker proteins', *Biomaterials*, 32(1), pp. 162-9.

Ingulli, E. (2010) 'Mechanism of cellular rejection in transplantation', *Pediatr Nephrol*, 25(1), pp. 61-74.

Islam, S.A., Ling, M.F., Leung, J., Shreffler, W.G. and Luster, A.D. (2013) 'Identification of human CCR8 as a CCL18 receptor', *J Exp Med*, 210(10), pp. 1889-98.

Iwasaki, A. and Medzhitov, R. (2015) 'Control of adaptive immunity by the innate immune system', *Nat Immunol*, 16(4), pp. 343-53.

Jang, K.J., Mehr, A.P., Hamilton, G.A., McPartlin, L.A., Chung, S., Suh, K.Y. and Ingber, D.E. (2013) 'Human kidney proximal tubule-on-a-chip for drug transport and nephrotoxicity assessment', *Integr Biol (Camb)*, 5(9), pp. 1119-29.

Jang, Y.C. and Van Remmen, H. (2009) 'The mitochondrial theory of aging: insight from transgenic and knockout mouse models', *Exp Gerontol*, 44(4), pp. 256-60.

Jiang, S.J., Liou, J.W., Chang, C.C., Chung, Y., Lin, L.F. and Hsu, H.J. (2015) 'Peptides derived from CXCL8 based on in silico analysis inhibit CXCL8 interactions with its receptor CXCR1', *Sci Rep*, 5, p. 18638.

Johnson, Z., Kosco-Vilbois, M.H., Herren, S., Cirillo, R., Muzio, V., Zaratin, P., Carbonatto, M., Mack, M., Smailbegovic, A., Rose, M., Lever, R., Page, C., Wells, T.N. and Proudfoot, A.E. (2004) 'Interference with heparin binding and oligomerization creates a novel anti-inflammatory strategy targeting the chemokine system', *J Immunol*, 173(9), pp. 5776-85.

Johnson, Z., Proudfoot, A.E. and Handel, T.M. (2005) 'Interaction of chemokines and glycosaminoglycans: a new twist in the regulation of chemokine function with opportunities for therapeutic intervention', *Cytokine Growth Factor Rev*, 16(6), pp. 625-36.

Jones, N.D., Brook, M.O., Carvalho-Gaspar, M., Luo, S. and Wood, K.J. (2010) 'Regulatory T cells can prevent memory CD8+ T-cell-mediated rejection following polymorphonuclear cell depletion', *Eur J Immunol*, 40(11), pp. 3107-16.

Jones, S.P. and Bolli, R. (2006) 'The ubiquitous role of nitric oxide in cardioprotection', *J Mol Cell Cardiol*, 40(1), pp. 16-23.

Joseph, P.R.B., Mosier, P.D., Desai, U.R. and Rajarathnam, K. (2015) 'Solution NMR characterization of chemokine CXCL8/IL-8 monomer and dimer binding to glycosaminoglycans: structural plasticity mediates differential binding interactions', *Biochemical Journal*, 472(1), pp. 121-133.

Kakkar, A.K. (2005) 'Low-molecular-weight heparin and survival in patients with malignant disease', *Cancer Control*, 12 Suppl 1, pp. 22-30.

Kalogeris, T., Baines, C.P., Krenz, M. and Korthuis, R.J. (2012) 'Cell biology of ischemia/reperfusion injury', *Int Rev Cell Mol Biol*, 298, pp. 229-317.

Kalogeris, T., Bao, Y. and Korthuis, R.J. (2014) 'Mitochondrial reactive oxygen species: a double edged sword in ischemia/reperfusion vs preconditioning', *Redox Biol*, 2, pp. 702-14.

Kasic, T., Colombo, P., Soldani, C., Wang, C.M., Miranda, E., Roncalli, M., Bronte, V. and Viola, A. (2011) 'Modulation of human T-cell functions by reactive nitrogen species', *European Journal of Immunology*, 41(7), pp. 1843-1849.

Kaths, J.M., Echeverri, J., Linares, I., Cen, J.Y., Ganesh, S., Hamar, M., Urbanellis, P., Yip, P., John, R., Bagli, D., Mucsi, I., Ghanekar, A., Grant, D., Robinson, L.A. and Selzner, M. (2017) 'Normothermic Ex Vivo Kidney Perfusion Following Static Cold Storage-Brief, Intermediate, or Prolonged Perfusion for Optimal Renal Graft Reconditioning?', *Am J Transplant*, 17(10), pp. 2580-2590.

Kaths, J.M., Spetzler, V.N., Goldaracena, N., Echeverri, J., Louis, K.S., Foltys, D.B., Strempel, M., Yip, P., John, R., Mucsi, I., Ghanekar, A., Bagli, D., Robinson, L. and Selzner, M. (2015) 'Normothermic Ex Vivo Kidney Perfusion for the Preservation of Kidney Grafts prior to Transplantation', *Jove-Journal of Visualized Experiments*, (101).

Kawai, T., Watanabe, N., Yokoyama, M., Arai, K., Oana, S., Harayama, S., Yasui, K., Oh-Ishi, T. and Onodera, M. (2013) 'Thalidomide attenuates excessive inflammation without interrupting lipopolysaccharide-driven inflammatory cytokine production in chronic granulomatous disease', *Clin Immunol*, 147(2), pp. 122-8.

'Keeping kidneys', (2012) *Bull World Health Organ*, 90(10), pp. 718-9.

Kleist, A.B., Getschman, A.E., Ziarek, J.J., Nevins, A.M., Gauthier, P.-A., Chevigné, A., Szpakowska, M. and Volkman, B.F. (2016) 'New paradigms in chemokine receptor signal transduction: moving beyond the two-site model', *Biochemical pharmacology*, 114, pp. 53-68.

Kobayashi, D., Endo, M., Ochi, H., Hojo, H., Miyasaka, M. and Hayasaka, H. (2017) 'Regulation of CCR7-dependent cell migration through CCR7 homodimer formation', *Sci Rep*, 7(1), p. 8536.

Kolaczkowska, E. and Kubes, P. (2013) 'Neutrophil recruitment and function in health and inflammation', *Nat Rev Immunol*, 13(3), pp. 159-75.

Kolb, H.J. (2017) 'Hematopoietic stem cell transplantation and cellular therapy', *HLA*, 89(5), pp. 267-277.

Kosieradzki, M. and Rowinski, W. (2008) 'Ischemia/reperfusion injury in kidney transplantation: mechanisms and prevention', *Transplant Proc*, 40(10), pp. 3279-88.

Kozek-Langenecker, S., Fenger-Eriksen, C., Thienpont, E., Barauskas, G. and Force, E.V.G.T. (2017) 'European guidelines on perioperative venous thromboembolism prophylaxis: Surgery in the elderly', *Eur J Anaesthesiol*.

Krieger, E., Geretti, E., Brandner, B., Goger, B., Wells, T.N. and Kungl, A.J. (2004) 'A structural and dynamic model for the interaction of interleukin-8 and glycosaminoglycans: Support from isothermal fluorescence titrations', *Proteins: Structure, Function, and Bioinformatics*, 54(4), pp. 768-775.

Krohn, S.C., Bonvin, P. and Proudfoot, A.E. (2013) 'CCL18 exhibits a regulatory role through inhibition of receptor and glycosaminoglycan binding', *PLoS One*, 8(8), p. e72321.

Kufareva, I., Salanga, C.L. and Handel, T.M. (2015) 'Chemokine and chemokine receptor structure and interactions: implications for therapeutic strategies', *Immunol Cell Biol*, 93(4), pp. 372-83.

Kunkel, S.L. (1999) 'Promiscuous chemokine receptors and their redundant ligands play an enigmatic role during HIV-1 infection', *American journal of respiratory cell and molecular biology*, 20(5), pp. 859-860.

Kurts, C., Panzer, U., Anders, H.J. and Rees, A.J. (2013) 'The immune system and kidney disease: basic concepts and clinical implications', *Nat Rev Immunol*, 13(10), pp. 738-53.

Kuschert, G.S., Coulin, F., Power, C.A., Proudfoot, A.E., Hubbard, R.E., Hoogewerf, A.J. and Wells, T.N. (1999) 'Glycosaminoglycans interact selectively with chemokines and modulate receptor binding and cellular responses', *Biochemistry*, 38(39), pp. 12959-68.

Kuschert, G.S.V., Hoogewerf, A.J., Proudfoot, A.E.I., Chung, C.-w., Cooke, R.M., Hubbard, R.E., Wells, T.N.C. and Sanderson, P.N. (1998) 'Identification of a glycosaminoglycan binding surface on human interleukin-8', *Biochemistry*, 37(32), pp. 11193-11201.

Lakschevitz, F.S., Hassanpour, S., Rubin, A., Fine, N., Sun, C. and Glogauer, M. (2016) 'Identification of neutrophil surface marker changes in health and inflammation using high-throughput screening flow cytometry', *Exp Cell Res*, 342(2), pp. 200-9.

Lamberti, G., Prabhakarpandian, B., Garson, C., Smith, A., Pant, K., Wang, B. and Kiani, M.F. (2014) 'Bioinspired microfluidic assay for in vitro modeling of leukocyte-endothelium interactions', *Anal Chem*, 86(16), pp. 8344-51.

Land, W. (1994) 'The potential impact of the reperfusion injury on acute and chronic rejection events following organ transplantation', *Transplant Proc*, 26(6), pp. 3169-71.

Lane, B.R., Lore, K., Bock, P.J., Andersson, J., Coffey, M.J., Strieter, R.M. and Markovitz, D.M. (2001) 'Interleukin-8 stimulates human immunodeficiency virus type 1 replication and is a potential new target for antiretroviral therapy', *J Virol*, 75(17), pp. 8195-202.

Larson, D.J., Middle, L., Vu, H., Zhang, W., Serianni, A.S., Duman, J. and Barnes, B.M. (2014) 'Wood frog adaptations to overwintering in Alaska: new limits to freezing tolerance', *J Exp Biol*, 217(Pt 12), pp. 2193-200.

Lee, J.W., Wang, P., Kattah, M.G., Youssef, S., Steinman, L., DeFea, K. and Straus, D.S. (2008) 'Differential Regulation of Chemokines by IL-17 in Colonic Epithelial Cells', *Journal of Immunology*, 181(9), pp. 6536-6545.

Leong, S.R., Kabakoff, R.C. and Hebert, C.A. (1994) 'Complete mutagenesis of the extracellular domain of interleukin-8 (IL-8) type A receptor identifies charged residues mediating IL-8 binding and signal transduction', *Journal of Biological Chemistry*, 269(30), pp. 19343-19348.

Lewis, J.K., Bischof, J.C., Braslavsky, I., Brockbank, K.G., Fahy, G.M., Fuller, B.J., Rabin, Y., Tocchio, A., Woods, E.J., Wowk, B.G., Acker, J.P. and Giwa, S. (2016) 'The Grand Challenges of Organ Banking: Proceedings from the first global summit on complex tissue cryopreservation', *Cryobiology*, 72(2), pp. 169-82.

Ley, K., Laudanna, C., Cybulsky, M.I. and Nourshargh, S. (2007) 'Getting to the site of inflammation: the leukocyte adhesion cascade updated', *Nat Rev Immunol*, 7(9), pp. 678-89.

Li, H.Y. and Nord, E.P. (2009) 'IL-8 amplifies CD40/CD154-mediated ICAM-1 production via the CXCR-1 receptor and p38-MAPK pathway in human renal proximal tubule cells', *American Journal of Physiology-Renal Physiology*, 296(2), pp. F438-F445.

Li, X., Liu, Y., Wang, L., Li, Z. and Ma, X. (2015) 'Unfractionated heparin attenuates LPS-induced IL-8 secretion via PI3K/Akt/NF-κB signaling pathway in human endothelial cells', *Immunobiology*, 220(3), pp. 399-405.

Lieberman-Blum, S.S., Fung, H.B. and Bandres, J.C. (2008) 'Maraviroc: a CCR5-receptor antagonist for the treatment of HIV-1 infection', *Clin Ther*, 30(7), pp. 1228-50.

Lo, D.J., Weaver, T.A., Kleiner, D.E., Mannon, R.B., Jacobson, L.M., Becker, B.N., Swanson, S.J., Hale, D.A. and Kirk, A.D. (2011) 'Chemokines and their receptors in human renal allotransplantation', *Transplantation*, 91(1), pp. 70-7.

Loos, T., Mortier, A., Gouwy, M., Ronsse, I., Put, W., Lenaerts, J.P., Van Damme, J. and Proost, P. (2008) 'Citrullination of CXCL10 and CXCL11 by peptidylarginine deiminase: a naturally occurring posttranslational modification of chemokines and new dimension of immunoregulation', *Blood*, 112(7), pp. 2648-56.

Loos, T., Opdenakker, G., Van Damme, J. and Proost, P. (2009) 'Citrullination of CXCL8 increases this chemokine's ability to mobilize neutrophils into the blood circulation', *Haematologica*, 94(10), pp. 1346-53.

Lopez-Otin, C. and Hunter, T. (2010) 'The regulatory crosstalk between kinases and proteases in cancer', *Nat Rev Cancer*, 10(4), pp. 278-92.

Lortat-Jacob, H., Grosdidier, A. and Imberty, A. (2002) 'Structural diversity of heparan sulfate binding domains in chemokines', *Proceedings of the National Academy of Sciences*, 99(3), pp. 1229-1234.

Lowman, H.B., Slagle, P.H., DeForge, L.E., Wirth, C.M., Gillece-Castro, B.L., Bourell, J.H. and Fairbrother, W.J. (1996) 'Exchanging interleukin-8 and melanoma growth-stimulating activity receptor binding specificities', *Journal of Biological Chemistry*, 271(24), pp. 14344-14352.

Lutz, J., Thurmel, K. and Heemann, U. (2010) 'Anti-inflammatory treatment strategies for ischemia/reperfusion injury in transplantation', *J Inflamm (Lond)*, 7, p. 27.

MacGregor, H.J., Kato, Y., Marshall, L.J., Nevell, T.G. and Shute, J.K. (2011) 'A copper-hydrogen peroxide redox system induces dityrosine cross-links and chemokine oligomerisation', *Cytokine*, 56(3), pp. 669-675.

Machuca, T.N. and Cypel, M. (2014) 'Ex vivo lung perfusion', *J Thorac Dis*, 6(8), pp. 1054-62.

MacMillan-Crow, L.A. and Crow, J.P. (2011) 'Does more MnSOD mean more hydrogen peroxide?', *Anticancer Agents Med Chem*, 11(2), pp. 178-80.

MacMillan-Crow, L.A., Crow, J.P., Kerby, J.D., Beckman, J.S. and Thompson, J.A. (1996) 'Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts', *Proc Natl Acad Sci U S A*, 93(21), pp. 11853-8.

Mahler, D.A., Huang, S., Tabrizi, M. and Bell, G.M. (2004) 'Efficacy and safety of a monoclonal antibody recognizing interleukin-8 in COPD: a pilot study', *Chest*, 126(3), pp. 926-34.

Mantovani, A., Bonecchi, R. and Locati, M. (2006) 'Tuning inflammation and immunity by chemokine sequestration: decoys and more', *Nat Rev Immunol*, 6(12), pp. 907-18.

Mantovani, A., Cassatella, M.A., Costantini, C. and Jaillon, S. (2011) 'Neutrophils in the activation and regulation of innate and adaptive immunity', *Nat Rev Immunol*, 11(8), pp. 519-31.

Marie, C., Pitton, C., Fitting, C. and Cavaillon, J.M. (1996) 'Regulation by anti-inflammatory cytokines (IL-4, IL-10, IL-13, TGFbeta)of interleukin-8 production by LPS- and/ or TNFalpha-activated human polymorphonuclear cells', *Mediators Inflamm*, 5(5), pp. 334-40.

Marine, A., Krager, K.J., Aykin-Burns, N. and Macmillan-Crow, L.A. (2014) 'Peroxynitrite induced mitochondrial biogenesis following MnSOD knockdown in normal rat kidney (NRK) cells', *Redox Biol*, 2, pp. 348-57.

Marletta, M.A. (1993) 'Nitric oxide synthase structure and mechanism', *J Biol Chem*, 268(17), pp. 12231-4.

Martinez Munoz, L., Lucas, P., Navarro, G., Checa, A.I., Franco, R., Martinez, A.C., Rodriguez-Frade, J.M. and Mellado, M. (2009) 'Dynamic regulation of CXCR1 and CXCR2 homo- and heterodimers', *J Immunol*, 183(11), pp. 7337-46.

Massena, S., Christoffersson, G., Hjertstrom, E., Zcharia, E., Vlodavsky, I., Ausmees, N., Rolny, C., Li, J.P. and Phillipson, M. (2010) 'A chemotactic gradient sequestered on endothelial heparan sulfate induces directional intraluminal crawling of neutrophils', *Blood*, 116(11), pp. 1924-31.

Matheson, P.J., Fernandez-Botran, R., Smith, J.W., Matheson, S.A., Downard, C.D., McClain, C.J. and Garrison, R.N. (2016) 'Association Between MC-2 Peptide and Hepatic Perfusion and Liver Injury Following Resuscitated Hemorrhagic Shock', *JAMA Surg*, 151(3), pp. 265-72.

McKinney, K.L., Dilwith, R. and Belfort, G. (1995) 'Optimizing antibody production in batch hybridoma cell culture', *J Biotechnol*, 40(1), pp. 31-48.

Meguro, M., Katsuramaki, T., Kimura, H., Isobe, M., Nagayama, M., Kukita, K., Nui, A. and Hirata, K. (2003) 'Apoptosis and necrosis after warm ischemia-reperfusion injury of the pig liver and their inhibition by ONO-1714', *Transplantation*, 75(5), pp. 703-710.

Meissner, E.G., Decalf, J., Casrouge, A., Masur, H., Kottilil, S., Albert, M.L. and Duffy, D. (2015) 'Dynamic Changes of Post-Translationally Modified Forms of CXCL10 and Soluble DPP4 in HCV Subjects Receiving Interferon-Free Therapy', *PLoS One*, 10(7), p. e0133236.

Meloni, F., Solari, N., Miserere, S., Morosini, M., Cascina, A., Klersy, C., Arbustini, E., Pellegrini, C., Vigano, M. and Fietta, A.M. (2008) 'Chemokine redundancy in BOS pathogenesis. A possible role also for the CC chemokines: MIP3-beta, MIP3-alpha, MDC and their specific receptors', *Transplant immunology*, 18(3), pp. 275-280.

Merhi, B., Bayliss, G. and Gohh, R.Y. (2015) 'Role for urinary biomarkers in diagnosis of acute rejection in the transplanted kidney', *World J Transplant*, 5(4), pp. 251-60.

Messer, S., Ardehali, A. and Tsui, S. (2015) 'Normothermic donor heart perfusion: current clinical experience and the future', *Transpl Int*, 28(6), pp. 634-42.

Mocellin, S., Panelli, M.C., Wang, E., Nagorsen, D. and Marincola, F.M. (2003) 'The dual role of IL-10', *Trends Immunol*, 24(1), pp. 36-43.

Moelants, E.A., Loozen, G., Mortier, A., Martens, E., Opdenakker, G., Mizgalska, D., Szmigielski, B., Potempa, J., Van Damme, J., Teughels, W. and Proost, P. (2014) 'Citrullination and proteolytic processing of chemokines by Porphyromonas gingivalis', *Infect Immun*, 82(6), pp. 2511-9.

Moelants, E.A., Mortier, A., Van Damme, J. and Proost, P. (2013) 'In vivo regulation of chemokine activity by post-translational modification', *Immunol Cell Biol*, 91(6), pp. 402-7.

Moelants, E.A., Van Damme, J. and Proost, P. (2011) 'Detection and quantification of citrullinated chemokines', *PLoS One*, 6(12), p. e28976.

Moers, C., Pirenne, J., Paul, A., Ploeg, R.J. and Machine Preservation Trial Study, G. (2012) 'Machine perfusion or cold storage in deceased-donor kidney transplantation', *N Engl J Med*, 366(8), pp. 770-1.

Molon, B., Ugel, S., Del Pozzo, F., Soldani, C., Zilio, S., Avella, D., De Palma, A., Mauri, P., Monegal, A., Rescigno, M., Savino, B., Colombo, P., Jonjic, N., Pecanic, S., Lazzarato, L., Fruttero, R., Gasco, A., Bronte, V. and Viola, A. (2011) 'Chemokine nitration prevents intratumoral infiltration of antigen-specific T cells', *J Exp Med*, 208(10), pp. 1949-62.

Montague, K. and Malcangio, M. (2016) 'The therapeutic potential of targeting chemokine signalling in the treatment of chronic pain', *Journal of Neurochemistry*.

Moreau, A., Varey, E., Anegon, I. and Cuturi, M.C. (2013) 'Effector mechanisms of rejection', *Cold Spring Harb Perspect Med*, 3(11).

Mortier, A., Berghmans, N., Ronsse, I., Grauwen, K., Stegen, S., Van Damme, J. and Proost, P. (2011) 'Biological activity of CXCL8 forms generated by alternative cleavage of the signal peptide or by aminopeptidase-mediated truncation', *PLoS One*, 6(8), p. e23913.

Mortier, A., Loos, T., Gouwy, M., Ronsse, I., Van Damme, J. and Proost, P. (2010) 'Posttranslational modification of the NH2-terminal region of CXCL5 by proteases or peptidylarginine Deiminases (PAD) differently affects its biological activity', *J Biol Chem*, 285(39), pp. 29750-9.

Mortier, A., Van Damme, J. and Proost, P. (2008) 'Regulation of chemokine activity by posttranslational modification', *Pharmacol Ther*, 120(2), pp. 197-217.

Mortier, A., Van Damme, J. and Proost, P. (2012) 'Overview of the mechanisms regulating chemokine activity and availability', *Immunol Lett*, 145(1-2), pp. 2-9.

Mowen, K.A. and David, M. (2014) 'Unconventional post-translational modifications in immunological signaling', *Nat Immunol*, 15(6), pp. 512-20.

Mukaida, N. (2003) 'Pathophysiological roles of interleukin-8/CXCL8 in pulmonary diseases', *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 284(4), pp. L566-L577.

Mulivor, A.W. and Lipowsky, H.H. (2004) 'Inflammation- and ischemia-induced shedding of venular glycocalyx', *Am J Physiol Heart Circ Physiol*, 286(5), pp. H1672-80.

Murphy, P.M. (1997) 'Neutrophil receptors for interleukin-8 and related CXC chemokines', *Semin Hematol*, 34(4), pp. 311-8.

Nagai, R., Unno, Y., Hayashi, M.C., Masuda, S., Hayase, F., Kinae, N. and Horiuchi, S. (2002) 'Peroxynitrite induces formation of N(epsilon)-(carboxymethyl) lysine by the cleavage of Amadori product and generation of glucosone and glyoxal from glucose: novel pathways for protein modification by peroxynitrite', *Diabetes*, 51(9), pp. 2833-9.

Nagata, K., Kumasaka, K., Browne, K.D., Li, S., St-Pierre, J., Cognetti, J., Marks, J., Johnson, V.E., Smith, D.H. and Pascual, J.L. (2016) 'Unfractionated heparin after TBI reduces in vivo cerebrovascular inflammation, brain edema and accelerates cognitive recovery', *Journal of Trauma and Acute Care Surgery*, 81(6), pp. 1088-1094.

Nasralla, D., Coussios, C.C., Mergental, H., Akhtar, M.Z., Butler, A.J., Ceresa, C.D.L., Chiocchia, V., Dutton, S.J., Garcia-Valdecasas, J.C., Heaton, N., Imber, C., Jassem, W., Jochmans, I., Karani, J., Knight, S.R., Kocabayoglu, P., Malago, M., Mirza, D., Morris, P.J., Pallan, A., Paul, A., Pavel, M., MTP, R.P., Pirenne, J., Ravikumar, R., Russell, L., Upponi, S., Watson, C.J.E., Weissenbacher, A., Ploeg, R.J., Friend, P.J. and Consortium for Organ Preservation in, E. (2018) 'A randomized trial of normothermic preservation in liver transplantation', *Nature*.

Nasser, M.W., Raghuwanshi, S.K., Grant, D.J., Jala, V.R., Rajarathnam, K. and Richardson, R.M. (2009) 'Differential activation and regulation of CXCR1 and CXCR2 by CXCL8 monomer and dimer', *J Immunol*, 183(5), pp. 3425-32.

Nojima, H., Konishi, T., Freeman, C.M., Schuster, R.M., Japtok, L., Kleuser, B., Edwards, M.J., Gulbins, E. and Lentsch, A.B. (2016) 'Chemokine Receptors, CXCR1 and CXCR2, Differentially Regulate Exosome Release in Hepatocytes', *PLoS One*, 11(8), p. e0161443.

Nuriel, T., Hansler, A. and Gross, S.S. (2011) 'Protein nitrotryptophan: formation, significance and identification', *J Proteomics*, 74(11), pp. 2300-12.

O'Boyle, G., Mellor, P., Kirby, J.A. and Ali, S. (2009) 'Anti-inflammatory therapy by intravenous delivery of non-heparan sulfate-binding CXCL12', *The FASEB Journal*, 23(11), pp. 3906-3916.

O'Donnell, M.A., Perez-Jimenez, E., Oberst, A., Ng, A., Massoumi, R., Xavier, R., Green, D.R. and Ting, A.T. (2011) 'Caspase 8 inhibits programmed necrosis by processing CYLD', *Nat Cell Biol*, 13(12), pp. 1437-42.

Pacher, P., Beckman, J.S. and Liaudet, L. (2007) 'Nitric oxide and peroxynitrite in health and disease', *Physiol Rev*, 87(1), pp. 315-424.

Pagliaro, P. and Penna, C. (2015) 'Redox signalling and cardioprotection: translatability and mechanism', *Br J Pharmacol*, 172(8), pp. 1974-95.

Palamaner Subash Shantha, G., Kumar, A.A., Sethi, M., Khanna, R.C. and Pancholy, S.B. (2015) 'Efficacy and safety of low molecular weight heparin compared to unfractionated heparin for chronic outpatient hemodialysis in end stage renal disease: systematic review and meta-analysis', *PeerJ*, 3, p. e835.

Pamies, D. and Hartung, T. (2017) '21st Century Cell Culture for 21st Century Toxicology', *Chem Res Toxicol*, 30(1), pp. 43-52.

Parish, C.R. (2006) 'The role of heparan sulphate in inflammation', *Nat Rev Immunol*, 6(9), pp. 633-43.

Perico, N., Cattaneo, D., Sayegh, M.H. and Remuzzi, G. (2004) 'Delayed graft function in kidney transplantation', *Lancet*, 364(9447), pp. 1814-1827.

Phillipson, M. and Kubes, P. (2011) 'The neutrophil in vascular inflammation', *Nat Med*, 17(11), pp. 1381-90.

Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., Squadrito, F., Altavilla, D. and Bitto, A. (2017) 'Oxidative Stress: Harms and Benefits for Human Health', *Oxid Med Cell Longev*, 2017, p. 8416763.

Pomfret, E.A., Sung, R.S., Allan, J., Kinkhabwala, M., Melancon, J.K. and Roberts, J.P. (2008) 'Solving the organ shortage crisis: the 7th annual American Society of Transplant Surgeons' State-of-the-Art Winter Symposium', *Am J Transplant*, 8(4), pp. 745-52.

Proost, P., Loos, T., Mortier, A., Schutyser, E., Gouwy, M., Noppen, S., Dillen, C., Ronsse, I., Conings, R., Struyf, S., Opdenakker, G., Maudgal, P.C. and Van Damme, J. (2008) 'Citrullination of CXCL8 by

peptidylarginine deiminase alters receptor usage, prevents proteolysis, and dampens tissue inflammation', *J Exp Med*, 205(9), pp. 2085-97.

Proudfoot, A.E. and Uguccioni, M. (2016) 'Modulation of Chemokine Responses: Synergy and Cooperativity', *Front Immunol*, 7, p. 183.

Proudfoot, A.E.I., Handel, T.M., Johnson, Z., Lau, E.K., LiWang, P., Clark-Lewis, I., Borlat, F., Wells, T.N.C. and Kosco-Vilbois, M.H. (2003) 'Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines', *Proceedings of the National Academy of Sciences*, 100(4), pp. 1885-1890.

Qin, L., Kufareva, I., Holden, L.G., Wang, C., Zheng, Y., Zhao, C., Fenalti, G., Wu, H., Han, G.W., Cherezov, V., Abagyan, R., Stevens, R.C. and Handel, T.M. (2015) 'Structural biology. Crystal structure of the chemokine receptor CXCR4 in complex with a viral chemokine', *Science*, 347(6226), pp. 1117-22.

Quan, J.M., Martin, T.R., Rosenberg, G.B., Foster, D.C., Whitmore, T. and Goodman, R.B. (1996) 'Antibodies against the N-terminus of IL-8 receptor A inhibit neutrophil chemotaxis', *Biochemical and biophysical research communications*, 219(2), pp. 405-411.

Quan, Q., Szeto, S.S., Law, H.C., Zhang, Z., Wang, Y. and Chu, I.K. (2015) 'Fully automated multidimensional reversed-phase liquid chromatography with tandem anion/cation exchange columns for simultaneous global endogenous tyrosine nitration detection, integral membrane protein characterization, and quantitative proteomics mapping in cerebral infarcts', *Anal Chem*, 87(19), pp. 10015-24.

Raghav, S.K., Gupta, B., Agrawal, C., Saroha, A., Das, R.H., Chaturvedi, V.P. and Das, H.R. (2006) 'Altered expression and glycosylation of plasma proteins in rheumatoid arthritis', *Glycoconj J*, 23(3-4), pp. 167-73.

Rajagopalan, L. and Rajarathnam, K. (2006) 'Structural basis of chemokine receptor function—a model for binding affinity and ligand selectivity', *Bioscience reports*, 26(5), pp. 325-339.

Ranjbaran, H., Wang, Y., Manes, T.D., Yakimov, A.O., Akhtar, S., Kluger, M.S., Pober, J.S. and Tellides, G. (2006) 'Heparin displaces interferon-gamma-inducible chemokines (IP-10, I-TAC, and Mig) sequestered in the vasculature and inhibits the transendothelial migration and arterial recruitment of T cells', *Circulation*, 114(12), pp. 1293-300.

Reitsma, S., Slaaf, D.W., Vink, H., van Zandvoort, M.A. and oude Egbrink, M.G. (2007) 'The endothelial glycocalyx: composition, functions, and visualization', *Pflugers Arch*, 454(3), pp. 345-59.

Richardson, R.M., Tokunaga, K., Marjoram, R., Sata, T. and Snyderman, R. (2003) 'Interleukin-8-mediated heterologous receptor internalization provides resistance to HIV-1 infectivity. Role of signal strength and receptor desensitization', *J Biol Chem*, 278(18), pp. 15867-73.

Riffo-Vasquez, Y., Somani, A., Man, F., Amison, R., Pitchford, S. and Page, C.P. (2016) 'A Non-Anticoagulant Fraction of Heparin Inhibits Leukocyte Diapedesis into the Lung by an Effect on Platelets', *American journal of respiratory cell and molecular biology*, 55(4), pp. 554-563.

Riva, A., Laird, M., Casrouge, A., Ambrozaitis, A., Williams, R., Naoumov, N.V., Albert, M.L. and Chokshi, S. (2014) 'Truncated CXCL10 is associated with failure to achieve spontaneous clearance of acute hepatitis C infection', *Hepatology*, 60(2), pp. 487-96.

Robinson, S., Zincuk, A., Larsen, U.L., Ekstrom, C. and Toft, P. (2014) 'A feasible strategy for preventing blood clots in critically ill patients with acute kidney injury (FBI): study protocol for a randomized controlled trial', *Trials*, 15, p. 226.

Romagnani, S. (1994) 'Lymphokine production by human T cells in disease states', *Annu Rev Immunol*, 12, pp. 227-57.

Rot, A. (1993) 'Neutrophil attractant/activation protein-1 (interleukin-8) induces in vitro neutrophil migration by haptotactic mechanism', *European journal of immunology*, 23(1), pp. 303-306.

Rot, A. (2009) 'Chemokine patterning by glycosaminoglycans and interceptors', *Frontiers in bioscience (Landmark edition)*, 15, pp. 645-660.

Rot, A., Hub, E., Middleton, J., Pons, F., Rabeck, C., Thierer, K., Wintle, J., Wolff, B., Zsak, M. and Dukor, P. (1996) 'Some aspects of IL-8 pathophysiology. III: Chemokine interaction with endothelial cells', *J Leukoc Biol*, 59(1), pp. 39-44.

Roy, I., Evans, D.B. and Dwinell, M.B. (2014) 'Chemokines and chemokine receptors: update on utility and challenges for the clinician', *Surgery*, 155(6), pp. 961-73.

Rudemiller, N.P. and Crowley, S.D. (2017) 'The role of chemokines in hypertension and consequent target organ damage', *Pharmacol Res*, 119, pp. 404-411.

Sadik, C.D., Kim, N.D. and Luster, A.D. (2011) 'Neutrophils cascading their way to inflammation', *Trends Immunol*, 32(10), pp. 452-60.

Sadir, R., Baleux, F., Grosdidier, A., Imberty, A. and Lortat-Jacob, H. (2001) 'Characterization of the stromal cell-derived factor-1alpha-heparin complex', *J Biol Chem*, 276(11), pp. 8288-96.

Saesen, E., Sarrazin, S., Laguri, C., Sadir, R., Maurin, D., Thomas, A., Imberty, A. and Lortat-Jacob, H. (2013) 'Insights into the mechanism by which interferon-gamma basic amino acid clusters mediate protein binding to heparan sulfate', *J Am Chem Soc*, 135(25), pp. 9384-90.

Salanga, C.L. and Handel, T.M. (2011) 'Chemokine oligomerization and interactions with receptors and glycosaminoglycans: the role of structural dynamics in function', *Exp Cell Res*, 317(5), pp. 590-601.

Salazar, N., Castellan, M., Shirodkar, S.S. and Lokeshwar, B.L. (2013) 'Chemokines and chemokine receptors as promoters of prostate cancer growth and progression', *Critical Reviews™ in Eukaryotic Gene Expression*, 23(1).

Salvadori, M., Rosso, G. and Bertoni, E. (2015) 'Update on ischemia-reperfusion injury in kidney transplantation: Pathogenesis and treatment', *World J Transplant*, 5(2), pp. 52-67.

Sato, E., Simpson, K.L., Grisham, M.B., Koyama, S. and Robbins, R.A. (1999) 'Effects of reactive oxygen and nitrogen metabolites on RANTES- and IL-5-induced eosinophil chemotactic activity in vitro', *Am J Pathol*, 155(2), pp. 591-8.

Sawant, K.V., Poluri, K.M., Dutta, A.K., Sepuru, K.M., Troshkina, A., Garofalo, R.P. and Rajarathnam, K. (2016) 'Chemokine CXCL1 mediated neutrophil recruitment: Role of glycosaminoglycan interactions', *Sci Rep*, 6, p. 33123.

Schauer, C., Janko, C., Munoz, L.E., Zhao, Y., Kienhofer, D., Frey, B., Lell, M., Manger, B., Rech, J., Naschberger, E., Holmdahl, R., Krenn, V., Harrer, T., Jeremic, I., Bilyy, R., Schett, G., Hoffmann, M. and Herrmann, M. (2014) 'Aggregated neutrophil extracellular traps limit inflammation by degrading cytokines and chemokines', *Nat Med*, 20(5), pp. 511-7.

Schnuelle, P., Gottmann, U., Hoeger, S., Boesebeck, D., Lauchart, W., Weiss, C., Fischereder, M., Jauch, K.W., Heemann, U., Zeier, M., Hugo, C., Pisarski, P., Kramer, B.K., Lopau, K., Rahmel, A., Benck, U., Birck, R. and Yard, B.A. (2009) 'Effects of donor pretreatment with dopamine on graft function after kidney transplantation: a randomized controlled trial', *JAMA*, 302(10), pp. 1067-75.

Scholten, D.J., Canals, M., Maussang, D., Roumen, L., Smit, M.J., Wijtmans, M., De Graaf, C., Vischer, H.F. and Leurs, R. (2012) 'Pharmacological modulation of chemokine receptor function', *British journal of pharmacology*, 165(6), pp. 1617-1643.

Segerer, S., Henger, A., Schmid, H., Kretzler, M., Draganovici, D., Brandt, U., Noessner, E., Nelson, P.J., Kerjaschki, D., Schlondorff, D. and Regele, H. (2006) 'Expression of the chemokine receptor CXCR1 in human glomerular diseases', *Kidney International*, 69(10), pp. 1765-1773.

Segerer, S. and Schlondorff, D. (2007) 'Role of chemokines for the localization of leukocyte subsets in the kidney', *Seminars in Nephrology*, 27(3), pp. 260-274.

Sekido, N., Mukaida, N., Harada, A., Nakanishi, I., Watanabe, Y. and Matsushima, K. (1993) 'Prevention of lung reperfusion injury in rabbits by a monoclonal antibody against interleukin-8', *Nature*, 365(6447), pp. 654-7.

Sellares, J., de Freitas, D.G., Mengel, M., Reeve, J., Einecke, G., Sis, B., Hidalgo, L.G., Famulski, K., Matas, A. and Halloran, P.F. (2012) 'Understanding the causes of kidney transplant failure: the dominant role of antibody-mediated rejection and nonadherence', *Am J Transplant*, 12(2), pp. 388-99.

Sepuru, K.M. and Rajarathnam, K. (2016) 'CXCL1/MGSA Is a Novel Glycosaminoglycan (GAG)-binding Chemokine: STRUCTURAL EVIDENCE FOR TWO DISTINCT NON-OVERLAPPING BINDING DOMAINS', *J Biol Chem*, 291(8), pp. 4247-55.

Shaw, C.A., Taylor, E.L., Fox, S., Megson, I.L. and Rossi, A.G. (2011) 'Differential susceptibility to nitric oxide-evoked apoptosis in human inflammatory cells', *Free Radical Biology and Medicine*, 50(1), pp. 93-101.

Shetty, S., Weston, C.J., Adams, D.H. and Lalor, P.F. (2014) 'A flow adhesion assay to study leucocyte recruitment to human hepatic sinusoidal endothelium under conditions of shear stress', *J Vis Exp*, (85).

Shimizu, K. and Mitchell, R.N. (2008) 'The role of chemokines in transplant graft arterial disease', *Arterioscler Thromb Vasc Biol*, 28(11), pp. 1937-49.

Simon Davis, D.A. and Parish, C.R. (2013) 'Heparan sulfate: a ubiquitous glycosaminoglycan with multiple roles in immunity', *Front Immunol*, 4, p. 470.

Sligh, J.E., Jr., Ballantyne, C.M., Rich, S.S., Hawkins, H.K., Smith, C.W., Bradley, A. and Beaudet, A.L. (1993) 'Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule 1', *Proc Natl Acad Sci U S A*, 90(18), pp. 8529-33.

Soehnlein, O. and Lindbom, L. (2010) 'Phagocyte partnership during the onset and resolution of inflammation', *Nat Rev Immunol*, 10(6), pp. 427-39.

Soehnlein, O., Steffens, S., Hidalgo, A. and Weber, C. (2017) 'Neutrophils as protagonists and targets in chronic inflammation', *Nat Rev Immunol*, 17(4), pp. 248-261.

Souza, D.G., Bertini, R., Vieira, A.T., Cunha, F.Q., Poole, S., Allegretti, M., Colotta, F. and Teixeira, M.M. (2004) 'Repertaxin, a novel inhibitor of rat CXCR2 function, inhibits inflammatory responses that follow intestinal ischaemia and reperfusion injury', *Br J Pharmacol*, 143(1), pp. 132-42.

Souza, J.M., Peluffo, G. and Radi, R. (2008) 'Protein tyrosine nitration--Functional alteration or just a biomarker?', *Free Radical Biology and Medicine*, 45(4), pp. 357-366.

Starr, A.E., Dufour, A., Maier, J. and Overall, C.M. (2012) 'Biochemical analysis of matrix metalloproteinase activation of chemokines CCL15 and CCL23 and increased glycosaminoglycan binding of CCL16', *J Biol Chem*, 287(8), pp. 5848-60.

Steele, A.D., Szabo, I., Bednar, F. and Rogers, T.J. (2002) 'Interactions between opioid and chemokine receptors: heterologous desensitization', *Cytokine Growth Factor Rev*, 13(3), pp. 209-22.

Stone, M.J., Hayward, J.A., Huang, C., Z, E.H. and Sanchez, J. (2017) 'Mechanisms of Regulation of the Chemokine-Receptor Network', *Int J Mol Sci*, 18(2).

Storey, K.B. and Storey, J.M. (2004) 'Metabolic rate depression in animals: transcriptional and translational controls', *Biol Rev Camb Philos Soc*, 79(1), pp. 207-33.

Storey, K.B. and Storey, J.M. (2007) 'Tribute to P. L. Lutz: putting life on 'pause'--molecular regulation of hypometabolism', *J Exp Biol*, 210(Pt 10), pp. 1700-14.

Storey, K.B. and Storey, J.M. (2010) 'Metabolic rate depression: the biochemistry of mammalian hibernation', *Adv Clin Chem*, 52, pp. 77-108.

Struyf, S., Noppen, S., Loos, T., Mortier, A., Gouwy, M., Verbeke, H., Huskens, D., Luangsay, S., Parmentier, M., Geboes, K., Schols, D., Van Damme, J. and Proost, P. (2009) 'Citrullination of CXCL12 differentially reduces CXCR4 and CXCR7 binding with loss of inflammatory and anti-HIV-1 activity via CXCR4', *J Immunol*, 182(1), pp. 666-74.

Struyf, S., Proost, P., Lenaerts, J.P., Stoops, G., Wuyts, A. and Van Damme, J. (2001) 'Identification of a blood-derived chemoattractant for neutrophils and lymphocytes as a novel CC chemokine, Regakine-1', *Blood*, 97(8), pp. 2197-204.

Sutton, A., Friand, V., Papy-Garcia, D., Dagouassat, M., Martin, L., Vassy, R., Haddad, O., Sainte-Catherine, O., Kraemer, M. and Saffar, L. (2007) 'Glycosaminoglycans and their synthetic mimetics inhibit RANTES-induced migration and invasion of human hepatoma cells', *Molecular cancer therapeutics*, 6(11), pp. 2948-2958.

Svensson, M., Yadav, M., Holmqvist, B., Lutay, N., Svanborg, C. and Godaly, G. (2011) 'Acute pyelonephritis and renal scarring are caused by dysfunctional innate immunity in mCxcr2 heterozygous mice', *Kidney Int*, 80(10), pp. 1064-72.

Szabo, C., Ischiropoulos, H. and Radi, R. (2007) 'Peroxynitrite: Biochemistry, pathophysiology and development of therapeutics', *Nature Reviews Drug Discovery*, 6(8), pp. 662-680.

Tabary, O., Zahm, J.M., Hinnrasky, J., Couetil, J.P., Cornillet, P., Guenounou, M., Gaillard, D., Puchelle, E. and Jacquot, J. (1998) 'Selective up-regulation of chemokine IL-8 expression in cystic fibrosis bronchial gland cells in vivo and in vitro', *Am J Pathol*, 153(3), pp. 921-30.

Takakura, K., Beckman, J.S., MacMillan-Crow, L.A. and Crow, J.P. (1999) 'Rapid and irreversible inactivation of protein tyrosine phosphatases PTP1B, CD45, and LAR by peroxynitrite', *Archives of Biochemistry and Biophysics*, 369(2), pp. 197-207.

Tan, J.H., Ludeman, J.P., Wedderburn, J., Canals, M., Hall, P., Butler, S.J., Taleski, D., Christopoulos, A., Hickey, M.J., Payne, R.J. and Stone, M.J. (2013) 'Tyrosine sulfation of chemokine receptor CCR2 enhances interactions with both monomeric and dimeric forms of the chemokine monocyte chemoattractant protein-1 (MCP-1)', *J Biol Chem*, 288(14), pp. 10024-34.

Tanino, Y., Coombe, D.R., Gill, S.E., Kett, W.C., Kajikawa, O., Proudfoot, A.E., Wells, T.N., Parks, W.C., Wight, T.N., Martin, T.R. and Frevert, C.W. (2010) 'Kinetics of chemokine-glycosaminoglycan interactions control neutrophil migration into the airspaces of the lungs', *J Immunol*, 184(5), pp. 2677-85.

Taylor, K.R. and Gallo, R.L. (2006) 'Glycosaminoglycans and their proteoglycans: host-associated molecular patterns for initiation and modulation of inflammation', *The FASEB Journal*, 20(1), pp. 9-22.

Thakar, D., Migliorini, E., Coche-Guerente, L., Sadir, R., Lortat-Jacob, H., Boturyn, D., Renaudet, O., Labbe, P. and Richter, R.P. (2014) 'A quartz crystal microbalance method to study the terminal functionalization of glycosaminoglycans', *Chem Commun (Camb)*, 50(96), pp. 15148-51.

Thompson, L.D., Pantoliano, M.W. and Springer, B.A. (1994) 'Energetic characterization of the basic fibroblast growth factor-heparin interaction: identification of the heparin binding domain', *Biochemistry*, 33(13), pp. 3831-40.

Thompson, S., Martinez-Burgo, B., Sepuru, K.M., Rajarathnam, K., Kirby, J.A., Sheerin, N.S. and Ali, S. (2017) 'Regulation of Chemokine Function: The Roles of GAG-Binding and Post-Translational Nitration', *Int J Mol Sci*, 18(8).

Thuraisingham, R.C., Nott, C.A., Dodd, S.M. and Yaqoob, M.M. (2000) 'Increased nitrotyrosine staining in kidneys from patients with diabetic nephropathy', *Kidney Int*, 57(5), pp. 1968-72.

Tittelbach-Helmrich, D., Bausch, D., Drognitz, O., Goebel, H., Schulz-Huotari, C., Kramer-Zucker, A., Hopt, U.T. and Pisarski, P. (2014) 'Hyperacute rejection of a living unrelated kidney graft', *Case Rep Med*, 2014, p. 613641.

Topley, N., Steadman, R. and Williams, J.D. (2005) 'Neutrophil activation and renal scarring', *Kidney Int*, 67(6), pp. 2504; author reply 2504-5.

Tripp, R.A., Jones, L. and Anderson, L.J. (2000) 'Respiratory syncytial virus G and/or SH glycoproteins modify CC and CXC chemokine mRNA expression in the BALB/c mouse', *J Virol*, 74(13), pp. 6227-9.

Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T. and Tada, K. (1980) 'Establishment and characterization of a human acute monocytic leukemia cell line (THP-1)', *Int J Cancer*, 26(2), pp. 171-6.

Uhlen, M., Oksvold, P., Fagerberg, L., Lundberg, E., Jonasson, K., Forsberg, M., Zwahlen, M., Kampf, C., Wester, K., Hober, S., Wernerus, H., Bjorling, L. and Ponten, F. (2010) 'Towards a knowledge-based Human Protein Atlas', *Nat Biotechnol*, 28(12), pp. 1248-50.

Urtasun, R., Cubero, F.J., Vera, M. and Nieto, N. (2009) 'Reactive nitrogen species switch on early extracellular matrix remodeling via induction of MMP1 and TNFalpha', *Gastroenterology*, 136(4), pp. 1410-22, e1-4.

Urtasun, R., de la Rosa, L.C. and Nieto, N. (2008) 'Oxidative and Nitrosative Stress and Fibrogenic Response', *Clinics in Liver Disease*, 12(4), pp. 769-790.

Uy, G.L., Rettig, M.P. and Cashen, A.F. (2008) 'Plerixafor, a CXCR4 antagonist for the mobilization of hematopoietic stem cells', *Expert Opin Biol Ther*, 8(11), pp. 1797-804.

Vanheule, V., Boff, D., Mortier, A., Janssens, R., Petri, B., Kolaczkowska, E., Kubes, P., Berghmans, N., Struyf, S., Kungl, A.J., Teixeira, M.M., Amaral, F.A. and Proost, P. (2017) 'CXCL9-Derived Peptides Differentially Inhibit Neutrophil Migration In Vivo through Interference with Glycosaminoglycan Interactions', *Front Immunol*, 8, p. 530.

Vanheule, V., Janssens, R., Boff, D., Kitic, N., Berghmans, N., Ronsse, I., Kungl, A.J., Amaral, F.A., Teixeira, M.M., Van Damme, J., Proost, P. and Mortier, A. (2015) 'The Positively Charged COOH-terminal Glycosaminoglycan-binding CXCL9(74-103) Peptide Inhibits CXCL8-induced Neutrophil

Extravasation and Monosodium Urate Crystal-induced Gout in Mice', *J Biol Chem*, 290(35), pp. 21292-304.

Vanheule, V., Vervaeke, P., Mortier, A., Noppen, S., Gouwy, M., Snoeck, R., Andrei, G., Van Damme, J., Liekens, S. and Proost, P. (2016) 'Basic chemokine-derived glycosaminoglycan binding peptides exert antiviral properties against dengue virus serotype 2, herpes simplex virus-1 and respiratory syncytial virus', *Biochem Pharmacol*, 100, pp. 73-85.

Verkaar, F., van Offenbeek, J., van der Lee, M.M.C., van Lith, L., Watts, A.O., Rops, A., Aguilar, D.C., Ziarek, J.J., van der Vlag, J., Handel, T.M., Volkman, B.F., Proudfoot, A.E.I., Vischer, H.F., Zaman, G.J.R. and Smit, M.J. (2014) 'Chemokine cooperativity is caused by competitive glycosaminoglycan binding', *J Immunol*, 192(8), pp. 3908-3914.

Verleden, G.M., Vanaudenaerde, B.M., Dupont, L.J. and Van Raemdonck, D.E. (2006) 'Azithromycin reduces airway neutrophilia and interleukin-8 in patients with bronchiolitis obliterans syndrome', *American journal of respiratory and critical care medicine*, 174(5), pp. 566-570.

Vielhauer, V., Eis, V., Schlondorff, D. and Anders, H.J. (2004) 'Identifying chemokines as therapeutic targets in renal disease: lessons from antagonist studies and knockout mice', *Kidney Blood Press Res*, 27(4), pp. 226-38.

Vogel, C.F.A., Li, W., Wu, D., Miller, J.K., Sweeney, C., Lazennec, G., Fujisawa, Y. and Matsumura, F. (2011) 'Interaction of aryl hydrocarbon receptor and NF-κB subunit RelB in breast cancer is associated with interleukin-8 overexpression', *Archives of biochemistry and biophysics*, 512(1), pp. 78-86.

Vogt, W. (1995) 'Oxidation of methionyl residues in proteins: Tools, targets, and reversal', *Free Radical Biology and Medicine*, 18(1), pp. 93-105.

Wada, T., Tomosugi, N., Naito, T., Yokoyama, H., Kobayashi, K., Harada, A., Mukaida, N. and Matsushima, K. (1994) 'Prevention of Proteinuria by the Administration of Anti-Interleukin-8 Antibody in Experimental Acute Immune Complex-Induced Glomerulonephritis', *Journal of Experimental Medicine*, 180(3), pp. 1135-1140.

Wang, J., Hu, W., Wang, K., Yu, J., Luo, B., Luo, G., Wang, W., Wang, H., Li, J. and Wen, J. (2016) 'Repertaxin, an inhibitor of the chemokine receptors CXCR1 and CXCR2, inhibits malignant behavior of human gastric cancer MKN45 cells in vitro and in vivo and enhances efficacy of 5-fluorouracil', *Int J Oncol*, 48(4), pp. 1341-52.

Wang, W. and Barger, S.W. (2012) 'Cross-linking of serine racemase dimer by reactive oxygen species and reactive nitrogen species', *Journal of Neuroscience Research*, 90(6), pp. 1218-1229.

Waugh, D.J. and Wilson, C. (2008) 'The interleukin-8 pathway in cancer', *Clin Cancer Res*, 14(21), pp. 6735-41.

Webb, L.M.C., Clark-Lewis, I. and Alcami, A. (2003) 'The gammaherpesvirus chemokine binding protein binds to the N terminus of CXCL8', *Journal of virology*, 77(15), pp. 8588-8592.

Weber, D., Stuetz, W., Bernhard, W., Franz, A., Raith, M., Grune, T. and Breusing, N. (2014) 'Oxidative stress markers and micronutrients in maternal and cord blood in relation to neonatal outcome', *Eur J Clin Nutr*, 68(2), pp. 215-22.

Weber, M., Hauschild, R., Schwarz, J., Moussion, C., de Vries, I., Legler, D.F., Luther, S.A., Bollenbach, T. and Sixt, M. (2013) 'Interstitial dendritic cell guidance by haptotactic chemokine gradients', *Science*, 339(6117), pp. 328-332.

White, J.R., Lee, J.M., Young, P.R., Hertzberg, R.P., Jurewicz, A.J., Chaikin, M.A., Widdowson, K., Foley, J.J., Martin, L.D., Griswold, D.E. and Sarau, H.M. (1998) 'Identification of a potent, selective non-peptide CXCR2 antagonist that inhibits interleukin-8-induced neutrophil migration', *J Biol Chem*, 273(17), pp. 10095-8.

Widdowson, K.L., Elliott, J.D., Veber, D.F., Nie, H., Rutledge, M.C., McCleland, B.W., Xiang, J.N., Jurewicz, A.J., Hertzberg, R.P., Foley, J.J., Griswold, D.E., Martin, L., Lee, J.M., White, J.R. and Sarau, H.M. (2004) 'Evaluation of potent and selective small-molecule antagonists for the CXCR2 chemokine receptor', *J Med Chem*, 47(6), pp. 1319-21.

Williams, G., Borkakoti, N., Bottomley, G.A., Cowan, I., Fallowfield, A.G., Jones, P.S., Kirtland, S.J., Price, G.J. and Price, L. (1996) 'Mutagenesis Studies of Interleukin-8 IDENTIFICATION OF A SECOND EPITOPE INVOLVED IN RECEPTOR BINDING', *Journal of Biological Chemistry*, 271(16), pp. 9579-9586.

Wilson, S., Wilkinson, G. and Milligan, G. (2005) 'The CXCR1 and CXCR2 receptors form constitutive homo- and heterodimers selectively and with equal apparent affinities', *J Biol Chem*, 280(31), pp. 28663-74.

Wisastra, R., Poelstra, K., Bischoff, R., Maarsingh, H., Haisma, H.J. and Dekker, F.J. (2011) 'Antibody-free detection of protein tyrosine nitration in tissue sections', *Chembiochem*, 12(13), pp. 2016-20.

Witko-Sarsat, V., Rieu, P., Descamps-Latscha, B., Lesavre, P. and Halbwachs-Mecarelli, L. (2000) 'Neutrophils: molecules, functions and pathophysiological aspects', *Lab Invest*, 80(5), pp. 617-53.

Wolf, M., Clark-Lewis, I., Buri, C., Langen, H., Lis, M. and Mazzucchelli, L. (2003) 'Cathepsin D specifically cleaves the chemokines macrophage inflammatory protein-1 alpha, macrophage inflammatory protein-1 beta, and SLC that are expressed in human breast cancer', *Am J Pathol*, 162(4), pp. 1183-90.

Wu, B., Zhang, K., Xu, B., Ji, D., Liu, Z. and Gong, D. (2015) 'Randomized Controlled Trial to Evaluate Regional Citrate Anticoagulation Plus Low-Dose of Dalteparin in Continuous Veno-Venous Hemofiltration', *Blood Purif*, 39(4), pp. 306-12.

Xu, D. and Esko, J.D. (2014) 'Demystifying heparan sulfate–protein interactions', *Annual review of biochemistry*, 83, pp. 129-157.

Yan, Z., Liu, J., Xie, L., Liu, X. and Zeng, Y. (2016) 'Role of heparan sulfate in mediating CXCL8-induced endothelial cell migration', *PeerJ*, 4, p. e1669.

Yoshimura, T., Matsushima, K., Oppenheim, J.J. and Leonard, E.J. (1987a) 'Neutrophil chemotactic factor produced by lipopolysaccharide (LPS)-stimulated human blood mononuclear leukocytes: partial characterization and separation from interleukin 1 (IL 1)', *J Immunol*, 139(3), pp. 788-93.

Yoshimura, T., Matsushima, K., Tanaka, S., Robinson, E.A., Appella, E., Oppenheim, J.J. and Leonard, E.J. (1987b) 'Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines', *Proc Natl Acad Sci U S A*, 84(24), pp. 9233-7.

Yuan, J.S., Reed, A., Chen, F. and Stewart Jr, C.N. (2006) 'Statistical analysis of real-time PCR data', *BMC Bioinformatics*, 7.

Yun, J.J., Fischbein, M.P., Laks, H., Fishbein, M.C., Espejo, M.L., Ebrahimi, K., Irie, Y., Berliner, J. and Ardehali, A. (2000) 'Early and late chemokine production correlates with cellular recruitment in cardiac allograft vasculopathy', *Transplantation*, 69(12), pp. 2515-24.

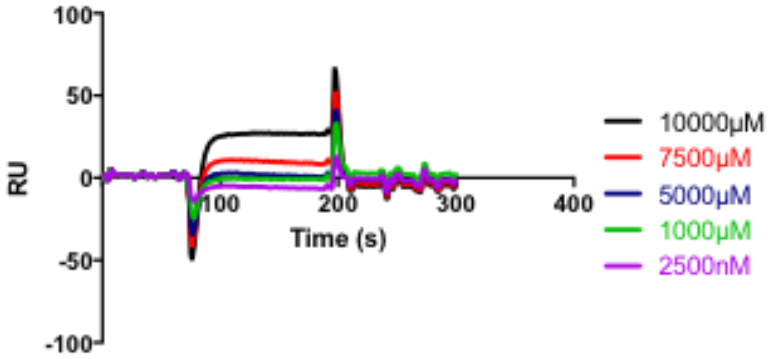
Zaslaver, A., Feniger-Barish, R. and Ben-Baruch, A. (2001) 'Actin filaments are involved in the regulation of trafficking of two closely related chemokine receptors, CXCR1 and CXCR2', *J Immunol*, 166(2), pp. 1272-84.

Zhai, Y., Shen, X.D., Hancock, W.W., Gao, F., Qiao, B., Lassman, C., Belperio, J.A., Strieter, R.M., Busuttil, R.W. and Kupiec-Weglinski, J.W. (2006) 'CXCR3+CD4+ T cells mediate innate immune function in the pathophysiology of liver ischemia/reperfusion injury', *J Immunol*, 176(10), pp. 6313-22.

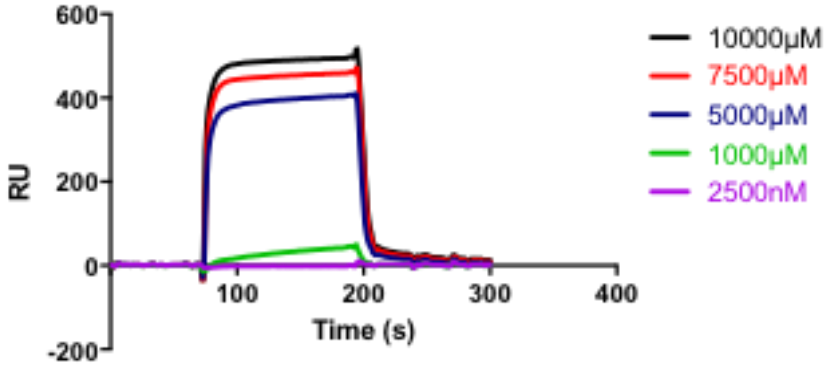
Zhang, Z., Zhong, W., Hall, M.J., Kurre, P., Spencer, D., Skinner, A., O'Neill, S., Xia, Z. and Rosenbaum, J.T. (2009) 'CXCR4 but not CXCR7 is mainly implicated in ocular leukocyte trafficking during ovalbumin-induced acute uveitis', *Exp Eye Res*, 89(4), pp. 522-31.

Zhou, Y., Kucik, D.F., Szalai, A.J. and Edberg, J.C. (2014) 'Human neutrophil flow chamber adhesion assay', *J Vis Exp*, (89).

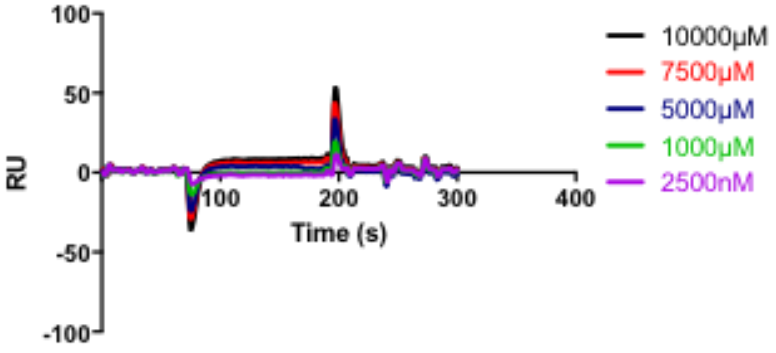
A.



B.



C.



Appendix I. Surface Plasmon Resonance of heparin-CXCL8 peptide.
SPR sensorgram shows heparin-CXCL8 peptide binding with magnified y-axis for WT peptide (A) and scrambled peptide (C), peptides which did not show significant binding at different concentrations, as opposed to E70K peptide (B). Data were analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. *$p<0.05$, **$P <0.01$, ***$P<0.001$. Data is representative of three independent experiments over a single heparin-coated SA chip.

PUBLICATIONS ARISING FROM THIS STUDY

Martínez-Burgo B., Ali S., Kirby J.A., Sheerin N.S., Cobb S.L., Kashanin D. Ischemia-Reperfusion Injury Following Transplantation: Implications for the Modulation of CXCL8 Chemokine Function. [abstract]. Am J Transplant. 2016; 16 (suppl 3).

Thompson, S.*, Martínez-Burgo, B.*, Sepuru, K.M., Rajarathnam, K., Kirby, J.A., Sheerin, N.S., Ali, S. Regulation of Chemokine Function: The Roles of GAG-Binding and Post-translational Nitration. *Int. J. Mol. Sci.* 2017, *18*, 1692. *These authors contributed equally to this work.

Martínez-Burgo B, Cobb S.L., Pohl E., Paul T., Kashanin D., Kirby J.A., Sheerin N.S., Ali S. Regulation of Chemokine Function: The Role of GAG Binding in Neutrophil-Mediated Inflammation. In preparation.

ORAL PRESENTATIONS

Durham University Chemistry Department, Durham UK, November 2014. "Synthesis and Biophysical Characterisation of CXCL8 chemokine peptides and peptides mutants: analysis of GAG-binding ability". Martínez-Burgo B., Cobb S.L., Pohl E., Kirby J., Sheerin N. S., Ali S.

Institute of Cellular Medicine Research Seminar, Newcastle UK, April 2015. "Modulation of chemokine function during ischaemia-reperfusion injury after transplantation". Martínez-Burgo B., Cobb S.L., Kirby J., Sheerin N. S., Ali S.

Cellix Ltd Research Partnership, Dublin, Ireland. October 2015. "Flow-based cell adhesion studies and analysis of chemokine function modulation". Martínez-Burgo B., Cobb S.L., Kashanin D., Paul T., Pohl E., Kirby J., Sheerin N. S., Ali S.

7th North-East Renal Research mini-symposium, Newcastle UK, April 2016. "Chemokine function Modulation: Implications in Ischaemia-Reperfusion Injury after Transplantation". Martínez-Burgo B., Cobb S.L., Kashanin D., Paul T., Pohl E., Kirby J., Sheerin N. S., Ali S.

Open Lab Book (OLB). Chemotaxis and Flow cytometry workshop. Newcastle University Graduate School PGR Innovation Fund 2016 (Proposers: Anna-Lena Dittrich, Irene del Molino del Barrio, Beatriz Martinez-Burgo, Shameem Ladak, Rachel Etherington, Erin Casey, Thomas McDaniel, Alistair Poll, Kate Fraser, Sam Logan). Newcastle UK, May 2016. Martinez-Burgo B, del-Barrio ID.

Institute of Cellular Medicine Research Seminar, Newcastle UK, April 2016. “Chemokine function modulation in the Acute Inflammatory Response: Implications in IRI after Transplantation". Martínez-Burgo B., Cobb S.L., Kashanin D., Paul T., Pohl E, Kirby J., Sheerin N. S., Ali S.

10th North-East Renal Research mini-symposium, Newcastle UK, March 2017. “Regulation of Chemokine Function by Post-translational Nitration and targeted Modulation during Inflammation”. Martínez-Burgo B., Cobb S.L., Kashanin D., Paul T., Pohl E., Kirby J., Sheerin N. S., Ali S.

British Society of Immunology (BSI) - Leukocyte Migration Group (LMG), Leeds UK, June 2017. “The Role of Glycosaminoglycans in CXCL8 Function Regulation during Inflammation”. Martínez-Burgo B., Cobb S.L., Kashanin D., Paul T., Pohl E., Kirby J., Sheerin N. S., Ali S.

Marie Curie Alumni Association (MCAA) – UK Chapter, General Assembly, University College London (UCL) London, March 2018. “Ice-breaker presentation: 2-minute PhD thesis.” Martínez-Burgo B., Cobb S.L., Kashanin D., Paul T., Pohl E., Kirby J., Sheerin N. S., Ali S.

POSTER PRESENTATIONS

Leukocyte Migration in Health and Disease Conference, Birmingham UK, February 2015. “Modulation of chemokine function during ischaemia-reperfusion injury after transplantation”. Martinez-Burgo B, Cobb S, Pohl E, Sheerin NS, Kirby JA, Ali S.

North East postgraduate conference (NEPG), Newcastle UK, 2015 and 2016. Open Lab Book event.

ICM Poster Evening “Blockade to chemokine function with peptides”, Newcastle University UK, April 2015. Martinez-Burgo B, Cobb S, Pohl E, Sheerin NS, Kirby JA, Ali S.

British Summer School in Immunology (BSI), York UK, July 2015. “Ischemia-Reperfusion Injury following transplantation: implications for the modulation of chemokine and S1P functions”. Martinez-Burgo B, Cobb S, Pohl E, Sheerin NS, Kirby JA, Ali S.

Gordon Conference on Chemotactic Cytokines (GRC), Spain, May 2016. “Targeting CXCL8 to Attenuate the Acute Inflammation – Implications of Biochemical Modulation in IRI”- Martinez-Burgo B, Cobb S, Pohl E, Sheerin NS, Kirby JA, Ali S.

Gordon Conference on Chemotactic Cytokines (GRC), Spain, May 2016. "Post-translational modification of chemokines during IRI"- Thompson S, Martinez-Burgo B, Sheerin NS, Kirby JA, Ali S.

American Transplant Congress (ATC), Boston USA, June 2016. Martinez-Burgo B, Ali S, Kirby J, Sheerin N, Cobb S, Kashanin D. Ischemia-Reperfusion Injury Following Transplantation: Implications for the Modulation of CXCL8 Chemokine Function. In: Am J Transplant. 2016; 16 (suppl 3).

European Chemokine and Cell Migration Congress (ECMC), Cardiff UK, September 2017. "Role of Glycosaminoglycans in the Regulation of CXCL8 function". Martínez-Burgo B., Cobb S.L., Kashanin D., Paul T., Pohl E., Kirby J., Sheerin N. S., Ali S.

AWARDS

Newcastle University Travel Award - £550 – allowed attendance of the Chemotactic Cytokine Gordon Seminar and Conference, Girona (Spain), July 2016.

Gordon Research Conference (GRC) Travel Award - £436 - allowed attendance of the Chemotactic Cytokine Gordon Seminar and Conference, Girona Spain, July 2016.

Laboratory commercial partners Travel Support - £200 - allowed attendance of the American Transplant Congress, Boston USA, July 2016.

British Society of Immunology Travel Award - £500 – allowed attendance of the 2nd European Chemokine and Cell Migration Congress, Cardiff UK, September 2017.

Marie Curie Alumni Association (MCAA) Travel Grant - €500 – allowed attendance of the joint event "Marie Sklodowska-Curie (MSC) Polish Chapter and Bridging Science and Business", Gdansk Poland, September 2017.

American Transplant Congress (ATC) 2016, Boston MA USA.

Congress Paper

Ischemia-Reperfusion Injury Following Transplantation: Implications for the Modulation of CXCL8 Chemokine Function

B. Martínez-Burgo[1], S. Cobb[2], D. Kashanin[3], J. Kirby[1], N. Sheerin[1], S. Ali[1]

[1]Institute of Cellular Medicine, Newcastle University, Newcastle-upon-Tyne, United Kingdom
[2]Chemistry Department, Durham University, Durham, United Kingdom
[3]Cellix Ltd., Dublin, Ireland.

Meeting: 2016 American Transplant Congress

Abstract number: D302

Keywords: Kidney transplantation, Neutrophils, Renal ischemia, Stem cells

Ischemia-Reperfusion Injury (IRI) is a major contributor to subsequent graft dysfunction. Chemokines play a key role in leukocyte recruitment towards to the injured tissue. CXCL8 is a critical inflammatory mediator of neutrophil migration, such as in kidney transplantation. Chemokines, in addition to binding with their G protein-coupled receptor (GPCR) on the leukocyte surface, also interact with endothelial glycosaminoglycan (GAG). Therefore, their activity can be modulated by inhibiting either of this system. Our aim was to design peptides targeting the receptor, or GAG-binding region of CXCL8 to block the chemokine activity.

Methods: A range of CXCL8 peptides were synthesised to target receptor-binding domain and GAG-binding domain. Chemical synthesis was carried out by Solid-Phase Peptide Synthesis (SPPS), MS and HPLC, and Analytical HPLC. Initial characterization of neutrophils was done through flow cytometry and qRT-PCR. *In vitro* analysis of peptides was performed through chemotaxis and flow-based cell adhesion assays with primary neutrophils.

Results: CXCL8 peptides and peptide mutants corresponding to the GAG-binding region of CXCL8, mainly its C-terminal α-helix, were chemically synthesised and peptide purity analysed (90%). Selected peptides were studied *in vitro* for their role in neutrophil migration. Flow-based adhesion studies showed very significant decrease in adhesion of primary neutrophils to TNF-α-stimulated HUVECs ($p<0.05$) in co-incubation of CXCL8 with WT peptide or E70K peptide (glutamic acid substituted with lysine), suggesting a competitive role of peptides in displacing CXCL8 from GAG. WT showed less ability than E70K mutant to decrease adhesion, potentially because lysine binds more strongly to polyanionic GAG. However, difference was not significant. Moreover, neutrophil migration was also shown to decrease in preliminary studies of CXCL8 in co-treatment with the peptides ($p<0.001$). Future *in vivo* analysis may help to unravel modulatory roles.

Conclusion: Better understanding of chemokine function and strategies for competitive modulation could offer therapeutic opportunities to protect from neutrophil-derived damage during IRI.

CITATION INFORMATION: Martinez Burgo B, Ali S, Kirby J, Sheerin N, Cobb S, Kashanin D. Ischemia-Reperfusion Injury Following Transplantation: Implications for the Modulation of CXCL8 Chemokine Function. *Am J Transplant.* 2016;16 (suppl 3).

European Chemokine and Cell Migration Conference (ECMC) 2017, UK.

Abstract

Role of glycosaminoglycans in CXCL8 function regulation during inflammation

Martínez-Burgo B.[1], Cobb S.L.[2], Kashanin D.[3], Paul T.[3], Pohl E.[2], Kirby J.[1], Sheerin N. S.[1], Ali S.[1]

[1]Applied Immunobiology and Transplantation Research Group, Institute of Cellular Medicine, Medical School, Newcastle University, UK; [2]Chemistry Department, Durham University, UK; [3]Cellix Ltd., Dublin, Ireland

Introduction:

Ischemia-Reperfusion Injury (IRI), unavoidable after transplantation, contributes to graft dysfunction. Chemokines play a key role in leukocyte recruitment to the injured tissue. CXCL8 is a critical inflammatory mediator of neutrophil migration in a range of transplant surgeries (e.g. kidney transplantation). In addition to binding to their G protein-coupled receptor (GPCR) on the leukocyte surface, chemokines also interact with endothelial surface glycosaminoglycan (GAG). Therefore, chemokine activity can be modulated by several means.

Methods and Results:

GAGs, present on cell surfaces, bind and immobilise chemokines at high concentrations localised to the site of injury, which directs leukocyte migration contributing to local inflammation. We aimed to interfere with this binding using CXCL8 peptides corresponding to the GAG binding C-terminus. CXCL8 peptides were chemically synthesised (90% purity) and their specific GAG-binding ability was shown by Surface Plasmon Resonance (SPR). *In vitro,* neutrophil trans-endothelial chemotaxis assays using either WT or E70K peptide showed significantly reduced CXCL8 mediated migration ($p<0.001$). This suggests a competitive role of the peptides displacing CXCL8 from GAG. Furthermore, flow-based adhesion assays using Cellix Venaflux microfluidic platform aimed to mimic physiological conditions also showed significantly decreased neutrophil adhesion to TNF-α-stimulated HUVECs ($p<0.05$) in presence of WT peptide (60%) or E70K peptide (70%). This effect on neutrophil adhesion highlights the role of the amino acid charge distribution in binding to polyanionic GAG.

Conclusion:

Our study has generated a better understanding of CXCL8 chemokine function and specifically the process involved in targeted competitive modulation. This could offer therapeutic opportunities to protect the organ from neutrophil-derived damage during IRI.

Molecular Sciences

Review

Regulation of Chemokine Function: The Roles of GAG-Binding and Post-translational Nitration

Sarah Thompson [1,†], Beatriz Martínez-Burgo [1,†], Krishna Mohan Sepuru [2], Krishna Rajarathnam [2], John A. Kirby [1], Neil S. Sheerin [1] and Simi Ali [1,*]

[1] Applied Immunobiology and Transplantation Group, Institute of Cellular Medicine, Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH, UK; s.thompson3@ncl.ac.uk (S.T.); b.martinez.burgo@ncl.ac.uk (B.M.-B.); john.kirby@ncl.ac.uk (J.A.K.); neil.sheerin@ncl.ac.uk (N.S.S.)

[2] Department of Biochemistry and Molecular Biology, The University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555, USA; kmsepuru@utmb.edu (K.M.S.); krrajara@utmb.edu (K.R.)

***** Correspondence: simi.ali@ncl.ac.uk; Tel.: +44-(0)191-208-7158

† These authors contributed equally to this work.

Received: 14 June 2017; Accepted: 30 July 2017; Published: 3 August 2017

Abstract: The primary function of chemokines is to direct the migration of leukocytes to the site of injury during inflammation. The effects of chemokines are modulated by several means, including binding to G-protein coupled receptors (GPCRs), binding to glycosaminoglycans (GAGs), and through post-translational modifications (PTMs). GAGs, present on cell surfaces, bind chemokines released in response to injury. Chemokines bind leukocytes via their GPCRs, which directs migration and contributes to local inflammation. Studies have shown that GAGs or GAG-binding peptides can be used to interfere with chemokine binding and reduce leukocyte recruitment. Post-translational modifications of chemokines, such as nitration, which occurs due to the production of reactive species during oxidative stress, can also alter their biological activity. This review describes the regulation of chemokine function by GAG-binding ability and by post-translational nitration. These are both aspects of chemokine biology that could be targeted if the therapeutic potential of chemokines, like CXCL8, to modulate inflammation is to be realised.

Keywords: chemokine-GAG interaction; synthetic peptide chemistry; PTM; chemokine nitration

1. Introduction

Chemokines are small cytokines (8–17 kDa) with chemoattractant properties that are involved in processes ranging from homeostasis to development and tissue repair. They also play essential roles in pathological conditions such as tumourigenesis, cancer metastasis and inflammatory or autoimmune disorders where they mediate the migration of leukocytes to the site of injury [1–4]. Chemokine biology also plays a role in generating immune tolerance [5]. Chemokines are classified into four subfamilies; C, CC, CXC and CX3C in relation to the location/spacing of cysteine residues within the N-terminal region.

The migration of immune cells is mediated through the formation of dynamic chemokine gradients, which are achieved by the binding of chemokines on glycosaminoglycans (GAGs) present on the surface of endothelial cells and in the extracellular matrix [6]. This creates an equilibrium of free and bound monomer and dimer in the proximity of the injury, resulting in haptotactic and chemotactic gradients. This allows directed movement of leukocytes from circulation to the site of injury via chemokine signaling through the G-protein coupled receptors (GPCR) [7,8]. One of many possible GAG-chemokine-receptor interaction scenarios is shown diagrammatically in Figure 1 below.

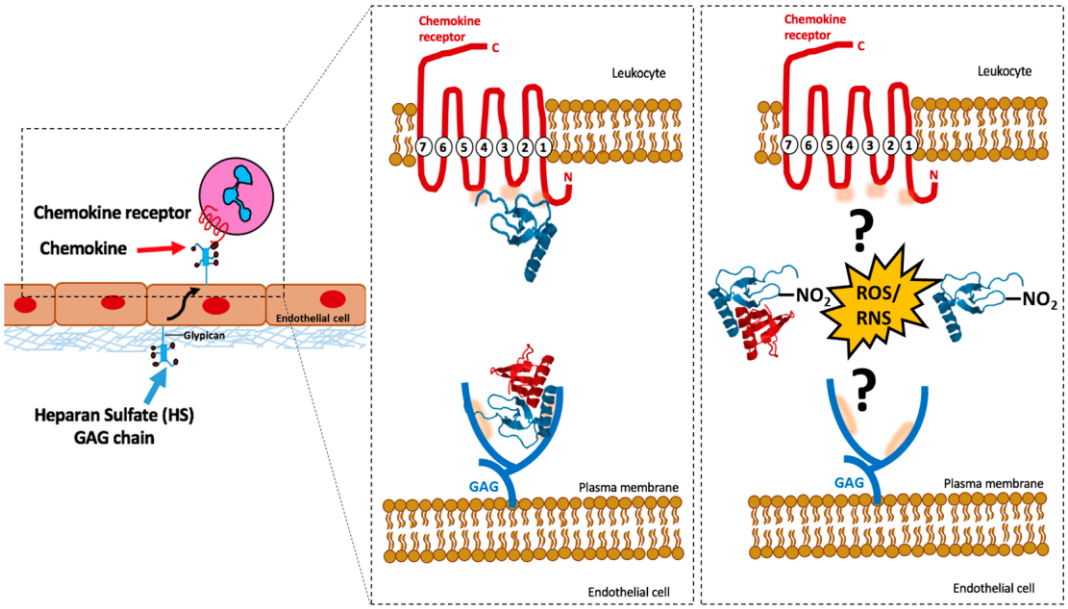


Figure 1. Chemokine interactions with G-protein coupled receptors (GPCRs) and glycosaminoglycans (GAGs). Chemokines bind to GAGs present on the surface of endothelial cells in a dynamic manner, creating a localised chemokine gradient and facilitating the recruitment of leukocytes. Leukocyte recruitment is a multistep process in which leukocytes tether to, roll along, and adhere to the endothelium before transmigrating out of the blood vessels. On the right, magnified image indicating specific chemokine regions involved in GPCR/GAG binding (shaded in orange), and potential consequences of stress (i.e., production of reactive oxygen species/reactive nitrogen species (ROS/RNS respectively)) on regulation of chemokine function. CXCL8 is used as an example chemokine, with the monomer shown in blue and the dimer depicted with one monomer in blue and the other in red.

Regulation of chemokine function is essential in order to prevent excessive inflammation and allow healing after injury. This regulation can occur at many levels and can involve different aspects of chemokine biology, including epigenetic modifications which can affect chemokine production [9], the concentration and oligomeric state of the chemokine (monomer/dimer), the steepness of the chemokine gradient [10,11], the ability of the chemokine to interact with GPCRs and GAGs [7,12], and receptor signaling bias [13,14]. Post-translational modifications (PTMs) such as nitration, glycosylation, phosphorylation, and citrullination also play a critical regulatory role on chemokine function.

In this review, we will describe how chemokine function can be regulated by GAG-binding and post-translational nitration, primarily focusing on CXCL8 as a model CXC chemokine.

2. Chemokine and Chemokine Receptor Interactions

Chemokine receptors all share a similar structure; an extracellular N-terminal domain, seven transmembrane-spanning segments, three extracellular loops, three cytoplasmic loops and a C-terminal segment [15]. Binding of chemokine ligands to their receptors initiates a signaling cascade involving the influx of calcium, which ultimately leads to chemotaxis [7].

Targeting the interaction between chemokines and their receptors is one potential method to regulate the recruitment of leukocytes and modulate inflammation. However, this is limited by the high level of promiscuity displayed by chemokines and their receptors [16]. While some receptor-ligand interactions are specific e.g., CX3CL1-CX3CR1 or CCL20-CCR6 [15], chemokines can often bind multiple receptors, and receptors may in turn be activated by many chemokines, making it difficult to achieve a selective and specific effect when targeting these interactions [17,18]. For example, whereas CXCR1 binds CXCL8 with high affinity

and CXCL6 with lower affinity, CXCR2 binds CXCL1/2/3/5/6/7/8 with high affinity [15,19,20]. In addition, there are atypical receptors (ACKR) such as ACKR1/D6 or ACKR2/DARC, that bind chemokines but do not induce G-protein signaling [21]. They act as chemokine scavengers and are thought to be involved in the regulation of the immune response. For instance, DARC present on erythrocytes is known to induce clearance of circulating CXCL8, affecting the chemokine's ability to stimulate neutrophil recruitment [22], hence having a significant role limiting the inflammatory response.

3. Chemokines and GAG Interactions

GAGs such as heparan sulphate (HS), are long linear polysaccharides consisting of a repeating disaccharide unit [23] frequently covalently attached to a core protein forming proteoglycans. The main classes of proteoglycans are defined according to their distribution, homologies, and function. Common examples of HS proteoglycans are glypican, syndecan and perlecan. GAGs display varying patterns of sulphation, which in addition to carboxyl groups, confer a negative charge which is a critical determinant of chemokine binding [24]. GAGs are located primarily on the surface of endothelial cells, as macromolecular complexes with matrix proteins in the extracellular matrix (ECM), and are also secreted/shed during active inflammation [25]. They can be divided into four groups: heparin/heparan sulphate, chondroitin sulphate/dermatan sulphate, keratan sulphate, and hyaluronic acid (a non-sulphated GAG, non-covalently attached to proteins) shown in Figure 2.

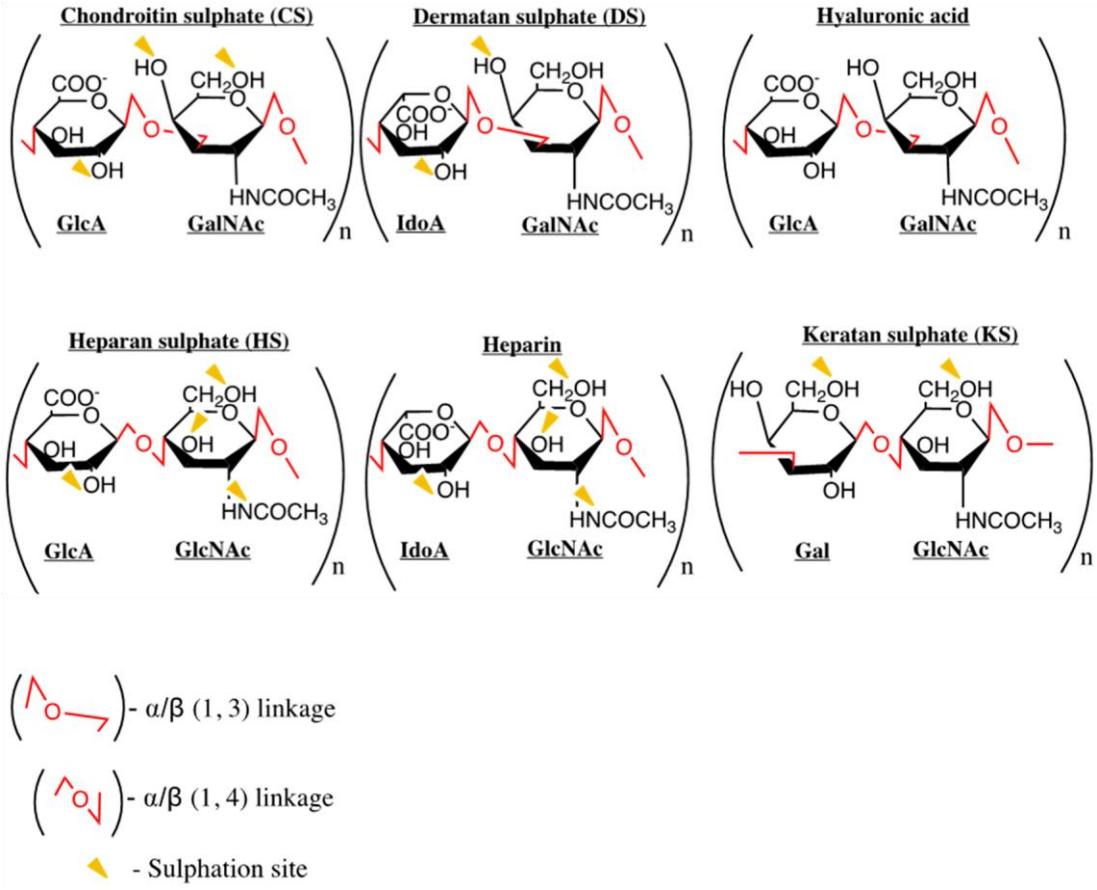


Figure 2. Structure and composition of GAGs. Linkages are shown in red, and sites of sulphation indicated by yellow triangles. The backbone is made up of repeating disaccharide blocks composed of uronic acid (glucuronic acid (GlcA) or iduronic acid (IdoA)), or galactose (Gal) and an amino sugar (*N*-acetyl-galactosamine (GalNAc) or *N*-acetyl-glucosamine (GlcNAc)).

Although chemokines are promiscuous to a degree in terms of receptor binding, data on GAG binding is beginning to show that chemokines interact with GAGs differently, and must be studied individually [26–28].

GAGs have the potential to modulate chemokine heterodimer formation and function, receptor binding and enhance stability [29–31]. GAG binding has been identified as essential for regulating chemotaxis in vivo [12], and could, therefore, be an aspect of chemokine biology to be targeted to modulate function. However, the system is intricate and complex, with the diversity of GAGs (which vary greatly in length, composition and sulphation pattern as shown in Figure 2), the oligomerisation state of the chemokine and the tissue microenvironment all affecting the chemokine-GAG interactions, and increasing the challenge of targeting this aspect of chemokine biology [32,33]. The presence/composition of other molecules beside GAGs also influences binding, for example, studies have shown that sialic acid and mannose-containing glycans are responsible (in addition to GAGs) for the binding of CCL5 to both CCR5+ and CCR5− cells [34]. Furthermore, data are beginning to show that chemokine residues that are involved in receptor interactions are also involved in GAG binding, suggesting GAG-bound chemokines may be unable to bind their receptors [27,29,35,36]. The affinity of the chemokine for different GAGs also changes depending upon whether the chemokine is in the monomer/dimer state, with dimers generally being the higher affinity GAG ligands [37–39]. The ratio of bound to free chemokine is therefore fine-tuned to modulate cellular recruitment.

The highly sulphated and acidic GAGs bind to basic residues within chemokines through electrostatic and H-bonding interactions. This usually involves residues such as arginine, lysine or histidine, which typically form the BBXB or (B)BXX(X/B)BXXB(B) peptide signature, where B is a basic amino acid residue and X a non-conserved amino acid, present in virtually all chemokines. Earlier studies revealed BBXB or (B)BXX(X/B)BXXB(B) as common heparin binding sequences for several chemokines, however, with the characterisation of more GAG-binding regions, it is suggested that GAG-binding motifs can be defined as sequential distant residues that form an optimal binding surface due to spatial orientation in the folded state [40]. This binding regulates the steepness and duration of chemokine gradients, which in turn regulates leukocyte adhesion and infiltration [41,42]. GAG binding has been identified as essential for the induction of chemotaxis, as chemokine mutants that bind receptor but not GAGs have impaired ability to recruit immune cells in vivo [12]. GAG binding could, therefore, be an aspect of chemokine biology to be targeted to modulate function.

Common GAGs: Heparan Sulphate and Heparin

Heparan Sulphate (HS) is an anionic GAG component of the glycocalyx, and the most abundant GAG on the surface of endothelial cells [43]. HS is initially synthesised as a repeating disaccharide composed of the monomeric units *N*-acetyl-glucosamine (GlcNAc) and glucuronic acid. These units may or may not then be modified by a series of biosynthetic reactions within the Golgi. These give rise to *N*-, 6-*O*, or (albeit rarely) 3-*O*-sulphation of the glucosamine (GlcNS), as well as epimerisation and subsequent 2-*O*-sulphation of the glucuronic acid. The family of enzymes responsible for these modifications includes *N*-deacetylase/*N*-sulphotranferases (NDSTs 1/2/3/4), 2-*O*-sulphotransferases (HS2ST), 6-*O*-sulphotransferases (HS6ST), and 3-*O*-sulphotransferases (HS3ST) [44,45]. Mature HS can also be modified on the cell surface glycocalyx by specific sulphatases (SULF1 and SULF2). Additionally, heparanase, an endo-glycosidase, can cleave the HS polymer releasing smaller fragments from the HS proteoglycan complex.

HS serves homeostatic functions, including maintenance of the endothelial barrier permeability and the activation of antithrombin III. During disease or stress, HS can present inflammatory molecules such as chemokines to leukocytes, facilitating selectin-mediated rolling along the endothelial surface, potentially leading to increased integrin adhesion, intravascular arrest and diapedesis [46] (Figure 1).

In the short term, inflammation such as ischaemia-reperfusion injury can induce the shedding of some HS proteoglycans from the endothelial cell surface, which can then bind and sequester chemokines in the blood and reduce leukocyte migration [47–49]. Upon regeneration of the glycocalyx, upregulation of the expression of NDST enzymes increases the extent of *N*-sulphation, which in turn enhances the potential of the endothelium to bind and present pro-inflammatory chemokines [50].
This highlights the flexibility and varied regulation of endothelial GAGs and their ability to modulate chemokine binding and subsequent leukocyte migration.

Heparin, a soluble GAG produced by mast cells [51], has essentially the same backbone structure as HS but a different (more uniform) sulphation pattern [52]. Due to heparin's uniform sulphation pattern, and the

commercial availability of size-fractionated oligosaccharides of many different sizes, heparin is commonly used for structure—function and chemokine-GAG interaction studies.

4. Post-translational Modification of Chemokines

The regulation of chemokines through post-translational modification can affect both receptor and GAG binding, and impact upon chemokine function and biological activity [53]. Many forms of modification can occur, such as cleavages by matrix metalloproteinases and other enzymes, as well as modifications of individual residues by citrullination or nitration [54–57].

The heterogeneous nature of post-translational modifications emphasises the need for better understanding, with some modifications enhancing or abrogating function, and others preventing detection using conventional methods [58,59]. This review article will focus on nitration, which occurs naturally during any situation that involves oxidative stress, such as myocardial infarction or organ transplantation.

5. Nitration of Chemokines

The reactive nitrogen species (RNS) peroxynitrite ($ONOO^-$) is formed from the reaction between nitric oxide (NO) with the superoxide anion (O_2^-) [60,61]. $ONOO^-$ has a very short half-life of around 10 ms at physiological pH, and can affect molecules within a 20 µm range of its production [62]. Effects of $ONOO^-$ include protein nitration, lipid peroxidation, DNA strand breakage and the inhibition of cell signaling and metabolism [63].

NO is produced by nitric oxide synthase enzymes present in many cell types and in all tissues [64–66]. O_2^- is produced by a range of enzymes present in many cell types, including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase within the mitochondria [67–69]. Production of both NO [70] and O_2^- [71,72] increases during inflammation and strategies to reduce production are protective in pre-clinical models of injury [73–75] and in human disease [76].

$ONOO^-$ nitrates tyrosine residues to form 3-nitrotyrosine (3NT), and also modifies tryptophan, cysteine, methionine, lysine and histidine, examples of which are shown in Figure 3 [77,78]. $ONOO^-$ has been implicated in the pathology of many diseases [79], including myocardial reperfusion injury [80], cardiac allograft rejection [81], Fabry disease [82] and kidney diseases including acute tubular necrosis and diabetic nephropathy [83]. An increase in 3NT was also detected in plasma and synovial fluid in osteoarthritis patients [84], in plasma from patients with interstitial lung disease [85] and type II diabetes mellitus [86].

One way that nitration could be affecting disease progression is through its effect on chemokines and leukocyte recruitment. Chemokine nitration usually results in a decrease in function [59] but for some proteins nitration can enhance function [87].

5.1. Effects of Nitration: Detection of Chemokines

Studies have shown that nitration may alter the ability of antibodies to detect proteins, presumably due to epitope modification by the addition of the NO_2 groups. This has been shown for nitrated CCL2 and CXCL12 [54,88]. This may limit the biological relevance of measuring chemokine concentrations as disease biomarkers if only unmodified chemokine is detected. The amount of unmodified chemokine may be a less informative indicator of disease activity than the ratio of modified to unmodified chemokine.

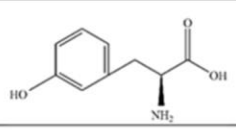
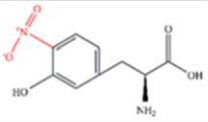
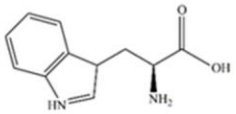
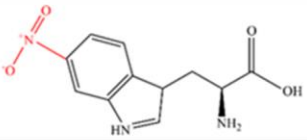
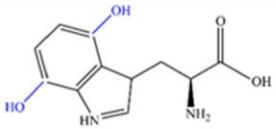
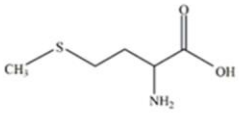
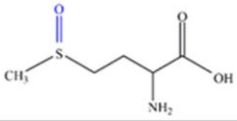
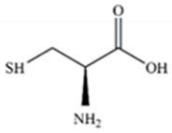
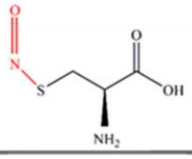
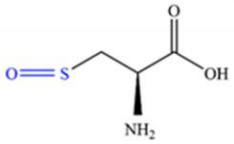
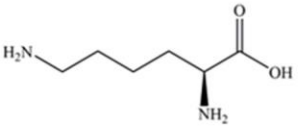
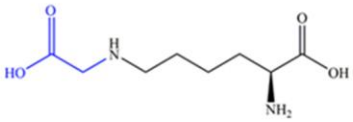
Unmodified Amino Acid	$ONOO^-$ -Modified Amino Acid	
Tyrosine	3-Nitrotyrosine	
		
Tryptophan	6-Nitrotryptophan	Dihydroxytryptophan
		
Methionine	Methionine Sulphoxide	
		
Cysteine	Nitrosylated Cysteine	Oxidized Cysteine
		
Lysine	Nε-(Carboxymethyl)Lysine	
		

Figure 3. Some examples of amino acid modifications by peroxynitrite ($ONOO^-$). Modifications involving oxidation are shown in blue, and modifications involving nitration are shown in red.

5.2. Effects of Nitration: Chemotaxis

Nitration affects the chemotactic function of several chemokines but the biological significance of this is not fully understood. Incubation of chemokine with $ONOO^-$ inhibits monocyte chemotaxis in response to CCL2 and eosinophil chemotaxis in response to CCL5 [89]. Another study found that CCL2 nitrated by intratumoural RNS was unable to induce CD8+ T cell recruitment to the tumour, but could still induce some recruitment of myeloid cells at high concentrations [88]. Nitration of tyrosine 7 in CXCL12 rendered the chemokine unable to induce lymphocyte chemotaxis both in vitro and in vivo [90]. Nitration could therefore be a negative regulator of inflammation; reducing the chemotactic functions of chemokines and thereby reducing leukocyte infiltration.

5.3. Effects of Nitration: Receptor Binding

The effect that nitration has on the ability of a chemokine to bind/signal through its receptor(s) is complex. Nitrated CCL2 was shown to have a reduced affinity for its receptor CCR2, which may explain its failure to induce chemotaxis of CD8+ T cells (as these cells express low levels of the CCR2 receptor), but retained ability to induce migration of myeloid cells (which express very high levels of CCR2) [88]. Nitration of CXCL12 does not affect its ability to bind the CXCR4 receptor, but does impair its ability to signal through this receptor [90]. In cases where nitration reduces receptor activation capacity, this could influence the receptor signaling bias mentioned previously, and increase the specificity of signaling in situations where many chemokines can bind to the same receptor.

To date, all research on nitration in chemokine biology appears to focus upon nitration of the chemokines themselves. The effect that nitration of the chemokine receptors may have is unknown. The Y188A CXCR1 mutant displayed a decreased affinity for CXCL8 compared with the wild type receptor, indicating the importance of this tyrosine residue in receptor-ligand interactions. As tyrosine is a potential target for nitration by $ONOO^-$, nitration of CXCR1 as well as CXCL8 could affect receptor-ligand interactions [91].

5.4. Effects of Nitration: GAG Binding

Whether or not nitration affects GAG-binding depends upon the chemokine in question. For example, nitrated CXCL12 binds GAGs with a similar affinity as wild type CXCL12 [90], but nitrated CCL2 has been shown to have reduced ability to bind both heparin and heparan sulphate when compared to wild type CCL2 [92].

It is worth noting that soluble/immobilized chemokines can initiate different downstream pathways affecting cell migration, as is the case of the CCR7-CCL19/CCL21 axis. This means that in cases where nitration affects GAG binding (i.e., ability of the chemokine to be immobilized), this can in turn affect receptor signaling and therefore regulation of receptor binding, GAG binding and post-translational modifications are all likely to be linked and influence each other [93].

6. GAGs, Nitration and CXCL8 Function

CXCL8 is a potent neutrophil chemoattractant protein released by many cell types in response to a wide range of stimuli including cytokines, microbial products and hypoxia [94,95]. CXCL8 has also been shown to act on other cell types such as lymphocytes and fibroblasts, and is known to promote angiogenesis [96] and leukocyte degranulation. CXCL8 is therefore implicated in both acute and chronic inflammation [97]. Its modulation could influence the pathology of a wide range of diseases and at multiple disease stages [98].

6.1. Targeting CXCL8-GAG Interactions

Studies have shown that while the CXCL8 monomer is the higher affinity receptor ligand, the CXCL8 dimer (which is the higher affinity GAG ligand) is far less competent at CXCR1 receptor activation (although quite active for CXCR2 [99]). This suggests that CXCL8, when GAG-bound, cannot access the receptor [36,100,101]. The C-terminal alpha helix of CXCL8, in addition to some basic residues located within the N-loop, is critical for GAG binding [102,103] due to its positive electrostatic charge. This binding is mediated by basic amino acids (Arg, Lys, His) core residues and by other secondary residues across its sequence (as shown in Figure 4) [41,104]. Targeted substitution of these basic residues for alanine residues reduced in vivo neutrophil recruitment to the peritoneum [8,32], but increased recruitment to the lungs [32,105]. These different recruitment patterns of neutrophils in response to CXCL8 in the mouse peritoneum compared to lung could be attributed to differences in chemokine gradients caused by different GAG structures and compositions between these tissues, and by differences in binding kinetics or diffusion rates, adding further complexity to this topic [32].

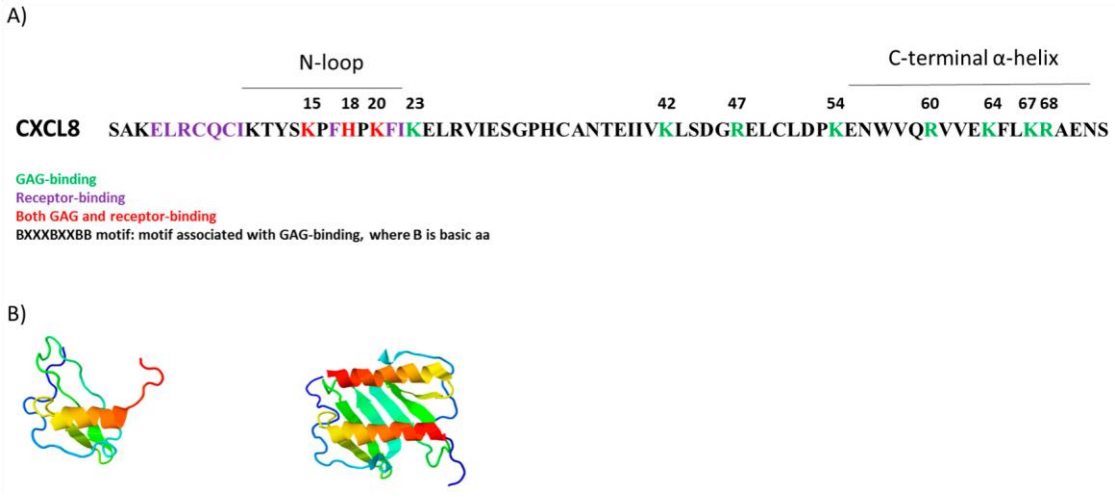


Figure 4. CXCL8 sequence and structure. (**A**) Diagrammatic representation of CXCL8 (72 amino acids long), showing the amino acid sequence. Purple: Receptor-binding residues. Green: GAG-binding residues. Red: residues implicated in both GAG and receptor binding; (**B**) CXCL8 in monomeric form (1KL, PDB) on the left, and dimeric form on the right (1CXCL8, PDB).

6.2. Competitive Displacement of Chemokines

The administration of a GAG, usually heparin, is a method that has been employed in pre-clinical models to modulate inflammation, and is thought to act through disruption of pre-formed chemokine gradients present on cell surface GAGs. Heparin in various forms inhibits leukocyte recruitment to mouse models of arthritis, traumatic brain injury and lipopolysaccharide (LPS) treatment [106–108], although its effectiveness depends upon the dose given and the duration of inflammation [109]. These studies show potential role of GAG mimetics on chemokine-mediated immunomodulation when administered, either local or systemically, however it should be noted that administered heparin is likely to interact with all cytokines due to its highly negative charge, and a more chemokine-specific gradient disruption method could be more beneficial.

Chemokine-GAG interactions also play an essential role in the antiviral immune response. Viruses can evade the chemokine-mediated immune response by expression of viral chemokine binding proteins (vCKBP), which interfere with the GAG binding, GPCR-binding, or both, thus modulating chemokine-mediated migration of leukocytes to the site of infection or tissue damage in vitro and in vivo [110].

6.3. Mutants with Altered GAG Binding

Substitution of basic residues for alanine residues in the GAG binding domain generates a non-GAG binding mutant. These mutant chemokines bind their cognate receptors normally and competitively inhibit binding of their wild type counterparts. Occupation of chemokine receptors by non-GAG binding chemokine variants prevents migration along a gradient and therefore inhibits chemotaxis, as has been shown with CCL5, CCL7 and CXCL12 amongst others [111,112]. Studies have shown that CXCL8 mutants with reduced GAG-binding abilities induced lower recruitment of neutrophils than wild type CXCL8 in the peritoneum but not the lung in vivo [32,105]. This work could be developed in order to create a non-GAG binding CXCL8 mutant with further impaired recruitment capabilities, although clearly biological activity effects in different tissues would need to be fully characterized. Studies conducted on CXCL11, however, showed that a mutant with reduced GAG binding in vitro could still induce cell migration in vivo, highlighting the need for each chemokine to be studied individually [113].

A variant of CXCL8 which has no ability to bind GPCRs but with increased GAG binding affinity inhibits transendothelial migration of neutrophils by displacing CXCL8 from the surface of endothelial cells [114]. A similar study by our group showed that a non-GPCR binding, increased-GAG binding CXCL12 variant showed a reduction in cell migration [115]. A CCL2 mutant with increased GAG binding was shown to displace multiple chemokines which could overcome the issues of redundancy [116], however high concentrations of

chemokine may be required to occupy binding sites on all GAGs [43,117]. This approach represents another potential method of regulating chemokine function.

6.4. Using Peptides to Block Chemokine-GAG Binding

In addition to whole chemokine mutants, small peptide fragments of chemokines, for example, a CXCL9 C-terminal peptide was successfully able to compete with CXCL8, CXCL11 and CCL2 for binding to heparin, HS or other GAGs [118]. This illustrates the therapeutic potential of peptides to inhibit chemokine function by disrupting the interaction between chemokines and GAGs. In addition, these short chemokine fragments might occur naturally, due to cleavage by proteases such as matrix metalloproteinases (MMPs). Unpublished data from our group suggests that both a synthesised wild type (KENWVQRVVEKFLKRAENS) and mutant E70K CXCL8 peptide (KENWVQRVVEKFLKRAKNS) can successfully inhibit the action of the full length wild type protein, and thereby reduce adhesion of leukocytes to an endothelial cell monolayer under physiological flow conditions.

6.5. Nitration and CXCL8 Function

Neutrophils recruited by CXCL8 produce NO and reactive species generating $ONOO^-$. Therefore nitration of CXCL8 is likely to occur at sites of inflammation. This could be a mechanism by which neutrophils limit further chemo-attraction to prevent tissue injury [119]. Unpublished data from our group suggests that nitration significantly reduces the ability of CXCL8 to induce neutrophil chemotaxis in vitro.

How nitration may affect the function of CXCL8 is as yet undetermined. Y13 is a residue in the *N*-loop that is known to be important for receptor signaling and a target for $ONOO^-$. Nitration alters the pK_a making tyrosine residues more acidic, increases the mass of the protein by 45 Da per residue nitrated [54], and is also likely to cause some steric hindrance through increasing the surface area of tyrosine's phenolic ring [120]. The nitration of tyrosine also affects its hydrophobicity, although there are conflicting reports in the literature as to whether this makes the residue more hydrophilic [70] or hydrophobic [120]. It is possible that the hydrophobicity of tyrosine is important in the function of CXCL8 in particular, as a Y13L mutant (which maintains hydrophobicity) showed similar if not slightly increased activity when compared to the wild type [121], but Y13E (hydrophilic) and Y13T (neutral) mutants both showed a decrease in receptor affinity [122]. As the core and secondary GAG-binding residues of CXCL8 described previously include histidines and lysines, which are potential targets of $ONOO^-$, it is likely that modification of CXCL8 by $ONOO^-$ could also affect its GAG binding properties [123].

Tyrosine has also been shown to be an important residue within the receptor CXCR1, as a Y188A mutant version showed decreased affinity for CXCL8 in comparison to the wild type receptor [91]. Therefore nitration of the receptors as well as the ligands (particularly tyrosine residues) could affect chemokine-mediated signal transduction and leukocyte chemotaxis. It is possible that the location and function of the aforementioned residues within any given chemokine (and/or receptor) will determine the specific effects of nitration on each one in turn, highlighting the need for further study.

7. Future Research Directions

Factors such as chemokine-GAG binding and post-translational protein modification are increasingly recognised as important determinants of chemokine function in vivo. How these factors affect chemokine function is only starting to emerge and the challenge is now to understand their effects at a whole organ/organism level during both normal tissue homeostasis and in disease. This is not only of biological interest but it may identify new treatment targets.

In this review we have discussed the importance of chemokine-GAG interactions and how this could be modified by soluble GAGs, mutant chemokines or peptide fragments. There is increasing evidence that this can be done in vitro and in pre-clinical disease models. However, we still do not know what the effect of disrupting chemokine gradients in injured tissues would be nor how this could be applied in the clinic. These are all important areas of future research.

The capacity to mount an effective inflammatory response is paramount. However, to maintain tissue integrity, this response has to be regulated. If we understand the natural mechanisms employed to control inflammation we may be able to exploit this to modify disease. One example discussed in this review is the nitration of chemokines, with resultant loss of activity. Currently, the best methods for detecting chemokine nitration involve NMR analysis or Nano-HPLC, however the development of antibodies specific for nitrated chemokines would better facilitate their study; something our group is currently investigating for nitrated CXCL8. This and similar chemokine modifications could be biological 'off switches', limiting unopposed leukocyte accumulation and tissue damage. Studies are beginning to find links between these different regulatory aspects of chemokine biology, and clearly further study is required to discover how post-translational modifications may affect GAG and GPCR binding in order to contribute to a more complete understanding of the biology of chemokine regulation.

Acknowledgments: This work was supported by The British Heart Foundation (FS/15/19/31327) and a Marie Curie Grant from the European Commission (POSAT 606979, FP7-PEOPLE-2013-ITN).

Author Contributions: Sarah Thompson and Beatriz Martinez-Burgo conceived and wrote the manuscript. Simi Ali, Neil S. Sheerin, Krishna Rajarathnam, Krishna Mohan Sepuru and John A. Kirby provided intellectual in-put and helped with the writing of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ACKR	Atypical chemokine receptor
ECM	Extracellular matrix
GAG	Glycosaminoglycan
Gal	Galactose
GalNAc	*N*-acetyl-galactosamine
GlcA	Glucuronic acid
GlcNAc	*N*-acetyl-glucosamine
GlcNS	Glucosamine
GPCR	G-protein coupled receptor
HS	Heparan sulphate
HS2ST	2-*O*-sulphotransferases
HS6ST	6-*O*-sulphotransferases
HS3ST	3-*O*-sulphotransferases
IdoA	Iduronic acid
LPS	Lipopolysaccharide
MnAb	Monoclonal Antibody
MMPs	Matrix metalloproteinases
NADPH	Nicotinamide adenine dinucleotide phosphate
NDSTs+	*N*-deacetylase/*N*-sulphotranferases
NO	Nitric oxide
O_2^-	Superoxide anion
$ONOO^-$	Peroxynitrite
PTM	Post-translational modifications
RNS	Reactive nitrogen species
SULF1/2	Sulphatases
vCKBP	Viral chemokine binding proteins
3NT	3-Nitrotyrosine

References

1. Lo, D.J.; Weaver, T.A.; Kleiner, D.E.; Mannon, R.B.; Jacobson, L.M.; Becker, B.N.; Swanson, S.J.; Hale, D.A.; Kirk, A.D. Chemokines and their receptors in human renal allotransplantation. *Transplantation* **2011**, *91*, 70–77. [CrossRef] [PubMed]

2. Meloni, F.; Solari, N.; Miserere, S.; Morosini, M.; Cascina, A.; Klersy, C.; Arbustini, E.; Pellegrini, C.; Vigano, M.; Fietta, A.M. Chemokine redundancy in BOS pathogenesis. A possible role also for the CC chemokines: MIP3-β, MIP3-α, MDC and their specific receptors. *Transpl. Immunol.* **2008**, *18*, 275–280. [CrossRef] [PubMed]
3. Collier, J.J.; Sparer, T.E.; Karlstad, M.D.; Burke, S.J. Pancreatic islet inflammation: An emerging role for chemokines. *J. Mol. Endocrinol.* **2017**, *59*, 33–46. [CrossRef] [PubMed]
4. Liao, X.; Pirapakaran, T.; Luo, X.M. Chemokines and chemokine receptors in the development of lupus nephritis. *Mediat. Inflamm.* **2016**, *2016*. [CrossRef] [PubMed]
5. Kulkarni, N.; Pathak, M.; Lal, G. Role of chemokine receptors and intestinal epithelial cells in the mucosal inflammation and tolerance. *J. Leukoc. Biol.* **2017**, *101*, 377–394. [CrossRef] [PubMed]
6. Weber, M.; Hauschild, R.; Schwarz, J.; Moussion, C.; de Vries, I.; Legler, D.F.; Luther, S.A.; Bollenbach, T.; Sixt, M. Interstitial dendritic cell guidance by haptotactic chemokine gradients. *Science* **2013**, *339*, 328–332. [CrossRef] [PubMed]
7. Kufareva, I.; Salanga, C.L.; Handel, T.M. Chemokine and chemokine receptor structure and interactions: Implications for therapeutic strategies. *Immunol. Cell Biol.* **2015**, *93*, 372–383. [CrossRef] [PubMed]
8. Kolaczkowska, E.; Kubes, P. Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* **2013**, *13*, 159–175. [CrossRef] [PubMed]
9. Takahashi, A.; de Andres, M.C.; Hashimoto, K.; Itoi, E.; Oreffo, R.O.C. Epigenetic regulation of interleukin-8, an inflammatory chemokine, in osteoarthritis. *Osteoarthr. Cartil.* **2015**, *23*, 1946–1954. [CrossRef] [PubMed]
10. Das, S.T.; Rajagopalan, L.; Guerrero-Plata, A.; Sai, J.; Richmond, A.; Garofalo, R.P.; Rajarathnam, K. Monomeric and dimeric CXCL8 are both essential for in vivo neutrophil recruitment. *PLoS ONE* **2010**, *5*, e11754. [CrossRef] [PubMed]
11. Dyer, D.P.; Salanga, C.L.; Volkman, B.F.; Kawamura, T.; Handel, T.M. The dependence of chemokine-glycosaminoglycan interactions on chemokine oligomerization. *Glycobiology* **2016**, *26*, 312–326. [CrossRef] [PubMed]
12. Proudfoot, A.E.I.; Handel, T.M.; Johnson, Z.; Lau, E.K.; LiWang, P.; Clark-Lewis, I.; Borlat, F.; Wells, T.N.C.; Kosco-Vilbois, M.H. Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 1885–1890. [CrossRef] [PubMed]
13. Zweemer, A.J.M.; Toraskar, J.; Heitman, L.H.; Ijzerman, A.P. Bias in chemokine receptor signaling. *Trends Immunol.* **2014**, *35*, 243–252. [CrossRef] [PubMed]
14. Zidar, D.A.; Violin, J.D.; Whalen, E.J.; Lefkowitz, R.J. Selective engagement of g protein coupled receptor kinases (GRKS) encodes distinct functions of biased ligands. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 9649–9654. [CrossRef] [PubMed]
15. Rajagopalan, L.; Rajarathnam, K. Structural basis of chemokine receptor function—A model for binding affinity and ligand selectivity. *Biosci. Rep.* **2006**, *26*, 325–339. [CrossRef] [PubMed]
16. Bromley, S.K.; Mempel, T.R.; Luster, A.D. Orchestrating the orchestrators: Chemokines in control of T cell traffic. *Nat. Immunol.* **2008**, *9*, 970–980. [CrossRef] [PubMed]
17. Kleist, A.B.; Getschman, A.E.; Ziarek, J.J.; Nevins, A.M.; Gauthier, P.-A.; Chevigné, A.; Szpakowska, M.; Volkman, B.F. New paradigms in chemokine receptor signal transduction: Moving beyond the two-site model. *Biochem. Pharmacol.* **2016**, *114*, 53–68. [CrossRef] [PubMed]
18. Kunkel, S.L. Promiscuous chemokine receptors and their redundant ligands play an enigmatic role during HIV-1 infection. *Am. J. Respir. Cell Mol. Biol.* **1999**, *20*, 859–860. [CrossRef] [PubMed]
19. Baggiolini, M.; Dewald, B.; Moser, B. Human chemokines: An update. *Annu. Rev. Immunol.* **1997**, *15*, 675–705. [CrossRef] [PubMed]
20. Gijsbers, K.; van Assche, G.; Joossens, S.; Struyf, S.; Proost, P.; Rutgeerts, P.; Geboes, K.; van Damme, J. CXCR1-binding chemokines in inflammatory bowel diseases: Down-regulated IL-8/CXCL8 production by leukocytes in crohn's disease and selective GCP-2/CXCL6 expression in inflamed intestinal tissue. *Eur. J. Immunol.* **2004**, *34*, 1992–2000. [CrossRef] [PubMed]
21. Bachelerie, F.; Graham, G.J.; Locati, M.; Mantovani, A.; Murphy, P.M.; Nibbs, R.; Rot, A.; Sozzani, S.; Thelen, M. New nomenclature for atypical chemokine receptors. *Nat. Immunol.* **2014**, *15*, 207–208. [CrossRef] [PubMed]
22. Loos, T.; Opdenakker, G.; van Damme, J.; Proost, P. Citrullination of CXCL8 increases this chemokine's ability to mobilize neutrophils into the blood circulation. *Haematologica* **2009**, *94*, 1346–1353. [CrossRef] [PubMed]

23. Fu, B.M.; Tarbell, J.M. Mechano-sensing and transduction by endothelial surface glycocalyx: Composition, structure, and function. *Wiley Interdiscip. Rev. Syst. Biol. Med.* **2013**, *5*, 381–390. [CrossRef] [PubMed]
24. Handel, T.M.; Johnson, Z.; Crown, S.E.; Lau, E.K.; Sweeney, M.; Proudfoot, A.E. Regulation of protein function by glycosaminoglycans-as exemplified by chemokines. *Annu. Rev. Biochem.* **2005**, *74*, 385–410. [CrossRef] [PubMed]
25. Mihov, D.; Spiess, M. Glycosaminoglycans: Sorting determinants in intracellular protein traffic. *Int. J. Biochem. Cell Biol.* **2015**, *68*, 87–91. [CrossRef] [PubMed]
26. Sepuru, K.M.; Rajarathnam, K. CXCL1/MGSA is a novel glycosaminoglycan (GAG)-binding chemokine structural evidence for two distinct non-overlapping binding domains. *J. Biol. Chem.* **2016**, *291*, 4247–4255. [CrossRef] [PubMed]
27. Sepuru, K.M.; Nagarajan, B.; Desai, U.R.; Rajarathnam, K. Molecular basis of chemokine CXCL5-glycosaminoglycan interactions. *J. Biol. Chem.* **2016**, *291*, 20539–20550. [CrossRef] [PubMed]
28. Liang, W.G.; Triandafillou, C.G.; Huang, T.Y.; Zulueta, M.M.L.; Banerjee, S.; Dinner, A.R.; Hung, S.C.; Tang, W.J. Structural basis for oligomerization and glycosaminoglycan binding of CCL5 and CCL3. *Proc.Natl. Acad. Sci. USA* **2016**, *113*, 5000–5005. [CrossRef] [PubMed]
29. Brown, A.J.; Joseph, P.R.B.; Sawant, K.V.; Rajarathnam, K. Chemokine CXCL7 heterodimers: Structural insights, CXCR2 receptor function, and glycosaminoglycan interactions. *Int. J. Mol. Sci.* **2017**, *18*, 748. [CrossRef] [PubMed]
30. Poluri, K.M.; Joseph, P.R.B.; Sawant, K.V.; Rajarathnam, K. Molecular basis of glycosaminoglycan heparin binding to the chemokine CXCL1 dimer. *J. Biol. Chem.* **2013**, *288*, 25143–25153. [CrossRef] [PubMed]
31. Crown, S.E.; Yu, Y.; Sweeney, M.D.; Leary, J.A.; Handel, T.M. Heterodimerization of CCR2 chemokines and regulation by glycosaminoglycan binding. *J. Biol. Chem.* **2006**, *281*, 25438–25446. [CrossRef] [PubMed]
32. Gangavarapu, P.; Rajagopalan, L.; Kolli, D.; Guerrero-Plata, A.; Garofalo, R.P.; Rajarathnam, K. The monomer-dimer equilibrium and glycosaminoglycan interactions of chemokine CXCL8 regulate tissue-specific neutrophil recruitment. *J. Leukoc. Biol.* **2012**, *91*, 259–265. [CrossRef] [PubMed]
33. Proudfoot, A.E. Chemokines and glycosaminoglycans. *Front. Immunol.* **2015**, *6*, 246. [CrossRef] [PubMed]
34. Mbemba, E.; Slimani, H.; Atemezem, A.; Saffar, L.; Gattegno, L. Glycans are involved in rantes binding to CCR5 positive as well as to CCR5 negative cells. *Biochim. Biophys. Acta Biomembr.* **2001**, *1510*, 354–366. [CrossRef]
35. Brown, A.J.; Sepuru, K.M.; Rajarathnam, K. Structural basis of native CXCL7 monomer binding to CXCR2 receptor n-domain and glycosaminoglycan heparin. *Int. J. Mol. Sci.* **2017**, *18*, 508. [CrossRef] [PubMed]
36. Singh, A.; Kett, W.C.; Severin, I.C.; Agyekum, I.; Duan, J.; Amster, I.J.; Proudfoot, A.E.I.; Coombe, D.R.; Woods, R.J. The interaction of heparin tetrasaccharides with chemokine CCL5 is modulated by sulfation pattern and PH. *J. Biol. Chem.* **2015**, *290*, 15421–15436. [CrossRef] [PubMed]
37. Sawant, K.V.; Poluri, K.M.; Dutta, A.K.; Sepuru, K.M.; Troshkina, A.; Garofalo, R.P.; Rajarathnam, K. Chemokine CXCL1 mediated neutrophil recruitment: Role of glycosaminoglycan interactions. *Sci. Rep.* **2016**, *6*. [CrossRef] [PubMed]
38. Ziarek, J.J.; Kleist, A.B.; London, N.; Raveh, B.; Montpas, N.; Bonneterre, J.; St-Onge, G.; DiCosmo-Ponticello, C.J.; Koplinski, C.A.; Roy, I. Structural basis for chemokine recognition by a g protein-coupled receptor and implications for receptor activation. *Sci. Signal.* **2017**, *10*, 5756. [CrossRef] [PubMed]
39. Drury, L.J.; Ziarek, J.J.; Gravel, S.; Veldkamp, C.T.; Takekoshi, T.; Hwang, S.T.; Heveker, N.; Volkman, B.F.; Dwinell, M.B. Monomeric and dimeric CXCL12 inhibit metastasis through distinct CXCR4 interactions and signaling pathways. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 17655–17660. [CrossRef] [PubMed]
40. Lortat-Jacob, H.; Grosdidier, A.; Imberty, A. Structural diversity of heparan sulfate binding domains in chemokines. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 1229–1234. [CrossRef] [PubMed]
41. Joseph, P.R.B.; Mosier, P.D.; Desai, U.R.; Rajarathnam, K. Solution NMR characterization of chemokine CXCL8/IL-8 monomer and dimer binding to glycosaminoglycans: Structural plasticity mediates differential binding interactions. *Biochem. J.* **2015**, *472*, 121–133. [CrossRef] [PubMed]
42. Rot, A. Chemokine patterning by glycosaminoglycans and interceptors. *Front. Biosci.* **2009**, *15*, 645–660. [CrossRef]
43. Ali, S.; Hardy, L.A.; Kirby, J.A. Transplant immunobiology: A crucial role for heparan sulfate glycosaminoglycans? *Transplantation* **2003**, *75*, 1773–1782. [CrossRef] [PubMed]
44. Hacker, U.; Nybakken, K.; Perrimon, N. Heparan sulphate proteoglycans: The sweet side of development. *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 530–541. [CrossRef] [PubMed]

45. Ferreras, L.; Sheerin, N.S.; Kirby, J.A.; Ali, S. Mechanisms of renal graft chronic injury and progression to interstitial fibrosis. *Curr. Transplant. Rep.* **2015**, *2*, 259–268. [CrossRef]
46. Bao, X.; Moseman, E.A.; Saito, H.; Petryanik, B.; Thiriot, A.; Hatakeyama, S.; Ito, Y.; Kawashima, H.; Yamaguchi, Y.; Lowe, J.B.; et al. Endothelial heparan sulfate controls chemokine presentation in recruitment of lymphocytes and dendritic cells to lymph nodes. *Immunity* **2010**, *33*, 817–829. [CrossRef] [PubMed]
47. Parish, C.R. The role of heparan sulphate in inflammation. *Nat. Rev. Immunol.* **2006**, *6*, 633–643. [CrossRef] [PubMed]
48. Marshall, L.J.; Ramdin, L.S.P.; Brooks, T.; Shute, J.K. Plasminogen activator inhibitor-1 supports IL-8-mediated neutrophil transendothelial migration by inhibition of the constitutive shedding of endothelial IL-8/heparan sulfate/syndecan-1 complexes. *J. Immunol.* **2003**, *171*, 2057–2065. [CrossRef] [PubMed]
49. Lipowsky, H.H.; Lescanic, A. Inhibition of inflammation induced shedding of the endothelial glycocalyx with low molecular weight heparin. *Microvasc. Res.* **2017**, *112*, 72–78. [CrossRef] [PubMed]
50. Carter, N.M.; Ali, S.; Kirby, J.A. Endothelial inflammation: The role of differential expression of *N*-deacetylase/*N*-sulphotransferase enzymes in alteration of the immunological properties of heparan sulphate. *J. Cell Sci.* **2003**, *116*, 3591–3600. [CrossRef] [PubMed]
51. Mulloy, B.; Lever, R.; Page, C.P. Mast cell glycosaminoglycans. *Glycoconj. J.* **2016**, *34*, 351–361. [CrossRef] [PubMed]
52. Doster, A.; Schwarzig, U.; Zygmunt, M.; Rom, J.; Schuetz, F.; Fluhr, H. Unfractionated heparin selectively modulates the expression of CXCL8, CCL2 and CCL5 in endometrial carcinoma cells. *Anticancer Res.* **2016**, *36*, 1535–1544. [PubMed]
53. Mortier, A.; Van Damme, J.; Proost, P. Regulation of chemokine activity by post-translational modification. *Pharmacol. Ther.* **2008**, *120*, 197–217. [CrossRef] [PubMed]
54. Barker, C.E.; Ali, S.; O'Boyle, G.; Kirby, J.A. Transplantation and inflammation: Implications for the modification of chemokine function. *Immunology* **2014**, *143*, 138–145. [CrossRef] [PubMed]
55. Loos, T.; Mortier, A.; Gouwy, M.; Ronsse, I.; Put, W.; Lenaerts, J.P.; Van Damme, J.; Proost, P. Citrullination of CXCL10 and CXCL11 by peptidylarginine deiminase: A naturally occurring post-translational modification of chemokines and new dimension of immunoregulation. *Blood* **2008**, *112*, 2648–2656. [CrossRef] [PubMed]
56. Proost, P.; Loos, T.; Mortier, A.; Schutyser, E.; Gouwy, M.; Noppen, S.; Dillen, C.; Ronsse, I.; Conings, R.; Struyf, S.; et al. Citrullination of CXCL8 by peptidylarginine deiminase alters receptor usage, prevents proteolysis, and dampens tissue inflammation. *J. Exp. Med.* **2008**, *205*, 2085–2097. [CrossRef] [PubMed]
57. Struyf, S.; Noppen, S.; Loos, T.; Mortier, A.; Gouwy, M.; Verbeke, H.; Huskens, D.; Luangsay, S.; Parmentier, M.; Geboes, K.; et al. Citrullination of CXCL12 differentially reduces CXCR4 and CXCR7 binding with loss of inflammatory and anti-HIV-1 activity via CXCR4. *J. Immunol.* **2009**, *182*, 666–674. [CrossRef] [PubMed]
58. Gole, M.D.; Souza, J.M.; Choi, I.; Hertkorn, C.; Malcolm, S.; Foust, R.F.; Finkel, B.; Lanken, P.N.; Ischiropoulos, H. Plasma proteins modified by tyrosine nitration in acute respiratory distress syndrome. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2000**, *278*, 961–967.
59. Greenacre, S.A.B.; Ischiropoulos, H. Tyrosine nitration: Localisation, quantification, consequences for protein function and signal transduction. *Free Radic. Res.* **2001**, *34*, 541–581. [CrossRef] [PubMed]
60. Lowenstein, C.J.; Snyder, S.H. Nitric oxide, a novel biologic messenger. *Cell* **1992**, *70*, 705–707. [CrossRef]
61. Lim, C.H.; Dedon, P.C.; Deen, W.M. Kinetic analysis of intracellular concentrations of reactive nitrogen species. *Chem. Res. Toxicol.* **2008**, *21*, 2134–2147. [CrossRef] [PubMed]
62. Szabó, C.; Ischiropoulos, H.; Radi, R. Peroxynitrite: Biochemistry, pathophysiology and development of therapeutics. *Nat. Rev. Drug Discov.* **2007**, *6*, 662–680. [CrossRef] [PubMed]
63. Beckman, J.S. Oxidative damage and tyrosine nitration from peroxynitrite. *Chem. Res. Toxicol.* **1996**, *9*, 836–844. [CrossRef] [PubMed]
64. Vanhoutte, P.M.; Zhao, Y.; Xu, A.; Leung, S.W.S. Thirty years of saying no. *Circ. Res.* **2016**, *119*, 375–396. [CrossRef] [PubMed]
65. Mount, P.F.; Power, D.A. Nitric oxide in the kidney: Functions and regulation of synthesis. *Acta Physiol.* **2006**, *187*, 433–446. [CrossRef] [PubMed]
66. De Oliveira, G.A.; Cheng, R.Y.S.; Ridnour, L.A.; Basudhar, D.; Somasundaram, V.; McVicar, D.W.; Monteiro, H.P.; Wink, D.A. Inducible nitric oxide synthase in the carcinogenesis of gastrointestinal cancers. *Antioxid. Redox Signal.* **2016**, *26*, 1059–1077. [CrossRef] [PubMed]
67. Epstein, F.H.; Weiss, S.J. Tissue destruction by neutrophils. *N. Engl. J. Med.* **1989**, *320*, 365–376. [CrossRef] [PubMed]

68. Inauen, W.; Suzuki, M.; Granger, D.N. Mechanisms of cellular injury: Potential sources of oxygen free radicals in ischemia/reperfusion. *Microcirc. Endothel. Lymphat.* **1988**, *5*, 143–155.
69. Biswas, S.K. Does the interdependence between oxidative stress and inflammation explain the antioxidant paradox? *Oxidative Med. Cell. Longev.* **2016**, *2016*, 1–9. [CrossRef] [PubMed]
70. Turko, I.V.; Murad, F. Protein nitration in cardiovascular diseases. *Pharmacol. Rev.* **2002**, *54*, 619–634. [CrossRef] [PubMed]
71. Thompson-Gorman, S.L.; Zweier, J.L. Evaluation of the role of xanthine oxidase in myocardial reperfusion injury. *J. Biol. Chem.* **1990**, *265*, 6656–6663. [PubMed]
72. Gondouin, B.; Jourde-Chiche, N.; Sallee, M.; Dou, L.; Cerini, C.; Loundou, A.; Morange, S.; Berland, Y.; Burtey, S.; Brunet, P.; et al. Plasma xanthine oxidase activity is predictive of cardiovascular disease in patients with chronic kidney disease, independently of uric acid levels. *Nephron* **2015**, *131*, 167–174. [CrossRef] [PubMed]
73. Choi, E.K.; Jung, H.; Kwak, K.H.; Yi, S.J.; Lim, J.A.; Park, S.H.; Park, J.M.; Kim, S.; Jee, D.L.; Lim, D.G. Inhibition of oxidative stress in renal ischemia-reperfusion injury. *Anesth. Anal.* **2017**, *124*, 204–213. [CrossRef] [PubMed]
74. Shin, J.H.; Chun, K.S.; Na, Y.G.; Song, K.H.; Kim, S.I.; Lim, J.S.; Kim, G.H. Allopurinol protects against ischemia/reperfusion-induced injury in rat urinary bladders. *Oxidative Med. Cell. Longev.* **2015**, *2015*, 1–8. [CrossRef] [PubMed]
75. Saavedra, W.F.; Paolocci, N.; John, M.E.S.; Skaf, M.W.; Stewart, G.C.; Xie, J.S.; Harrison, R.W.; Zeichner, J.; Mudrick, D.; Marbán, E.; et al. Imbalance between xanthine oxidase and nitric oxide synthase signaling pathways underlies mechanoenergetic uncoupling in the failing heart. *Circ. Res.* **2002**, *90*, 297–304. [CrossRef] [PubMed]
76. Cappola, T.P.; Kass, D.A.; Nelson, G.S.; Berger, R.D.; Rosas, G.O.; Kobeissi, Z.A.; Marbán, E.; Hare, J.M. Allopurinol improves myocardial efficiency in patients with idiopathic dilated cardiomyopathy. *Circulation* **2001**, *104*, 2407–2411. [CrossRef] [PubMed]
77. Pacher, P.; Beckman, J.S.; Liaudet, L. Nitric oxide and peroxynitrite in health and disease. *Physiol. Rev.* **2007**, *87*, 315–424. [CrossRef] [PubMed]
78. Nagai, R.; Unno, Y.; Hayashi, M.C.; Masuda, S.; Hayase, F.; Kinae, N.; Horiuchi, S. Peroxynitrite induces formation of Nε-(carboxymethyl) lysine by the cleavage of amadori product and generation of glucosone and glyoxal from glucose. *Diabetes* **2002**, *51*, 2833–2839. [CrossRef] [PubMed]
79. Batthyány, C.; Bartesaghi, S.; Mastrogiovanni, M.; Lima, A.; Demicheli, V.; Radi, R. Tyrosine-nitrated proteins: Proteomic and bioanalytical aspects. *Antioxid. Redox Signal.* **2016**, *26*, 313–328. [CrossRef] [PubMed]
80. Lee, W.H.; Gounarides, J.S.; Roos, E.S.; Wolin, M.S. Influence of peroxynitrite on energy metabolism and cardiac function in a rat ischemia-reperfusion model. *Am. J. Physiol. Heart Circ. Physiol.* **2003**, *285*, 1385–1395. [CrossRef] [PubMed]
81. Sakurai, M.; Fukuyama, N.; Iguchi, A.; Akimoto, H.; Ohmi, M.; Yokoyama, H.; Nakazawa, H.; Tabayashi, K. Quantitative analysis of cardiac 3-L-nitrotyrosine during acute allograft rejection in an experimental heart transplantation1. *Transplantation* **1999**, *68*, 1818–1822. [CrossRef] [PubMed]
82. Chimenti, C.; Scopelliti, F.; Vulpis, E.; Tafani, M.; Villanova, L.; Verardo, R.; de Paulis, R.; Russo, M.A.; Frustaci, A. Increased oxidative stress contributes to cardiomyocyte dysfunction and death in patients with fabry disease cardiomyopathy. *Hum. Pathol.* **2015**, *46*, 1760–1768. [CrossRef] [PubMed]
83. Thuraisingham, R.C.; Nott, C.A.; Dodd, S.M.; Yaqoob, M.M. Increased nitrotyrosine staining in kidneys from patients with diabetic nephropathy. *Kidney Int.* **2000**, *57*, 1968–1972. [CrossRef] [PubMed]
84. Ahmed, U.; Anwar, A.; Savage, R.S.; Thornalley, P.J.; Rabbani, N. Protein oxidation, nitration and glycation biomarkers for early-stage diagnosis of osteoarthritis of the knee and typing and progression of arthritic disease. *Arthritis Res. Ther.* **2016**, *18*, 250. [CrossRef] [PubMed]
85. Pennathur, S.; Vivekanandan-Giri, A.; Locy, M.L.; Kulkarni, T.; Zhi, D.; Zeng, L.; Byun, J.; de Andrade, J.A.; Thannickal, V.J. Oxidative modifications of protein tyrosyl residues are increased in plasma of human subjects with interstitial lung disease. *Am. J. Respir. Crit. Care Med.* **2016**, *193*, 861–868. [CrossRef] [PubMed]
86. Aydın, A.; Orhan, H.; Sayal, A.; Özata, M.; S¸ahin, G.; Is¸ımer, A. Oxidative stress and nitric oxide related parameters in type II diabetes mellitus: Effects of glycemic control. *Clin. Biochem.* **2001**, *34*, 65–70. [CrossRef]
87. Balafanova, Z.; Bolli, R.; Zhang, J.; Zheng, Y.; Pass, J.M.; Bhatnagar, A.; Tang, X.-L.; Wang, O.; Cardwell, E.; Ping, P. Nitric oxide (NO) induces nitration of protein kinase Cε (PKCE), facilitating PKCE translocation via enhanced PKCE–

RACK2 interactions a novel mechanism of no-triggered activation of PKCE. *J. Biol. Chem.* **2002**, *277*, 15021–15027. [CrossRef] [PubMed]

88. Molon, B.; Ugel, S.; Del Pozzo, F.; Soldani, C.; Zilio, S.; Avella, D.; De Palma, A.; Mauri, P.; Monegal, A.; Rescigno, M.; et al. Chemokine nitration prevents intratumoural infiltration of antigen-specific T cells. *J. Exp. Med.* **2011**, *208*, 1949–1962. [CrossRef] [PubMed]
89. Sato, E.; Simpson, K.L.; Grisham, M.B.; Koyama, S.; Robbins, R.A. Effects of reactive oxygen and nitrogen metabolites on rantes and IL-5-induced eosinophil chemotactic activity in vitro. *Am. J. Pathol.* **1999**, *155*, 591–598. [CrossRef]
90. Janssens, R.; Mortier, A.; Boff, D.; Vanheule, V.; Gouwy, M.; Franck, C.; Larsen, O.; Rosenkilde, M.M.; Van Damme, J.; Amaral, F.A.; et al. Natural nitration of CXCL12 reduces its signaling capacity and chemotactic activity in vitro and abrogates intra-articular lymphocyte recruitment in vivo. *Oncotarget* **2016**, *7*, 62439–62459. [CrossRef] [PubMed]
91. Leong, S.R.; Kabakoff, R.C.; Hebert, C.A. Complete mutagenesis of the extracellular domain of interleukin-8 (IL-8) type a receptor identifies charged residues mediating IL-8 binding and signal transduction. *J. Biol. Chem.* **1994**, *269*, 19343–19348. [PubMed]
92. Barker, C.E.; Thompson, S.; O'boyle, G.; Lortat-Jacob, H.; Sheerin, N.S.; Ali, S.; Kirby, J.A. CCL2 nitration is a negative regulator of chemokine-mediated inflammation. *Sci. Rep.* **2017**, *7*, 44384. [CrossRef] [PubMed]
93. Hauser, M.A.; Legler, D.F. Common and biased signaling pathways of the chemokine receptor CCR7 elicited by its ligands CCL19 and CCL21 in leukocytes. *J. Leukoc. Biol.* **2016**, *99*, 869–882. [CrossRef] [PubMed]
94. De Oliveira, S.; Reyes-Aldasoro, C.C.; Candel, S.; Renshaw, S.A.; Mulero, V.; Calado, Â. CXCL8 (IL-8) mediates neutrophil recruitment and behavior in the zebrafish inflammatory response. *J. Immunol.* **2013**, *190*, 4349–4359. [CrossRef] [PubMed]
95. Rot, A. Neutrophil attractant/activation protein-1 (interleukin-8) induces in vitro neutrophil migration by haptotactic mechanism. *Eur. J. Immunol.* **1993**, *23*, 303–306. [CrossRef] [PubMed]
96. Mehrad, B.; Keane, M.P.; Strieter, R.M. Chemokines as mediators of angiogenesis. *Thromb. Haemost.* **2007**, *97*, 755. [CrossRef] [PubMed]
97. Kendrick, A.A.; Holliday, M.J.; Isern, N.G.; Zhang, F.; Camilloni, C.; Huynh, C.; Vendruscolo, M.; Armstrong, G.; Eisenmesser, E.Z. The dynamics of interleukin-8 and its interaction with human CXC receptor I peptide. *Protein Sci.* **2014**, *23*, 464–480. [CrossRef] [PubMed]
98. Ranganathan, P.; Jayakumar, C.; Manicassamy, S.; Ramesh, G. CXCR2 knockout mice are protected against DSS-colitis-induced acute kidney injury and inflammation. *Am. J. Physiol. Ren. Physiol.* **2013**, *305*, 1422–1427. [CrossRef] [PubMed]
99. Nasser, M.W.; Raghuwanshi, S.K.; Grant, D.J.; Jala, V.R.; Rajarathnam, K.; Richardson, R.M. Differential activation and regulation of CXCR1 and CXCR2 by CXCL8 monomer and dimer. *J. Immunol.* **2009**, *183*, 3425–3432. [CrossRef] [PubMed]
100. Fernando, H.; Chin, C.; Rösgen, J.; Rajarathnam, K. Dimer dissociation is essential for interleukin-8 (IL-8) binding to CXCR1 receptor. *J. Biol. Chem.* **2004**, *279*, 36175–36178. [CrossRef] [PubMed]
101. Rajarathnam, K.; Prado, G.N.; Fernando, H.; Clark-Lewis, I.; Navarro, J. Probing receptor binding activity of interleukin-8 dimer using a disulfide trap. *Biochemistry* **2006**, *45*, 7882–7888. [CrossRef] [PubMed]
102. Webb, L.M.C.; Clark-Lewis, I.; Alcami, A. The gammaherpesvirus chemokine binding protein binds to the N terminus of CXCL8. *J. Virol.* **2003**, *77*, 8588–8592. [CrossRef] [PubMed]
103. Falsone, A.; Wabitsch, V.; Geretti, E.; Potzinger, H.; Gerlza, T.; Robinson, J.; Adage, T.; Teixeira, M.M.; Kungl, A.J. Designing CXCL8-based decoy proteins with strong anti-inflammatory activity in vivo. *Biosci. Rep.* **2013**, *33*. [CrossRef] [PubMed]
104. Kuschert, G.S.V.; Hoogewerf, A.J.; Proudfoot, A.E.I.; Chung, C.-W.; Cooke, R.M.; Hubbard, R.E.; Wells, T.N.C.; Sanderson, P.N. Identification of a glycosaminoglycan binding surface on human interleukin-8. *Biochemistry* **1998**, *37*, 11193–11201. [CrossRef] [PubMed]
105. Tanino, Y.; Coombe, D.R.; Gill, S.E.; Kett, W.C.; Kajikawa, O.; Proudfoot, A.E.I.; Wells, T.N.C.; Parks, W.C.; Wight, T.N.; Martin, T.R.; et al. Kinetics of chemokine-glycosaminoglycan interactions control neutrophil migration into the airspaces of the lungs. *J. Immunol.* **2010**, *184*, 2677–2685. [CrossRef] [PubMed]
106. Al Faruque, H.; Kang, J.H.; Hwang, S.R.; Sung, S.; Alam, M.M.; Sa, K.H.; Nam, E.J.; Byun, Y.R.; Kang, Y.M. Stepwise inhibition of T cell recruitment at post-capillary venules by orally active desulfated heparins in inflammatory arthritis. *PLoS ONE* **2017**, *12*. [CrossRef] [PubMed]

107. Nagata, K.; Kumasaka, K.; Browne, K.D.; Li, S.; St-Pierre, J.; Cognetti, J.; Marks, J.; Johnson, V.E.; Smith, D.H.; Pascual, J.L. Unfractionated heparin after TBI reduces in vivo cerebrovascular inflammation, brain edema and accelerates cognitive recovery. *J. Trauma Acute Care Surg.* **2016**, *81*, 1088–1094. [CrossRef] [PubMed]
108. Riffo-Vasquez, Y.; Somani, A.; Man, F.; Amison, R.; Pitchford, S.; Page, C.P. A non-anticoagulant fraction of heparin inhibits leukocyte diapedesis into the lung by an effect on platelets. *Am. J. Respir. Cell Mol. Biol.* **2016**, *55*, 554–563. [CrossRef] [PubMed]
109. Arimateia, D.S.; da Silva Brito, A.; de Azevedo, F.M.; de Andrade, G.P.V.; Chavante, S.F. Heparin fails to inhibit the leukocyte recruitment for an extended time following inflammatory stimulus. *Pharm. Biol.* **2015**, *53*, 72–77. [CrossRef] [PubMed]
110. Gonzalez-Motos, V.; Kropp, K.A.; Viejo-Borbolla, A. Chemokine binding proteins: An immunomodulatory strategy going viral. *Cytokine Growth Factor Rev.* **2016**, *30*, 71–80. [CrossRef] [PubMed]
111. O'Boyle, G.; Mellor, P.; Kirby, J.A.; Ali, S. Anti-inflammatory therapy by intravenous delivery of non-heparan sulfate-binding CXCL12. *FASEB J.* **2009**, *23*, 3906–3916. [CrossRef] [PubMed]
112. Johnson, Z.; Kosco-Vilbois, M.H.; Herren, S.; Cirillo, R.; Muzio, V.; Zaratin, P.; Carbonatto, M.; Mack, M.; Smailbegovic, A.; Rose, M.; et al. Interference with heparin binding and oligomerization creates a novel anti-inflammatory strategy targeting the chemokine system. *J. Immunol.* **2004**, *173*, 5776–5785. [CrossRef] [PubMed]
113. Severin, I.C.; Gaudry, J.-P.; Johnson, Z.; Kungl, A.; Jansma, A.; Gesslbauer, B.; Mulloy, B.; Power, C.; Proudfoot, A.E.I.; Handel, T. Characterization of the chemokine CXCL11-heparin interaction suggests two different affinities for glycosaminoglycans. *J. Biol. Chem.* **2010**, *285*, 17713–17724. [CrossRef] [PubMed]
114. Gschwandtner, M.; Strutzmann, E.; Teixeira, M.M.; Anders, H.J.; Diedrichs-Möhring, M.; Gerlza, T.; Wildner, G.; Russo, R.C.; Adage, T.; Kungl, A.J. Glycosaminoglycans are important mediators of neutrophilic inflammation in vivo. *Cytokine* **2017**, *91*, 65–73. [CrossRef] [PubMed]
115. Gschwandtner, M.; Trinker, M.U.; Hecher, B.; Adage, T.; Ali, S.; Kungl, A.J. Glycosaminoglycan silencing by engineered CXCL12 variants. *FEBS Lett.* **2015**, *589*, 2819–2824. [CrossRef] [PubMed]
116. Gerlza, T.; Winkler, S.; Atlic, A.; Zankl, C.; Konya, V.; Kitic, N.; Strutzmann, E.; Knebl, K.; Adage, T.; Heinemann, A.; et al. Designing a mutant CCL2–HSA chimera with high glycosaminoglycan-binding affinity and selectivity. *Protein Eng. Des. Sel.* **2015**, *28*, 231–240. [CrossRef] [PubMed]
117. Bedke, J.; Nelson, P.J.; Kiss, E.; Muenchmeier, N.; Rek, A.; Behnes, C.-L.; Gretz, N.; Kungl, A.J.; Gröne, H.-J. A novel CXCL8 protein-based antagonist in acute experimental renal allograft damage. *Mol. Immunol.* **2010**, *47*, 1047–1057. [CrossRef] [PubMed]
118. Vanheule, V.; Janssens, R.; Boff, D.; Kitic, N.; Berghmans, N.; Ronsse, I.; Kungl, A.J.; Amaral, F.A.; Teixeira, M.M.; Van Damme, J.; et al. The positively charged COOH-terminal glycosaminoglycan-binding CXCL9 (74–103) peptide inhibits CXCL8-induced neutrophil extravasation and monosodium urate crystal-induced gout in mice. *J. Biol. Chem.* **2015**, *290*, 21292–21304. [CrossRef] [PubMed]
119. Greenacre, S.A.B.; Rocha, F.A.C.; Rawlingson, A.; Meinerikandathevan, S.; Poston, R.N.; Ruiz, E.; Halliwell, B.; Brain, S.D. Protein nitration in cutaneous inflammation in the rat: Essential role of inducible nitric oxide synthase and polymorphonuclear leukocytes. *Br. J. Pharmacol.* **2002**, *136*, 985–994. [CrossRef] [PubMed]
120. Souza, J.M.; Peluffo, G.; Radi, R. Protein tyrosine nitration—Functional alteration or just a biomarker? *Free Radic. Biol. Med.* **2008**, *45*, 357–366. [CrossRef] [PubMed]
121. Hammond, M.E.W.; Shyamala, V.; Siani, M.A.; Gallegos, C.A.; Feucht, P.H.; Abbott, J.; Lapointe, G.R.; Moghadam, M.; Khoja, H.; Zakel, J.; et al. Receptor recognition and specificity of interleukin-8 is determined by residues that cluster near a surface-accessible hydrophobic pocket. *J. Biol. Chem.* **1996**, *271*, 8228–8235. [CrossRef] [PubMed]
122. Clark-Lewis, I.; Dewald, B.; Loetscher, M.; Moser, B.; Baggiolini, M. Structural requirements for interleukin-8 function identified by design of analogs and CXC chemokine hybrids. *J. Biol. Chem.* **1994**, *269*, 16075–16081. [PubMed]
123. Alvarez, B.; Ferrer-Sueta, G.; Freeman, B.A.; Radi, R. Kinetics of peroxynitrite reaction with amino acids and human serum albumin. *J. Biol. Chem.* **1999**, *274*, 842–848. [CrossRef] [PubMed]