University of Glasgow

Hewit, Kay D (2014) *Biochemical features important for D6 function.* PhD thesis.

http://theses.gla.ac.uk/5573/

Copyright and moral rights for this thesis are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the Author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the Author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Glasgow Theses Service
http://theses.gla.ac.uk/
theses@gla.ac.uk

Biochemical Features Important for D6 Function

Kay Deborah Hewit B.Sc. (Hons)

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Institute of Infection, Immunity and Inflammation
College of Medical, Veterinary and Life Sciences
University of Glasgow

May 2014

Abstract

Chemokines are the principle regulators of leukocyte migration *in vivo* and function during both normal (homeostatic) and inflammatory conditions to direct leukocytes to appropriate tissue locales. Chemokines mediate their affects by binding to their cognate G-protein coupled receptors (GPCRs) which are expressed on the surface of cells, and generate a signal upon ligand binding resulting in the initiation of a response such as chemotaxis. As well as the classical chemokine receptors which generate a conventional GPCR signal upon ligand binding, there exists a small family of atypical chemokine receptors that are characterised by an inability to mount classical receptor signalling. One of the most prominent members of this family is the atypical chemokine receptor, D6, which can bind at least 14 inflammatory CC chemokines with high affinity, but instead of the generation of a classical G-protein signalling response, D6 internalises ligands and targets them for lysosomal-mediated degradation. This functional attribute makes D6 a highly efficient binder, internaliser and scavenger of inflammatory CC chemokines that has been shown to be important for the resolution of inflammatory responses *in vivo*. Despite its well-studied biological role, very little is known about the structure/function relationships within and around D6 which regulate ligand binding and scavenging.

Glycosaminoglycans have been demonstrated to be important for chemokine sequestration and presentation to many of the conventional chemokine receptors. Consequently, the role of glycosaminoglycans (GAGs) in chemokine presentation to D6 was studied using a cell line which is deficient in the synthesis of proteoglycans (CHO 745). Transfection of these cells with D6 and comparison to transfected WT CHO cells revealed that D6-mediated uptake and internalisation of chemokine is significantly reduced in the absence of GAGs.

The *N*-terminus of D6 is thought to be the principle site for ligand binding, and the ability of D6 to bind all inflammatory CC chemokines makes this region an attractive target for therapeutic manipulation. Therefore a sulphated peptide representing the first 35 amino acids of D6 (D6-N (s)) was synthesised and investigated for its ability to bind D6 ligands. D6-N (s) was shown to neutralise the activity of the inflammatory CC chemokine CCL2 and prevent its interaction with its cognate receptor CCR2 *in vitro*. Importantly D6-N (s) was active, only in a specifically sulphated form, highlighting the importance of sulphated tyrosines for ligand binding.

Considering the functional significance of the synthetic D6 peptide, attempts were made to identify a naturally ‘shed’ D6 *N*-terminal peptide which had been reported previously. Further study demonstrated the ability of the bacterial protease staphopain A, released from *Staphylococcus aureus,* to cleave the *N*-terminus of D6 and suppress its ligand internalisation activity.

Finally, the conserved tyrosine motif present on the *N*-terminus of D6 was investigated more closely. Site-directed mutagenesis and sulphation inhibition of this region revealed the importance of post-translational tyrosine sulphation for ligand binding, internalisation and scavenging of inflammatory chemokines and alluded to the existence of an optimal sulphation pattern for ligand binding.

Overall the results presented in this thesis shed new light on the nature of the molecules around, and the structural features within D6 that contribute to ligand binding and function of this extraordinary receptor. Furthermore, it was shown that a sulphated peptide derived from the *N*-terminus of D6 has the potential to be used therapeutically as a broad-based chemokine scavenger, which may be useful for dampening the effects of excessive chemokine production in chronic inflammatory conditions.

Contents

List of Tables

Chapter 1:

Chapter 2:

Chapter 6:

List of Figures

Chapter 1:

Chapter 3:

Chapter 4:

Chapter 7:

List of accompanying material

Appendix 1: Publications arising from this work:

MCKIMMIE, C. S., SINGH, M. D., **HEWIT, K**., LOPEZ-FRANCO, O., LE BROCQ, M., ROSE-JOHN, S., LEE, K. M., BAKER, A. H., WHEAT, R., BLACKBOURN, D. J., NIBBS, R. J. B. & GRAHAM, G. J. (2013) An analysis of the function and expression of D6 on lymphatic endothelial cells. *Blood*, 121**,** 3768-77.

HEWIT, K. D., FRASER, A., NIBBS, R. J. & GRAHAM, G. J. (2014) The N-terminal region of the atypical chemokine receptor ACKR2 is a key determinant of ligand binding. *J Biol Chem*. 289, 12330-42.

LE BROCQ, M. L., FRASER, A. R., COTTON, G., WOZNICA, K., MCCULLOCH, C. V., **HEWIT, K. D**., MCKIMMIE, C. S., NIBBS, R. J. B., CAMPBELL, J. D. M. & GRAHAM, G. J. (2014) Chemokines as novel and versatile reagents for flow cytometry and cell sorting. *J Immun*, 192, 6120-30.

Acknowledgements

Firstly I would like to thank my supervisor, Professor Gerry Graham, for giving me the great opportunity to do a PhD in the Chemokine Research Group. Thank you for showing me your support both academically and personally through good and difficult times, for keeping me focussed and motivated throughout my research, and for providing me with the opportunity to work in the group post-PhD. I am extremely grateful for all your help, advice and mentorship. I would also like to thank my advisor Professor Rob Nibbs for your interest in my work and for your critique and encouragement. Thank you to my assessors Professor Iain McInnes and Dr Pasquale Maffia for reading my reports and for offering your support and advice. I must also say a huge thank you to my lab mentor and friend Dr Alasdair Fraser. Thank you so much for taking the time to listen to my ideas and for offering your words of wisdom and assistance throughout my PhD.

I would also like to thank my family; firstly my mum Greta for always believing in me and supporting me throughout my university career. I know times have been tough through the years but you have done your best and I hope I have made you proud. Thanks to my dad Billy for always showing an interest in my work and for your encouragement and support. Special thanks to my little sister Cara, and big brother Craig for all your help along the way, I'm proud to be your sister. Lastly thank you to Jamie for being such a special little person in my life!

I'd also like to thank my friends, both within the GBRC and out with. Thanks especially to my oldest and dearest friend Jade, for being there for me day and night, year after year, drama after drama. Thanks to my best friend in the lab; Kenny Pallas. I would not have had nearly as much fun doing my PhD if you had not been here! Hopefully we will stay friends even when you're a golfing mega-star! I also have to thank Dr Samuel Curran for your exceptional and entirely bizarre friendship. You have been sorely missed since you left to become a corporate colossus!

I'd also like to thank every member of the Chemokine Research Group (past and present) and my friends from the other labs on level 3, for your friendship, help and advice. You are a great bunch of people to work with, and I will miss you all when I leave..... Tea and lunch breaks will never be the same!

Lastly I’d like to thank my boyfriend and best friend Zander; without your belief in me, and your unconditional love and support, I would not be the person I am today. I am so grateful to have you in my life, and I know it would have been a thousand times more difficult without you!

‘The future belongs to those who believe in the beauty of their dreams’ Eleanor Roosevelt

Author's declaration

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

Signature .....

Printed name: Kay Deborah Hewit

Abbreviations

ACKR	Atypical chemokine receptor
AF-CCL2	Alexafluor labelled CCL2
AF-CCL22	Alexafluor labelled CCL22
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
APC dye	Allophycocyanin
bp	base pairs
Bio-	Biotinylated
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
cDNA	Complimentary deoxyribonucleic acid
CHBP	Chemokine binding protein
CHO	Chinese hamster ovary
CIA	Type II collagen-induced arthritis
CNS	Central nervous system
CT	Cycle threshold
D6-N (s)	Sulphated D6 *N*-terminal peptide
D6-N (s) NEW	Sulphated D6 *N*-terminal peptide (New batch)
D6-N (non-s)	Non-sulphated D6 *N*-terminal peptide
DARC	Duffy antigen receptor for chemokines
DC	Dendritic cell
DMEM	Dulbecco’s minimal essential medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
EAE	Experimental autoimmune encephalitis
ECL	Extracellular loop
EDC	ethyl(dimethylaminopropyl) carbodiimide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
GAG	Glycosaminoglycan
GCSF	Granulocyte colony stimulating factor
GDP	Guanosine diphosphate
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
HA	Haemagglutinin

HD LEC	Human dermal lymphatic endothelial cell
HEK	Human embryonic kidney (cells)
HEPA	High efficiency particulate air
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEV	High endothelial venule
HHV6	Human herpes virus 6
HIS	Histidine
HIV	Human immunodeficiency virus
HPC	Haematopoietic progenitor cell
HPLC	High-performance liquid chromatography
IBD	Irritable bowel disease
IFNγ	Interferon gamma
IP	Immunoprecipitation
iDC	Immature dendritic cell
IgG	Immunoglobulin G
IL-6	Interleukin 6
IL-12	Interleukin 12
IL-17A	Interleukin 17A
JAK/STAT	Janus kinase / signal transducer and activator of transcription
KO	Knock-out
LEC	Lymphatic endothelial cell
LN	Lymph node
LPS	Lipopolysaccharide
MCS	Multiple cloning site
MCV	*Molluscum contagiosum*
MFI	Mean fluorescence intensity
MI	Myocardial infarction
MMP	Matrix metalloproteinase
NFκB	Nuclear factor kappa B
NHS	*N*-Hydroxysuccinimide
NMR	Nuclear magnetic resonance
NK	Natural killer
NS	Not significantly different
NTP	Non-template control
PAD	Peptidylarginine deiminase
PAPS	3’-phosphoadenosine 5’-phosphosulfate
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.05% Tween 20
PCR	Polymerase chain reaction
PE	Phycoerythrin
Ptx	*Pertussis toxin*
QPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
RBC	Red blood cell
RNA	Ribonucleic acid
RPM	Rotations per minute

RPMI	Roswell park memorial institute (media)
RT	Reverse transcription
RU	Response units
SDS PAGE	Sodium Dodecyl sulphate polyacrylamide gel electrophoresis
siRNA	Small interfering ribonucleic acid
SIV	Simian immunodeficiency virus
SPR	Surface Plasmon Resonance
TAE	Tris acetate ethylenediaminetetraacetic acid
TBP	Tata binding protein
Th1	Type 1 T helper
Th2	Type 2 T helper
Th17	Type 17 T helper
THP-1	Tamm-horsfall protein 1 (cells)
TNFα	Tumour necrosis factor alpha
TPA	12-O-tetradecanoylphorbol-13-acetate
TPST-1	Tyrosylprotein sulphotransferase 1
TPST-2	Tyrosylprotein sulphotransferase 2
UV	Ultra violet
WT	Wildtype
(w/v)	Weight/volume

Chapter 1

Introduction

1.1 Chemokines

The immune and inflammatory response works as an intricately regulated system involving a wide array of different signalling molecules and receptors. In the late 1980's, a specific subset of cytokines was discovered that regulate immune cell migration (Rot and von Andrian, 2004) . These chemotactic molecules were originally referred to as 'chemotactic cytokines' but are now collectively known as chemokines (Luster, 1998). One way in which chemokines can be distinguished from other cytokines is that they work through G-protein coupled receptors (GPCRs). The primary immunological role of chemokines is to co-ordinate the migration of leukocytes in both physiological and pathological contexts (Rollins, 1997). Chemokines and their receptors are therefore essential to the orchestration and regulation of immune cell movement, and it comes as no great surprise that they play an important role in several diseases characterised by inflammation and cell infiltration, including many autoimmune diseases and cancer (Rossi and Zlotnik, 2000).

Despite their function as trafficking molecules, chemokines have also been assigned many other important functional attributes, such as the regulation of angiogenesis (Belperio et al., 2000), cell proliferation (Coussens and Werb, 2002), apoptosis susceptibility (Luster, 1998), and stem cell mobilisation and quiescence (Lapidot and Petit, 2002, Graham et al., 1996, Graham et al., 1990). These pleiotropic molecules are therefore essential to normal immune function, yet there is much about their roles still to be elucidated.

1.1.1 Structure and Characterization of Chemokines

Chemokines are a group of small (8-12kDa) proteins which can be divided into four subfamilies based upon their structure, and specifically, the composition of a conserved cysteine motif that is present in the mature sequence of all chemokines. Most chemokines have two cysteine residues in the N-terminal motif, however there are exceptions to this rule. Chemokine genes possess a high degree of homology, suggesting that they arose by duplication of an ancestral gene. For example, in humans, the inflammatory CC chemokine genes are mostly located on chromosome 17, whereas the inflammatory CXC chemokine genes are found on chromosome 4, suggesting a translocation event resulting in divergence into the two main chemokine subfamilies (Baggiolini et al., 1994, Murphy et al., 2000).

Family Name	Description	Nomenclature of Chemokines in Family
CC	Juxtaposition of the first two cysteines	CCL1 to CCL28
CXC	Single variable amino acid between first two cysteines	CXCL1 to CXCL17
CX3C	Three amino acids between the first two cysteines	CX3CL1
XC	Lacks the first and third cysteines seen in the other family members.	XCL1 and XCL2

Table 1-1: Structural categorization and nomenclature of the four chemokine subfamilies. Created using information from (Zlotnik et al., 2006).

Table 1-1 describes the four chemokine subfamilies detailing the nature of the cysteine motif in each. Disulphide bonding between the first and third cysteine residues and the second and fourth cysteine residues respectively stabilise the tertiary structure of chemokines, which accounts for the exceptionally analogous tertiary structure observed throughout the chemokine family, despite their limited amino acid sequence similarity (Zlotnik et al., 2006). **Figure 1-1** illustrates the structure of the chemokine classes.

1.1.1.1 Post-translational modification of chemokines

Isoforms, splice variants, polymorphisms and enzymatically processed forms all increase the number of different chemokine molecules that can naturally influence the activity of immune and stromal cells (Struyf et al., 2003).

The flexible *N*-terminal region of the chemokine contains both activation and binding domains, critical for effective chemokine interaction with their cognate receptors. Mutagenesis studies have demonstrated the importance of the *N*-terminal region for binding to receptors, as well as an ‘N-loop’ which has also been emphasized as a receptor binding region (Campanella et al., 2003, Clarklewis et al., 1995). See **Figure 1-2**.

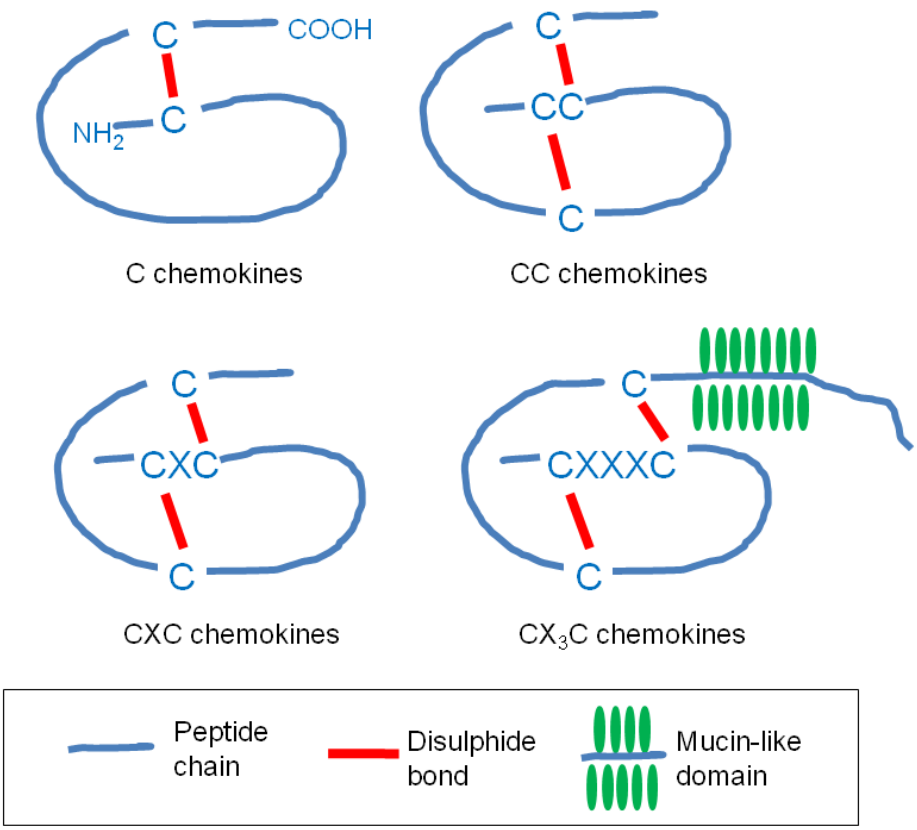


Figure 1-1: Two-dimensional representation of chemokine structure within the four subfamilies.
Note the structural features of CX_3C chemokines are described in section **1.1.2.3.** Created using information from (Zlotnik et al., 2006).

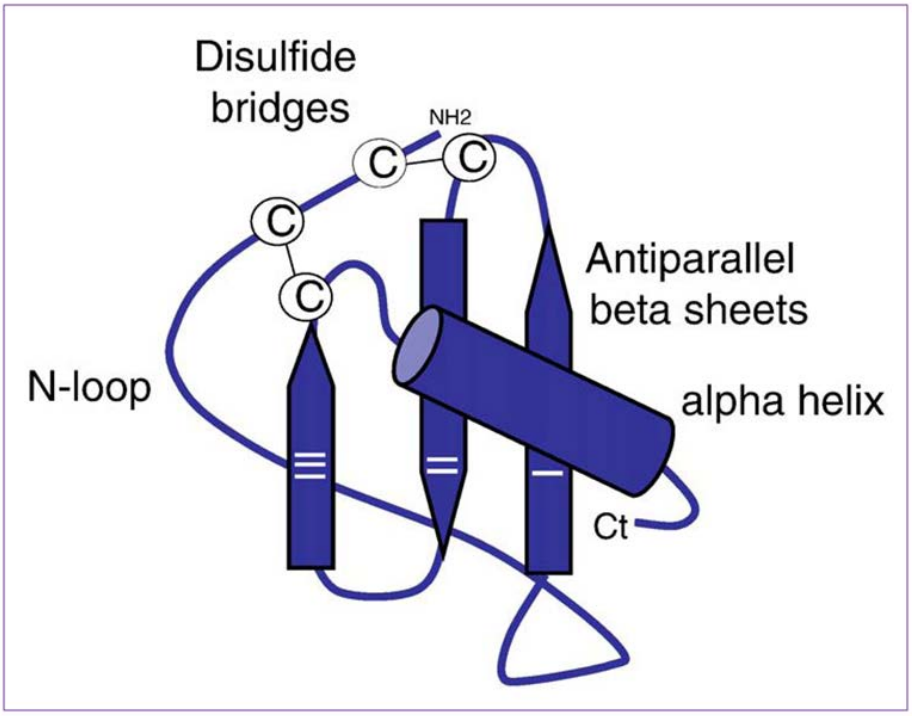


Figure 1-2: Tertiary structure of a chemokine
Diagrammatical representation of a chemokine which highlights typical structural features including the positioning of the disulphide bridges and the N-loop. Taken from (Galzi et al., 2010).

Proteolytic cleavage of the *N*-terminal region by proteases including matrix metalloproteinases (MMPs) has been shown to exert specific functional effects on many chemokines (Parks et al., 2004, Mortier et al., 2011). For example MMP2 can act antagonistically by cleaving the first four amino acids from the *N*-terminal of CCL7, creating a truncated form that is still able to bind to, but not activate, its cognate receptors (McQuibban et al., 2000). In contrast, CXCL8 processing by MMP9 has been shown to distinctly increase its chemotactic activity (Van den Steen et al., 2000). Proteolytic cleavage of CCL4 and CCL5 by another protease, CD26; which is a leukocyte activation marker that possesses dipeptidyl peptidase IV activity, also results in changes in chemokine activity. Truncation of CCL4 expands its receptor reactivity, rendering it capable of activating CCR1 and CCR2, as well as its natural receptor CCR5 (Guan et al., 2002), whereas truncation of CCL5 results in reduced activity via CCR1, but not CCR5 (Oravecz et al., 1997).

Citrullination is also a natural post-translational modification of many chemokines which can alter their biological activities (Mortier et al., 2011). This involves the conversion of a specific arginine residue to citrulline by the enzyme peptidylarginine deiminase (PAD). Importantly, citrullination also occurs on peptides which are implicated as autoantigens in driving the pathology of rheumatoid arthritis (RA) (Suzuki et al., 2003). Citrullination was first demonstrated in the chemokine system with CXCL8, whereby site-specific citrullination of arginine in position 5 of the CXCL8 amino acid sequence inhibits proteolytic processing by MMP9. This reduces the affinity of CXCL8 for binding to glycosaminoglycans (GAGs), its efficiency to signal through CXCR2, and ultimately its ability to induce neutrophil extravasation, therefore dampening the inflammatory response (Proost et al., 2008). Immunoregulation by citrullination of CXCL10 and CXCL11 (Loos et al., 2008) and more recently CXCL5 (Mortier et al., 2010) has also been demonstrated, suggesting the possibility that many more chemokines are also processed in this way.

1.1.1.2 Chemokine dimerisation and aggregation

Chemokines, although often thought to exert their functions while in monomeric form, are also capable of forming dimers and higher order oligomers. This was first shown with CCL3 which can form extensive higher order aggregates (Graham et al., 1994). Notably, chemokine dimerisation and aggregation often involves their initial mobilisation to glycosaminoglycans (GAGs), a topic which will be discussed further in section **1.1.4**. For

example CCL5 forms higher-order oligomers after its initial immobilisation by glycosaminoglycans (GAGs) on the surface of endothelium, and this is crucial for CCR1-mediated leukocyte arrest (Proudfoot et al., 2003, Baltus et al., 2003). Chemokine dimerisation is thought to play specific and differential roles related to leukocyte function. Recent studies have demonstrated that CCL2 dimers, which were created artificially by the introduction of a disulphide bond at the dimer interface, cannot bind nor activate CCR2 (Tan et al., 2012). Conversely, CXCL1 dimers, that were created using similar methods, can potently activate CXCR2 (Ravindran et al., 2013). Interestingly oligomeric CXCL12 variants, are able to halt chemotaxis due to inducing differential signalling pathways upon binding to CXCR4. This had consequences in a murine metastasis model whereby CXCR4-mediated metastasis was inhibited by exogenous administration of high concentrations of dimeric CXCL12 (Drury et al., 2011). Following this, synthetic covalently locked dimers of CXCL12 have been engineered which show enhanced stability in serum compared to wtCXCL12 and which have been suggested as potential anti-metastatic drugs (Takekoshi et al., 2012).

Heterodimerisation, whereby two different chemokines join, has also been shown to occur with important functional significance. In an atherosclerosis mouse model, CXCL4 and CCL5 heterodimers enhanced CCL5's ability to attract monocytes. This effect was shown to be abrogated with the introduction of peptides to block the CXCL4-CCL5 heterodimerisation interface, thereby attenuating monocyte recruitment (Koenen et al., 2009). Conversely numerous studies have reported that mutating sites on chemokines to abrogate dimer formation does not alter their ability to bind and activate their cognate receptors (Gong and Clarklewis, 1995, Gong et al., 1996, Fernando et al., 2004). Therefore this aspect of chemokine biology is still an area of debate and may be chemokine specific.

1.1.2 Chemokine nomenclature and classification

Chemokines were originally named based upon their observed functional characteristics; for example CCL3 was originally termed Macrophage inflammatory protein 1 alpha (Mip-1α) because of its ability to elicit macrophage movement towards sites of inflammation (Sherry et al., 1988). However as the number of newly discovered chemokines increased, the variety of different names to describe them became increasingly confusing. The situation was exacerbated still during the 'data-mining' era when single chemokines were given multiple names by different research groups. The systematic nomenclature system

described in **Table 1-1** and **Table 1-2** was implemented in 2000 to simplify the considerable, and confusing, collection of names that were being employed to define chemokines. The number at the end of the name corresponds to the order of classification of that chemokine family member; consequently CXCL1 is the first CXC chemokine to be recognized and CXCL17 is the latest. The nomenclature is based upon structure, so chemokines are defined independently of their function (Graham, 2009, Lukacs and Harrison, 2007). **Table 1-2** summarises each chemokine and cognate receptor.

1.1.2.1 The CC chemokines

The CC chemokine subfamily contains the largest number of members, with 28 identified to date. The majority of CC chemokines are functionally associated with inflammatory conditions, e.g. are secreted by damaged or infected tissue to encourage migration of leukocytes to the site of insult. For instance, CCL3 and CCL4 have been shown to be important during a murine model of cutaneous granuloma formation. During this model, neutrophils are recruited to sites of skin injury and release CCL3 and CCL4 which in turn were observed to be crucial for the influx of macrophages to the site of inflammation (von Stebut et al., 2003). These chemokines, along with CCL5, have also been implicated in inflammatory responses against infectious pathogens. For example, in *Toxoplasma gondii* infections, the binding of CCL3, CCL4 and CCL5 to CCR5 promotes the release of the cytokine IL-12 from dendritic cells, which results in an enhanced Th1 immune response. This response is important for the development of cellular immunity to infectious agents and demonstrates the importance of the chemokine system, not just for the recruitment of immune cells, but also for the initiation of adaptive immunity (Aliberti et al., 2000).

Homeostatic CC chemokines include CCL19 and CCL21, which are constitutively produced and are not normally induced by inflammation. Both are produced by stromal cells within T-cell areas of lymphoid tissue, and CCL21 is also produced by high endothelial venules (HEVs) and lymphatic endothelial cells (LECs). Together with their receptor CCR7, CCL19 and CCL21 promote the homing of T cells and dendritic cells (DCs) to lymphoid T zones where T cell priming occurs. Therefore their function is critical for bringing antigen presenting cells (APCs) and lymphocytes together for the initiation of adaptive immune responses (Luther et al., 2002, Comerford et al., 2013).

1.1.2.2 The CXC chemokines

The CXC chemokine subfamily is the second largest, with 17 members to date. Some of the CXC chemokines display diverse roles, one of which is the regulation of angiogenesis. In keeping with this, the CXC chemokines can be divided into two groups based on the presence, or absence, of a specific sequence motif known as the 'ELR motif'. This motif occurs before the first *N*-terminal cysteine residue and is a sequence of 3 amino acids; glutamic acid (E), leucine (L) and arginine (R). CXC chemokines that contain this motif (CXCL1, 2, 3, 5, 6, 7 and 8) tend to be angiogenic, whereas members that lack the motif (CXCL4, 9, 10, 11 and 14) tend to be angiostatic (Belperio et al., 2000). Mutagenic studies on CXCL8 have demonstrated loss of angiogenic function when the ELR motif is removed. Conversely a mutant of CXCL9 expressing the ELR motif is able to induce angiogenic activity (Strieter et al., 1995). It must be noted, however, that not all CXC chemokines follow the rule that ELR motif presence/absence equals angiogenic/angiostatic activity. CXCL12 lacks the ELR motif but can still act as an angiogenic factor, however the way in which it does so differs from classical angiogenic activity exerted through CXCR2 (Strieter et al., 2005). The presence of the ELR motif in CXC chemokines is also required for neutrophil activation and recruitment. This was demonstrated experimentally when a CXCL4 ELR^+ mutant was shown to be capable of initiating neutrophil recruitment by binding to CXCR2, whereas wildtype CXCL4 could not (Clarklewis et al., 1993).

1.1.2.3 XC and CX_3C chemokines

Despite being thought of as minor components of the large chemokine family, XC and CX_3C chemokines possess unique features and generally have less overlapping functionality than the other chemokine subfamilies (Stievano et al., 2004). The XC subfamily has two members; XCL1 and XCL2, which are highly homologous and only differ in two amino acids at positions 7 and 8 of the full length protein (Yoshida et al., 1996). XCL1 is produced by T lymphocytes and natural killer (NK) cells in response to infection or inflammation (Lei and Takahama, 2012). CX_3CL1 is the only member of the CX_3C chemokine family and unlike the majority of chemokine proteins, with the exception of CXCL16 (Matloubian et al., 2000), CX_3CL1 is not typically secreted, but rather expressed on the cell surface, anchored to the membrane via a mucin-like stalk which contains a transmembrane domain (Bazan et al., 1997). CX_3CL1 therefore acts as a cell-adhesion receptor, able to arrest cells under physiological flow conditions (Haskell et al.,

1999, Fong et al., 1998). However, a soluble chemo-attractant version of CX_3CL1 can also be released from the cell surface following enzymatic cleavage of the mucin stalk (Tsou et al., 2001). Soluble CX_3CL1 triggers migration of natural killer (NK) cells, cytotoxic T lymphocytes and macrophages, whereas the membrane-bound form arrests and captures these cells, which in turn augments their migratory response upon secondary stimulation by other chemokines (Umehara et al., 2004).

1.1.2.4 Viral chemokines and chemokine receptor blockers

Viral genomes often express proteins which act as chemokine homologs and this is usually advantageous to the virus and enhances its ability to cause disease (Alcami, 2003). The human herpesvirus 6 (HHV6) infects mononuclear cells and encodes a CCL3 homolog; U83 which is capable of calcium mobilization and chemotactic activation (Zou et al., 1999). This has been postulated to aid virus production by activating and trafficking mononuclear cells to sites of viral replication. Conversely, the human pox virus *Molluscum contagiosum* (MCV), encodes a highly selective viral CC chemokine homolog; MC148 that binds to CCR8 but does not induce activation or migration and antagonistically blocks CCL1 (Luttichau et al., 2000). This emphasizes the importance of normal chemokine-induced receptor signalling for host defence against viral pathogens.

1.1.2.5 Chemokine binding proteins (CHBPs)

Interestingly, various pathogens have developed broad specificity chemokine antagonists that are able to destabilize the natural host immune and inflammatory responses. For example the murine gamma herpesvirus MHV-68 encodes a protein named M3 which binds chemokines from all four subfamilies. Such binding blocks the interaction of host chemokines with their receptors (Parry et al., 2000, Epperson et al., 2012). A variety of chemokine binding proteins (CHBPs) are also secreted in the saliva of blood-sucking parasites such as ticks. These proteins have been termed Evasins, and subsequent structural analysis has revealed novel folds in these proteins which are distinct from those of viral CHBPs. Importantly, *in vivo* analyses provided evidence that such chemokine blockers are capable of repressing inflammatory disease (Deruaz et al., 2008).

CC Chemokine Family

Systematic Name	Alternative name(s)	Receptor(s)
CCL1	I-309, TCA-3	CCR8
CCL2	MCP-1, MCAF, JE	CCR2
CCL3	MIP-1αS, MIP-1α	CCR1, CCR5
hCCL3L1	MIP-1αP, MIP-1α	CCR1, CCR3, CCR5
CCL4	MIP-1β	CCR5
CCL5	RANTES	CCR1, CCR3, CCR5
mCCL6	C10, MRP-1	CCR1
CCL7	MCP-3, MARC	CCR1, CCR2, CCR3, CCR5
CCL8	MCP-2	CCR2, CCR3
mCCL9	MIP-1γ, MRP-2, CCF18	CCR1
mCCL10	MIP-1γ, MRP-2, CCF18	CCR1
CCL11	Eotaxin	CCR3, *CXCR3*
mCCL12	MCP-5	CCR2
hCCL13	MCP-4	CCR2, CCR3
hCCL14	HCC-1	CCR1
hCCL15	HCC-2, Lkn-1, MIP-1δ	CCR1, CCR3
hCCL16	HCC-4, LEC, LCC-1	CCR1, CCR2, CCR5
CCL17	TARC	CCR4
hCCL18	DC-CK1, PARC	CCR8
CCL19	ELC, MIP-3β, exodus-3	CCR7
CCL20	MIP-3α, LARC, exodus-1	CCR6
CCL21	SLC, 6Ckine, exodus-2	CCR7
CCL22	MDC, STCP-1, ABCD-1	CCR4
hCCL23	MPIF-1	CCR1
CCL24	Eotaxin-2, MPIF-2	CCR3
CCL25	TECK	CCR9
CCL26	Eotaxin-3	CCR3, *CCR1*, *CCR2*, *CCR5*, *CX_3CR1*
CCL27	C-TACK, PESKY, Eskine	CCR10
CCL28	MEC	CCR3, CCR10

CXC Chemokine Family

Systematic Name	Alternative name(s)	Receptor(s)
CXCL1	GROα, KC	CXCR1, CXCR2
CXCL2	GROβ, MIP-2	CXCR2
CXCL3	GROγ, DCIP-1	CXCR2
CXCL4	PF4	CXCR3B
CXCL5	ENA-78, GCP-2, LIX	CXCR2
hCXCL6	GCP-2	CXCR1, CXCR2
hCXCL7	NAP-2	CXCR2
hCXCL8	IL-8	CXCR1, CXCR2
CXCL9	Mig	CXCR3, *CCR3*
CXCL10	IP-10	CXCR3, *CCR3*
CXCL11	I-TAC	CXCR3, CXCR7, *CCR3*
CXCL12	SDF-1	CXCR4, CXCR7
CXCL13	BCA-1, BLC	CXCR5
CXCL14	BRAK, Bolekine	unknown

mCXCL15	Lungkine, WECHE	unknown
CXCL16	SCYB16, SRNM-PSOX	CXCR6
CXCL17	VCC-1, DMC	unknown

XC Chemokine Family

Systematic Name	Alternative name(s)	Receptor(s)
XCL1	Lymphotactin, SCM-1α	XCR1
hXCL2	SCM-1β	XCR1

CX_3C Chemokine Family

Systematic Name	Alternative name(s)	Receptor(s)
CX_3CL1	Fractalkine, neurotactin	CX_3CR1

Table 1-2: Systematic nomenclature and classification for chemokines
Names of chemokines as per systematic nomenclature, with alternative/previous name(s) and cognate receptor(s). Chemokines present only in humans are prefixed with 'h'. Chemokines present only in mice are prefixed with 'm'. Chemokines are also classified as inflammatory (pink), homeostatic (blue) function as both (green), or currently unknown or conflicting classification (white). Adapted from (Nomiyama et al., 2013, Comerford and Nibbs, 2005, Moser et al., 2004, Burkhardt et al., 2014)

1.1.3 Homeostatic vs. Inflammatory Chemokines

As well as classification according to the cysteine motif, chemokines can also be classified according to the context in which they function, and are thus often described as being either homeostatic or inflammatory, although some can fall into both categories in different contexts.

1.1.3.1 Homeostatic chemokines

Homeostatic chemokines, as the name suggests, are expressed at fairly constant levels and expression does not generally increase in response to infection or inflammation. They, along with other molecules such as selectins (capture receptors) and integrins (adhesion receptors), operate to control basal leukocyte movement and can form parts of a very specific tissue 'address code' to instruct the delivery of particular leukocytes to specific *in vivo* sites. This constitutive expression of specific chemokines ensures the correct organisation and compartmentalisation of immune cells during development and homeostasis (Springer, 1995, Zlotnik and Yoshie, 2000).

Naive B and T lymphocytes can gain entry into lymph nodes by expressing the correct entry code reader (including chemokine receptor CCR7), which will recognize the entry signals CCL19 and CCL21; homeostatic chemokines that are expressed on the luminal side of high endothelial venules (HEVs) of the lymph node. Conversely, antigen-experienced B cells leave the lymph node and migrate to specific niches such as the spleen and bone marrow, via up-regulation of CXCR4. In this context, CXCR4 acts as an endothelial entry code reader (Parsonage et al., 2005).

The CXCL12/CXCR4 pairing is perhaps the most studied chemokine ligand/receptor combination. Knock-out mice, lacking either CXCL12 or its receptor CXCR4, die during embryogenesis, or shortly after birth, and exhibit marked defects in B lymphopoiesis, myelopoieisis, and heart and brain development (Ma et al., 1998, Nagasawa et al., 1996). CXCL12 is the most ancient chemokine and is often referred to as the primordial chemokine (Mithal et al., 2012). Homeostatic chemokine activity is epitomized by CXCL12, as it is constitutively produced by stroma in the bone marrow, supporting the proliferation of B cell progenitors and acting to recruit haematopoietic precursors to the bone marrow (Nagasawa et al., 1998). The CXCL12/CXCR4 axis is also required for the normal migration and survival of primordial germ cells in development (Molyneaux et al., 2003).

1.1.3.2 Inflammatory chemokines

In contrast, inflammatory chemokines are only expressed during the response to a stimulus such as infection or damage within the tissue. They are expressed by many different cell types and in various tissues in order to generate a quick and effective inflammatory response and to direct leukocytes to the site of infection or damage (Rollins, 1997). There exists an array of different inflammatory chemokines. Most bind to more than one receptor, therefore individual functions for each are difficult to define. For example, a multiplicity of CXC chemokines, including CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8 attract neutrophils to infected sites by binding to either CXCR1 or CXCR2, the major neutrophil receptors. Likewise, monocytes are recruited into inflamed tissues by various CC chemokines, including CCL2, CCL3, CCL4, CCL6, CCL7, CCL8, CCL9, CCL15 and CCL20 (Charo and Ransohoff, 2006), which target many different CC chemokine receptors.

It is thought that this extensive collection of inflammatory chemokines is an example of biological redundancy, and exists to increase the robustness of the chemokine system and ensure that if one mechanism fails, another is available that can exert the equivalent response (Mantovani, 1999). However, with a scarcity of examples of true redundancy in biology, it may also be possible that the extensive inflammatory chemokine network is not redundant, and exists to provide a complex and exquisite regulation of leukocyte movement during inflammation (Proudfoot, 2002, Johnson et al., 2005, Schall and Proudfoot, 2011). This conundrum has perplexed chemokine biologists since chemokines were discovered, and it seems only with revolutionary experimental techniques, will the individual roles for inflammatory chemokines, or indeed their redundancy, be established. **Table 1-2** lists the chemokines and defines them as being homeostatic, inflammatory or both.

1.1.4 Glycosaminoglycans and chemokine presentation

Chemokines are secreted by many cell types and are often found attached to endothelial cells through association with glycosaminoglycans (GAGs). GAGs are long, linear and highly negatively charged heterogeneous polysaccharides which are generally sulphated. They can be expressed as soluble GAGs, such as heparin and hyaluronic acid, or attached to a protein core to form structures called proteoglycans, which decorate the surface of most mammalian cells. As well as chemokines, these GAGs interact with a variety of proteins, including growth factors, enzymes and cytokines (Proudfoot et al., 2003).

It has been demonstrated that the composition of different GAGs coating the cells and tissues of an organism can reflect a high degree of specificity, and is dependent on a number of factors, including cell type, developmental stage and the pathophysiological state of the cell (Turnbull et al., 2001, Couchman and Pataki, 2012). Indeed GAGs exhibit the largest diversity among biological macromolecules, vastly exceeding that of protein and DNA (Laine, 1994), and chemokines themselves display a degree of selectivity when binding to different types of GAGs (Kuschert et al., 1999, Adage et al., 2012). This makes carbohydrate biology notoriously difficult to study and, as a consequence, the importance of GAGs has been historically overlooked (Johnson et al., 2005).

Initially chemokine binding to GAGs was thought to be the result of a non-specific electrostatic interaction; however mutagenesis studies have defined specific GAG binding

sites on many chemokines (Proudfoot et al., 2001, Lau et al., 2004, Koopmann and Krangel, 1997, Campanella et al., 2003). GAG binding epitopes vary depending on the chemokine and some epitopes overlap with sites required for the interaction with chemokine receptors (Graham et al., 1996). It is not known whether chemokines can simultaneously bind to GAGs and their cognate receptors, or whether the interactions involve a sequential 'hand-over' method; however the general model for GAG presentation of chemokines encompasses to both possibilities. The involvement of GAGs in the process of leukocyte migration from blood to extravascular tissue is summarised in **Figure 1-3**. Many different molecules, expressed on both leukocytes and endothelial cells are involved in this multistep process. Selectins expressed on the surface of leukocytes interact with addressins on endothelial cells and allow them to 'roll' along blood vessel walls. The presentation of chemokines by GAGs on the apical surface of endothelial cells prevents the diffusion of chemokines away from sites of production and is thought to encourage the binding of chemokines to chemokine receptors expressed on the leukocyte. This interaction between receptor and chemokine leads to a change in conformation of surface integrins on leukocyes, which can then bind cell adhesion molecules on endothelial cells. This interaction promotes firm adhesion of rolling leukocytes in the blood vessel and allows the extravasation and entrance of leukocytes into the tissues secreting the chemokines (Johnson et al., 2005, Proudfoot, 2006).

The interaction between chemokines and immobilised GAGs has been shown to have crucial importance for the migration of inflammatory leukocytes. Mutants of CCL2, CCL3, CCL5 and CCL7 engineered to have impaired GAG-binding capacity, have all demonstrated an inability to induce cell migration *in vivo* (Proudfoot et al., 2003, Ali et al., 2005), despite showing only minor effects *in vitro*, where blood and tissue flow forces are not operative (Ali et al., 2000). Likewise lymphocyte homing to lymph nodes relies on GAG presentation of CCL21 and this was demonstrated *in vivo* by a murine model whereby heparan sulphate GAGs were temporarily removed from endothelial cells enzymatically by injecting a mixture of heparinase and heparitinase intravenously (Bao et al., 2010, Alon, 2010).

The idea that GAG-bound chemokines form gradients to direct the movement of leukocytes is considered crucial for chemokine-directed migration. This phenomenon has been shown *in vitro* (Rot, 1993, Haessler et al., 2011), and more recently *in vivo*, with evidence from quantitative imaging showing the presence of endogenous CCL21 gradients within mouse skin that lead dendritic cells towards lymphatic vessels (Weber et al., 2013).

As touched upon previously, the oligomerisation of particular chemokines, such as CCL5, is important for cell recruitment activity. Many studies have focused on the possibility of modulating the inflammatory process therapeutically through administration of variant chemokines that are incapable of GAG-binding. For example, generation of a CCL5 variant by mutating the heparin binding site abrogated its ability to form higher order oligomers, and in turn, impaired cellular recruitment to sites of inflammation in a range of murine inflammation models (Johnson et al., 2004).

In some cases chemokine interactions with soluble GAGs may prevent inappropriate engagement of chemokines with their receptors. For example particular T lymphocytes secrete CCL3, CCL4 and CCL5 as a complex with proteoglycans *in vivo* (Wagner et al., 1998). Indeed this type of system has been utilized artificially for some time, with the use of soluble heparin to inhibit the biological effects of chemokines, thereby creating anti-inflammatory effects both in animal models and in patients suffering from inflammatory conditions such as allergies and irritable bowel disease (Lider et al., 1989, Ahmed et al., 2000, Lever and Page, 2002).

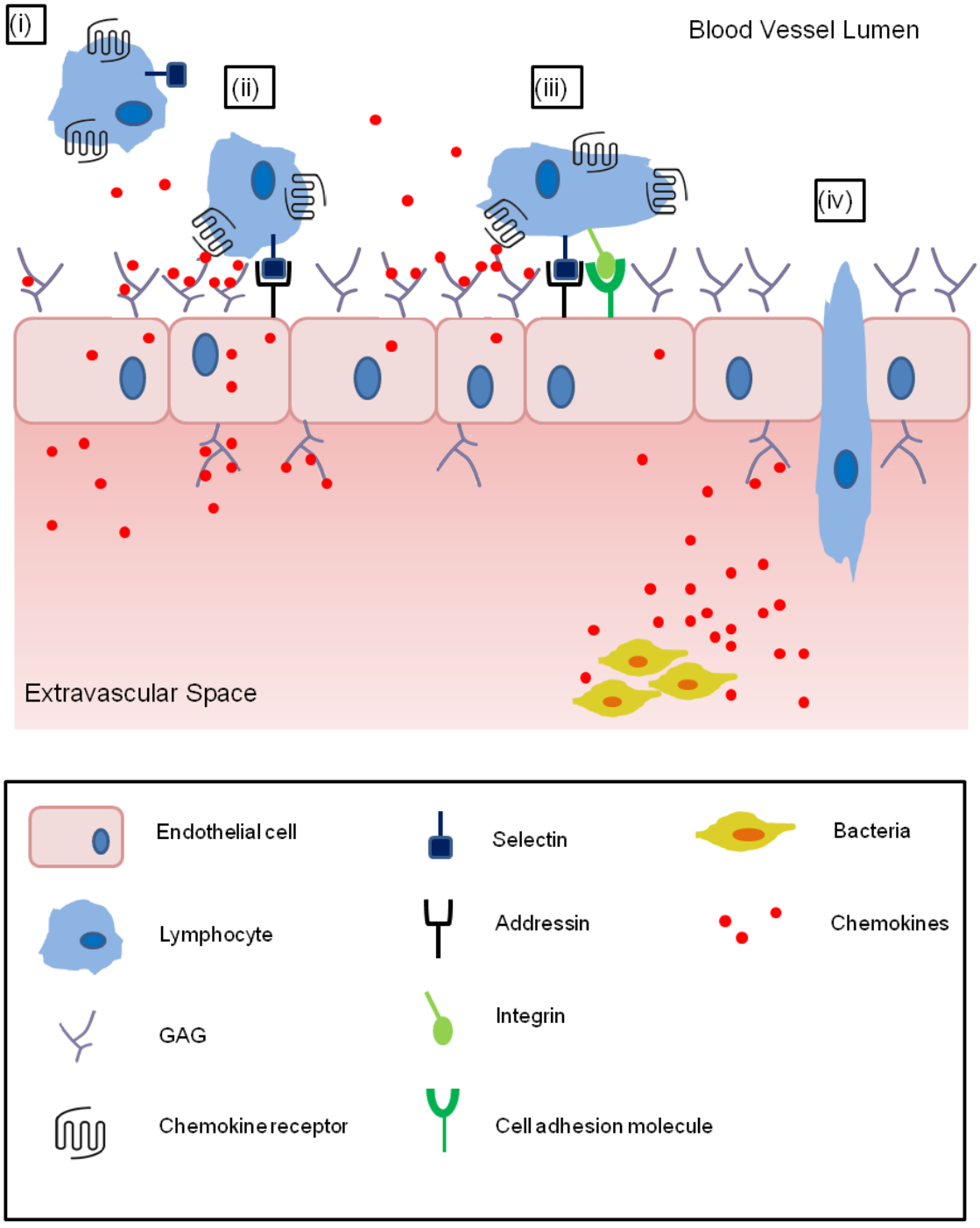


Figure 1-3: Leukocyte migration from blood to extravascullar tissue.
Migration of leukocytes from the blood into tissue in response to infection or inflammation is a multi-step process involving many different molecules. **(i)** Selectins and chemokine receptors are expressed on the endothelial cell surface. **(ii)** Selectins interact with mucin-like addressins on the leukocyte causing reversible tethering and a 'rolling' behaviour of the leukocyte. The chemokine receptor comes into contact with its chemokine ligand, presented on endothelial surfaces via GAGs. **(iii)** The interaction between receptor and chemokine leads to a change in conformation of surface integrins, which can then bind cell adhesion molecules. **(iv)** This causes leukocyte arrest and extravasion through either paracellular (as depicted) or transcellular routes. Figure adapted from (Handel et al., 2005, Johnson et al., 2005).

1.2 The Chemokine Receptors

1.2.1 General structure and characterisation

Chemokine receptors belong to the large superfamily of G protein-coupled, seven-transmembrane spanning receptors, forming part of the Rhodopsin family of GPCRs. These molecules span the plasma membrane of cells and share a common structure of seven transmembrane alpha helices, an extracellular *N*-terminus, an intracellular *C*-terminus, and three inter-helical loops on each side of the membrane. Other important shared structural features of chemokine receptors relate to signalling, including the presence of a DRYLAIV amino acid sequence motif, or a similar variation, on the second intracellular loop which is the main site for G protein-coupling, and many serine and threonine residues on the *C*-terminus which act as phosphorylation sites (see section 1.2.4.2) (Oldham and Hamm, 2008, Murphy et al., 2000, Bachelerie et al., 2014). To date, 18 signalling chemokine receptors, as well as 4 ‘non-signalling’ or atypical chemokine receptors (see section 1.4) have been identified, all of which help to govern immune and developmental processes.

Recently three-dimensional structures have been elucidated for CXCR4, CXCR1 and CCR5. The CXCR4 structure was determined by X-ray crystallography, with CXCR4 bound to either an antagonistic small molecule, or a cyclic peptide, and confirmed many supposed structural characteristics, including its tendency to form homodimers (Wu et al., 2010). Likewise the CCR5 structure was determined by X-ray crystallography while in complex with the HIV drug Maraviroc (Tan et al., 2013b) (see **section 1.3.5** for more details on pharmaceuticals). In contrast, the CXCR1 structure was determined by NMR spectroscopy, whereby the receptor is contained within crystalline phospholipid bilayers, under physiological conditions (Park et al., 2012). These studies have provided invaluable templates that should facilitate the clarification of yet more chemokine receptors, and improve our understanding of the nature of interactions between chemokine receptors, ligands, and indeed antagonistic therapeutics designed to modulate the chemokine system.

1.2.1.1 Evolutionary origin

Members of the chemokine receptor family share 25 to 80% amino acid homology, however many other G protein-coupled receptors also have around 25% amino acid

identity with chemokine receptors, indicating a hazy structural boundary. Their origin seems fairly recent in evolutionary terms, with chemokine receptor-like sequences having been identified in mammals, birds and fish (Gupta et al., 1998, Daniels et al., 1999, Nomiyama et al., 2013, DeVries et al., 2006). Phylogenetic analysis indicates that, similar to its ligand CXCL12, CXCR4 likely represents the earliest ancestral chemokine receptor. Surprisingly, it seems that the ancestral role of CXCR4 pre-dates the evolution of a sophisticated immune system, and instead, is thought to be within the central nervous system, as determined by comparison of the phylogenies of fish and mammals (Huising et al., 2003). CXCL12 and CXCR4 are also crucial to the migration of primordial germ cells to the gonads, as determined by studies in mice and zebrafish (Doitsidou et al., 2002, Knaut et al., 2003). An ancestral role for CXCR4 in stem cell trafficking during embryogenesis has also been established (Mithal et al., 2012).

1.2.1.2 Ligand binding

Studies investigating the mode of binding between chemokines and chemokine receptors support a two-site model involving the *N*-terminal region of the chemokine receptor along with one or more of the extracellular domains (Monteclaro and Charo, 1996, Rajagopalan and Rajarathnam, 2006). The *N*-terminal region of the chemokine receptor is crucial for high-affinity ligand binding (Blanpain et al., 1999a, Monteclaro and Charo, 1997), whereas one or more extracellular domains are thought to strengthen ligand binding and are also necessary for transmembrane signalling (Han et al., 1999, Peeters et al., 2011). The presence of two disulphide bonds between the *N*-terminus and the extracellular loops of GPCRs are important for the conformational integrity of the extracellular domains and are thought to create a ‘binding pocket’ to which the ligand can attach (Allen et al., 2007) (**Figure 1-4**).

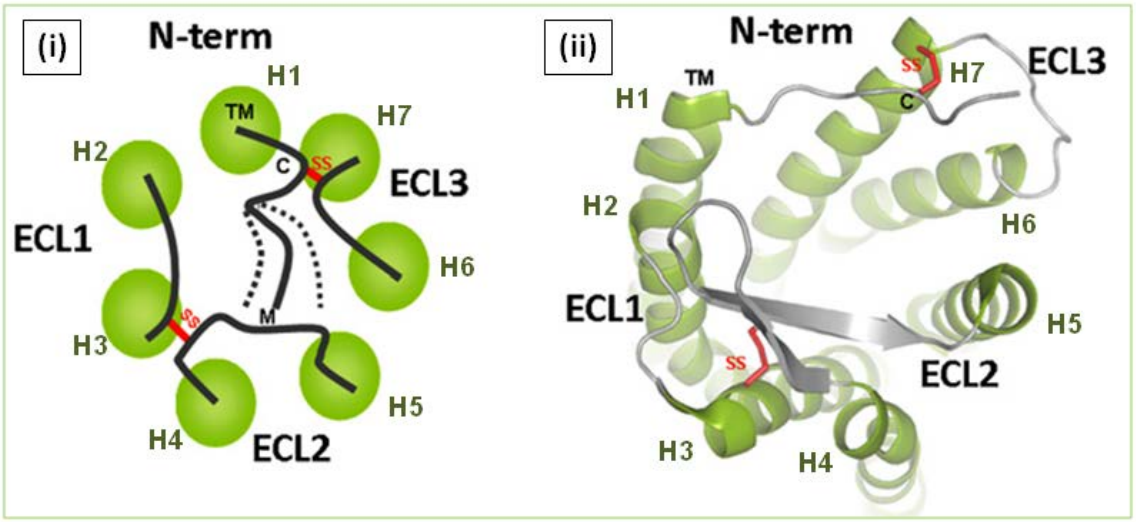


Figure 1-4: Birds-eye representation of chemokine receptor structure.
(i) Schematic diagram illustrating transmembrane domains as green circles and extracellular domains (labelled ELC1, ELC2 and ELC3) as grey lines. The two disulphide bonds are represented as red lines. The grey dots characterize the flexible nature of the M-C region of the *N*-terminus. **(ii)** X-ray structure of CXCL12 detailing the alpha-helices of the transmembrane domains and again the disulphide bonds (in red). H1 – H7 – alpha helices 1 to 7. Figure adapted from: (Szpakowska et al., 2012).

On every chemokine receptor, except for CXCR6, the *N*-terminus contains a conserved cysteine residue which is likely to be engaged with the third extracellular loop (ECL3) in a disulphide bond. This demarcates two regions in the *N*-terminus; the M-C domain from the first methionine residue to the cysteine residue, and the C-TM domain, from the cysteine residue to the first transmembrane segment. The M-C domain is the least conserved domain of chemokine receptors and has a fairly flexible structure. It also holds a net negative charge and typically contains multiple tyrosine and asparagine residues that can be post-translationally modified. These different features are thought to contribute to the high degree of ligand selectivity exhibited by chemokine receptors (Szpakowska et al., 2012).

In addition to chemokines, there is evidence that other types of molecule have chemokine receptor binding affinity, including pathogen-derived peptides (**section 1.1.2.5**), viral chemokines (**section 1.1.2.4**) and autoantigens (Aliberti et al., 2003, Howard et al., 2005). Furthermore, numerous studies examining HIV pathogenesis demonstrate the importance of chemokine receptors for viral entry into cells (Berger et al., 1999) (**section 1.3.1**).

1.2.1.3 Dimerisation

A single cell may express various chemokine receptors during different stages of its life cycle, or indeed, simultaneously. Although historically assumed to exist, and function, as monomeric species, it is now the general consensus that chemokine receptors, analogous to other GPCRs, function primarily as dimers or even higher-order functional units (Springael et al., 2007, Thelen et al., 2010). Furthermore the current hypothesis suggests that GPCRs assemble into dimers shortly after synthesis in the endoplasmic reticulum (ER) and traffic as such throughout their lifespan within the cell, suggesting that receptor oligomerisation has a role in receptor trafficking and may also regulate receptor expression at the cell surface (Bulenger et al., 2005, Thelen et al., 2010).

Experimental evidence, although somewhat contentious, supports chemokine receptor dimerisation, whereby a number of chemokine receptors have been shown to form both homo- and heterodimers. For example CCR2 and CCR5 are preferentially expressed as homodimers (Rodriguez-Frade et al., 1999, Issafras et al., 2002), however co-expression of CCR2 and CCR5 in transfected cell lines can result in heterodimerisation of these two CC chemokine receptors and this has been shown to have functional consequences for cross-ligand competition and prevention of activation of these receptors (El-Asmar et al., 2005). Similarly stabilisation of CCR2-CCR5 and CCR2-CXCR4 heterodimers was reported to inhibit HIV-1 infection through trans-inhibition of CCR5 and CXCR4 (Rodriguez-Frade et al., 2004), although this effect has been disputed elsewhere (Percherancier et al., 2005). It has also been demonstrated that chemokine receptor oligomerisation is not specific to just the signalling receptors, with heterodimerisation having also been shown between signalling receptors and atypical chemokine receptors, and even other GPCR family members such as glutamate receptors (Chakera et al., 2008, Vinet et al., 2013, Levoye et al., 2009, Lax et al., 2002). These interactions often exhibit antagonistic signalling effects and add further complexity to an already complicated system.

Altogether the true consequences of receptor dimerisation need more complete characterisation, with the existence of many conflicting data sets and limitations on experimental techniques making assessment difficult. The recent advancements in the elucidation of CXCR4, CXCR1 and CCR5 structures (Wu et al., 2010, Park et al., 2012, Tan et al., 2013b) (see **section 1.2.1**), and the models that are likely to be available in the future, should provide novel structural information about different chemokine receptors and their natural stoichiometry within the cell membrane. This will be fundamental in

unravelling the significance of chemokine receptor dimerisation and its effects on ligand binding, intracellular signalling and function.

1.2.2 Post-translational modification

The expression and function of many molecules, including chemokine receptors, is controlled at both transcriptional and translational levels, as well as by post-translational modification. Post-translational modifications of chemokine receptors include *N*-linked and *O*-linked glycosylation and tyrosine sulphation on the extracellular domains, as well as the possibility of palmitoylation, phosphorylation and ubiquitination on the intracellular domains. Receptors can also be cleaved once expressed on the surface of cells. All such modifications can play major roles in receptor functionality and a select few will be discussed in this thesis.

1.2.2.1 Tyrosine sulphation

Tyrosine residues, particularly those following a neutral or acidic amino acid, can be post-translationally sulphated, in the Golgi apparatus, by two broadly expressed enzymes called tyrosyl-protein sulphotransferases-1 and -2 (TPST-1 and TPST-2) (Kehoe and Bertozzi, 2000). These enzymes catalyze the transfer of a sulphate group from the universal donor 3'-phosphoadenosine 5'-phosphosulphate (PAPS) to the hydroxyl group of a tyrosine residue (**Figure 1-5**). Approximately 1% of the eukaryotic genome is thought to encode proteins which undergo tyrosine sulphation, including many GPCRs and adhesion molecules required for cell recruitment (Danan et al., 2008). Studies in which TPST-1 and TPST-2 were disrupted in mice demonstrate important and distinct roles for these enzymes in different biological processes such as cardiovascular development, reproduction and metabolism. TPST-1 KO mice display reduced body weight and increased foetal death post-implantation (Ouyang et al., 2002), whereas TPST-2 KO males have reduced fertility (Borghei et al., 2006). TPST-1 and TPST-2 double KO mice are born alive, however most pups die in the early postnatal period with cardiopulmonary deficiencies and also display disrupted retinal anatomy and function (Westmuckett et al., 2008, Sherry et al., 2010).

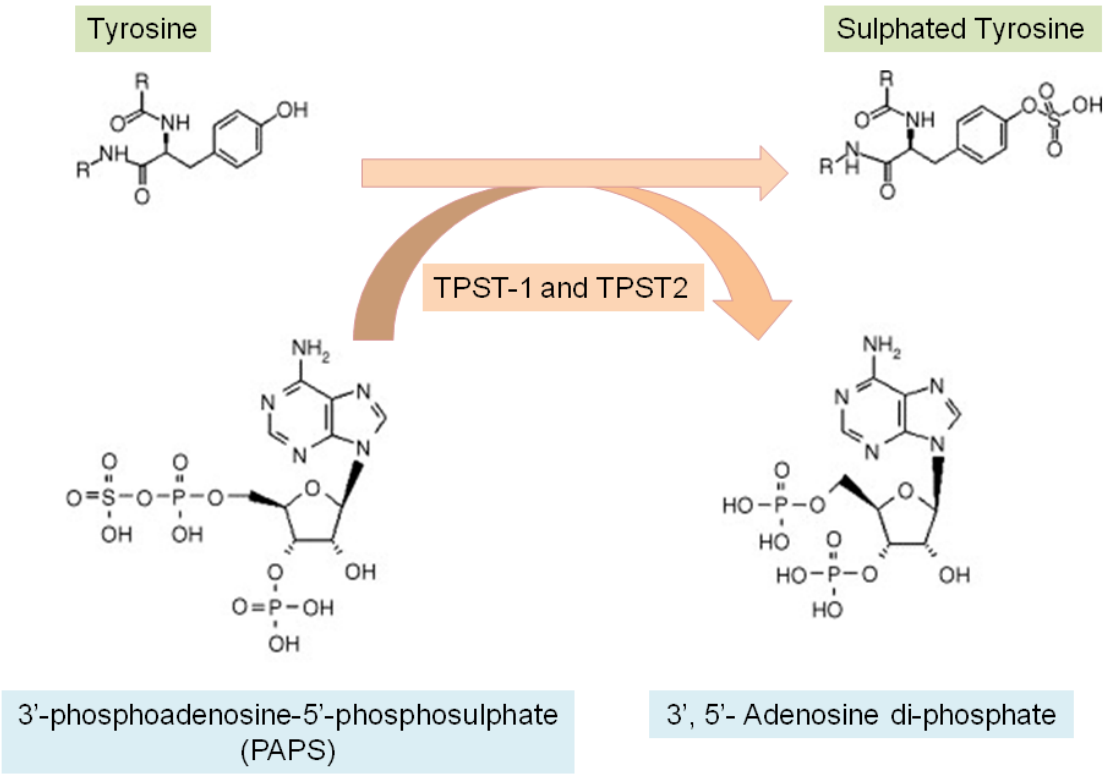


Figure 1-5: Sulphation of a tyrosine residue
Transfer of a sulphate group from the universal donor PAPS to the hydroxyl group of a tyrosine residue catalysed by either TPST-1 or TPST-2. Adapted from (Monigatti et al., 2006).

A conserved structural feature of the chemokine receptors is the incidence of clusters of tyrosine residues in the M-C domain (the domain preceding the first cysteine on the *N*-terminus). Evidence from studies on various chemokine receptors suggests that these tyrosine residues can be sulphated and that this sulphation is important, and, often essential, for activation and function (Seibert et al., 2002). This is exemplified by CCR5, where sulphation of *N*-terminal tyrosine residues is essential for ligand binding, and importantly, the entry of HIV into the cell (Cormier et al., 2000, Farzan et al., 1999). Mutation of tyrosine residues in the *N*-terminal regions of numerous chemokine receptors, including CCR2, CCR5, mCCR8, CXCR3, CXCR4, CX3CR1 and Duffy antigen receptor for chemokines (DARC) (see section 1.5.1), has been shown to reduce the level of metabolic sulphate labelling of these receptors, which subsequently reduces the binding affinity for many of their chemokine ligands (Preobrazhensky et al., 2000, Seibert et al., 2002, Gutierrez et al., 2004, Colvin et al., 2006, Farzan et al., 1999, Fong et al., 2002, Choe et al., 2005). Similarly, the chemokine binding affinities of *N*-terminal peptides from receptors CCR2, CCR3, CCR5 and CXCR4 are enhanced by sulphation of tyrosine

residues in these peptides (Tan et al., 2012, Veldkamp et al., 2006, Simpson et al., 2009, Duma et al., 2007). Interestingly, studies using sulphated CCR2 *N*-terminal peptides also highlighted a preference to bind monomeric CCL2, as opposed to its dimeric form. This has been suggested to assist in the destabilisation of the inactive dimeric form of CCL2 and induce dissociation to its active monomeric state (Tan et al., 2013a), implicating tyrosine sulphation of chemokine receptors as a prerequisite for chemokine activation and subsequent downstream consequences, such as an inflammatory response.

Indeed tyrosine sulphation of chemokine receptors has been implicated in inflammatory conditions including atherosclerosis and lung disease (Liu et al., 2008, Westmuckett and Moore, 2009). **Figure 1-6** illustrates the possible mechanisms whereby tyrosine sulphation of chemokine receptors and cell adhesion molecules may influence disease pathology in inflammatory diseases.

Metabolic sulphate labelling studies have indicated that the sulphation of particular tyrosine residues is incomplete and therefore there may not be an exact sulphation pattern of tyrosines in chemokine receptors, rather a heterogeneous assortment of different sulphation states. In addition TPST-1 and TPST-2 catalyse at different rates depending upon the sequence they are working on, and gene expression of the two TPST isoforms varies significantly between different tissues (Mishiro et al., 2006). As a result it is possible that differentially sulphated forms of the same chemokine receptor can exist in different cell types, and in different environments (Stone et al., 2009). In this way the sulphation state of a chemokine receptor corresponding to the cell it is expressed in could regulate the responsiveness of the chemokine receptor to its cognate chemokines (Zhu et al., 2011).

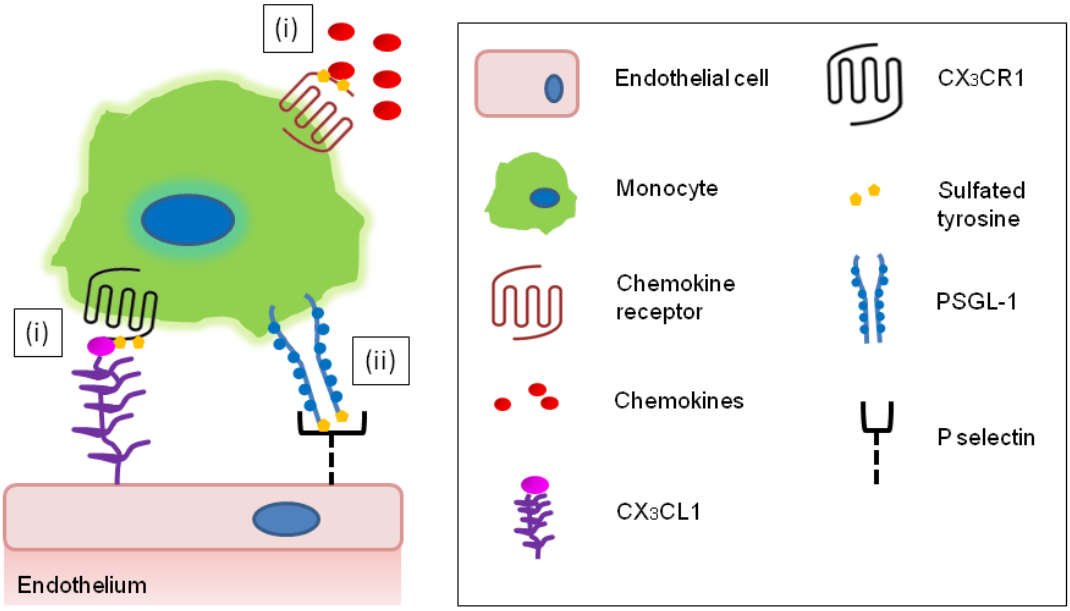


Figure 1-6: Immune cell recruitment to sites of inflammation requires post-translationally sulfated proteins.
During inflammatory cell infiltration, immune cells such as monocytes express chemokine receptors such as CX_3CR1 and CCR2 and migrate towards ligands such as CX_3CL1 and CCL2 respectively. The expression of the adhesion molecule; P-selectin glycoprotein ligand-1 (PSGL-1), allows the infiltrating monocyte to form transitory bonds with P-selectin molecules expressed on endothelial cells, inducing leukocyte 'rolling'. Post-translational tyrosine sulphation is crucial for both **(i)** chemokine/chemokine receptor binding and **(ii)** rolling adhesion. Both processes lead to infiltration of leukocytes during the inflammatory response. Adapted from: (Koltsova and Ley, 2009).

1.2.2.2 Palmitoylation

Many GPCRs are palmitoylated on cysteine residues located on the *C*-terminus of the receptor. This lipid-based post translational modification is a result of the esterification of cysteine residues by palmitate and leads to structural changes of the cytoplasmic tail and the formation of a fourth cytoplasmic loop. Palmitoylation is thought to be involved in various different functional attributes, including G protein-coupling efficiency, control of receptor phosphorylation and desensitization, and regulation of intracellular trafficking of the receptor (Qanbar and Bouvier, 2003). Many chemokine receptors have clusters of cysteine residues in their *C*-terminal regions and therefore have the potential to be palmitoylated. This is the case with CCR5, which has been shown to be palmitoylated at a three-cysteine cluster in its *C*-terminus. Studies whereby CCR5 palmitoylation was inhibited resulted in deficient receptor trafficking to the cell membrane (Blanpain et al., 2001). Palmitoylation of CCR5 has also been implicated in the regulation of the receptor

life span (Percherancier et al., 2001), although there is currently no evidence that this post-translational modification has functional significance in other chemokine receptors.

1.2.2.3 Cleavage by proteases

The regulation of chemokine receptors by post-translational modification also includes the proteolytic cleavage of some chemokine receptors after expression on the cell surface. The receptors CXCR1, CXCR2 and CXCR4 have all been reported to be cleaved extracellularly in response to the enzymatic action of proteases, thereby removing the ligand binding site and rendering the receptor inactive.

In the case of CXCR4, specific serine proteases released by neutrophils; namely neutrophil elastase and cathepsin G, cleave the receptor at its *N*-terminus and leave it unresponsive to its ligand, CXCL12 (Valenzuela-Fernandez et al., 2002). This was thought to coincide with the mobilization of hematopoietic progenitor cells (HPCs) in response to treatment with either granulocyte colony stimulating factor (G-CSF) or cyclophosphamide (Levesque et al., 2003). It was hypothesized that treatment with G-CSF or cyclophosphamide results in the accumulation of active neutrophil proteases in bone marrow tissue, thereby inducing cleavage of CXCR4 expressed on HPCs and allowing mobilization from the bone marrow. This study was performed using human samples, but the same results could not be repeated using a murine model (Levesque et al., 2004).

Interestingly a study, again involving neutrophil elastase and cathepsin G, demonstrated their ability to cleave two distinct, but nearby, sites on the second extracellular loop of CXCR1. Cleavage of CXCR1 is particularly noteworthy, as the resulting cleaved peptide is itself bioactive, and its downstream effects include stimulation of the production of CXCL8, a CXCR1 ligand (Hartl et al., 2007). This process was implicated in chronic neutrophilic lung diseases such as cystic fibrosis, whereby the lung environment is characterised by elevated proteolytic activity and is often colonized by many different bacterial pathogens (Greene and McElvaney, 2009).

The other main neutrophil chemokine receptor, CXCR2 can also be cleaved at the *N*-terminus by the enzyme staphopain A; a cysteine protease secreted by the bacterial pathogen *Staphylococcus aureus*. Staphopain A is thought to act as an immune evasion factor by inactivating CXCR2 expressed on neutrophils, thus increasing bacterial virulence (Laarman et al., 2012).

These separate examples highlight the importance of chemokine receptor modulation and its functional consequences in disease. It seems likely that as further studies are undertaken, additional proteases may be implicated in the cleavage of various other chemokine receptors.

1.2.3 Viral chemokine receptors

Virus-encoded 7-transmembrane-spanning chemokine receptors have been identified in the genomes of many large DNA viruses, in particular herpes- and poxviruses (Alcami, 2003). These molecules are thought to have been duplicated from host genomes long ago, and have been through intense rates of mutation, resulting in molecular mimics that can subvert and exploit the host immune system, as well as influencing other important biological processes that are crucial to the virus life-cycle (Rosenkilde, 2005). Viral chemokine receptors share general features, including high promiscuity for both host and virally expressed chemokines, the ability to activate an exhaustive number of signalling pathways, and constitutive activity (McLean et al., 2004, Holst et al., 2003). For example the γ2-Herpesvirus HHV8, encodes a viral chemokine receptor that shares homology with mammalian CXCR2 and can bind a plethora of CXC chemokines as well as an endogenously expressed chemokine – vCXCL2. This receptor is able to exploit various types of G-protein, with the potential to activate an abundance of down-stream signalling pathways, which ultimately contributes to the pathogenicity of the virus (Arvanitakis et al., 1997, Bais et al., 1998, Rosenkilde et al., 1999).

1.2.4 Chemokine receptor signalling

1.2.4.1 Ligand binding and signalling

Classical chemokine receptors are coupled to G proteins which act as molecular switches to activate intracellular signalling cascades in response to ligand binding. Heterotrimeric G proteins are made up of three subunits α, β and γ. In its inactive state, guanosine diphosphate (GDP) remains bound to the Gα subunit, however upon ligand binding, the conformation of the receptor changes leading to the release of GDP and the formation of a stable complex between the receptor and the G protein. This interaction is rapidly destabilised by the binding of guanosine triphophate (GTP) to the Gα subunit. The

activated Gα-GTP subunit and the Gβγ subunit are now capable of triggering or modulating the activity of many downstream signalling cascades (Oldham and Hamm, 2008). These events are summarized in **Figure 1-7**.

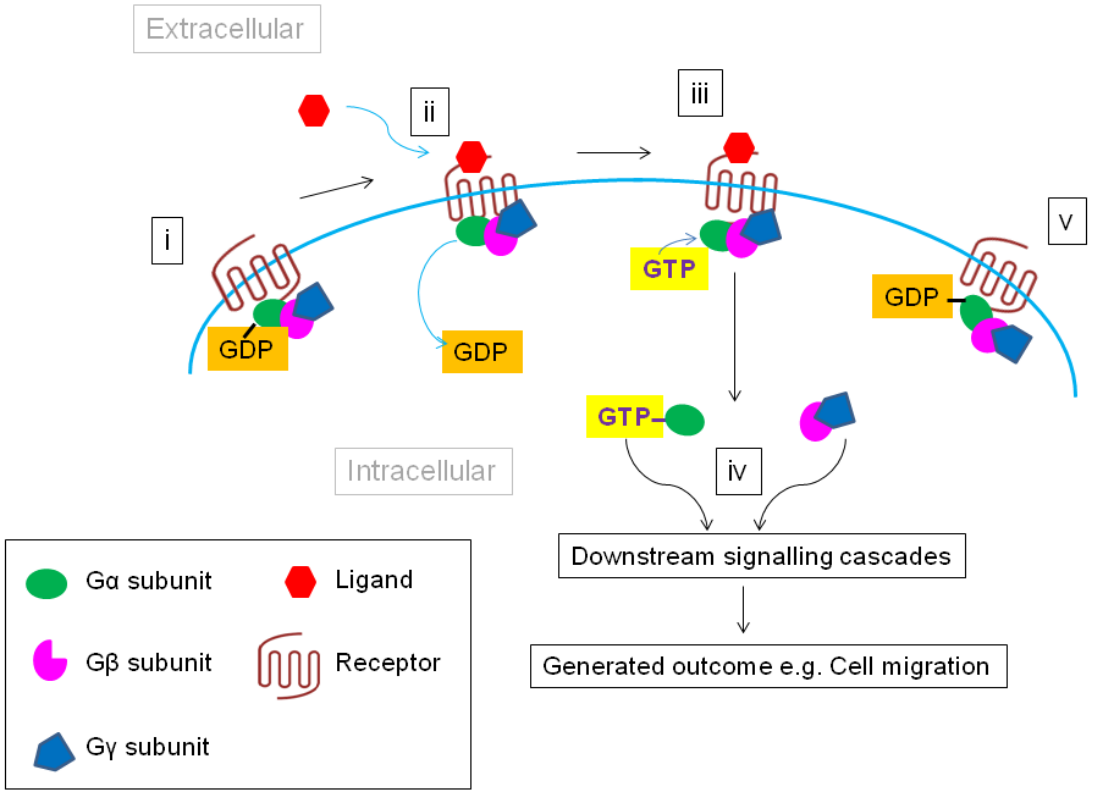


Figure 1-7: G protein coupled receptor signalling
(i) Before activation, the G protein heterotrimer, composed of the Gα, Gβ and Gγ subunits, is associated with the receptor. GDP is bound to the Gα subunit. **(ii)** Ligand binding results in release of GDP and the formation of a stable, high-affinity complex between the receptor and the G protein complex. **(iii)** GTP rapidly binds to the Gα subunit leading to **(iv)** destabilisation of the heterotrimer complex into Gα-GTP and Gβγ subunits. Both subunits can interact with downstream effector proteins. **(v)** The signal is terminated on hydrolysis of GTP to GDP by Gα. The Gα subunit re-binds to the Gβγ, re-forming the inactive heterotrimer complex that can again associate with the receptor. Created using information from (Oldham and Hamm, 2008).

The signalling pathways induced and the resulting changes in the cell which are brought about by chemokine receptor signalling can differ depending on the chemokine receptor, the ligand, the cell and the context of the interaction (Thelen, 2001). Ligand binding can induce activation of a multitude of downstream effector proteins including members of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) family (Soriano et al., 2003, Wong and Fish, 2003). There are also four main classes of G protein heterotrimer based on the primary sequence similarity of the Gα subunit, these are denoted G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$ (Cotton and Claing, 2009). Cell migration is usually facilitated through $G_{i/o}$ activation and interestingly the various α subunits of this G protein subtype are

all inactivated by *pertussis* toxin (Ptx), which irreversibly blocks the α subunit from coupling to the receptor (Katada, 2012).

1.2.4.2 Receptor internalisation and desensitisation

Chemokine receptors undergo a basal level of internalisation and either recycling or lysosome-mediated degradation, however ligand-induced activation can greatly enhance the internalisation and trafficking of chemokine receptors. Internalisation after ligand-induced activation is thought of as a feedback mechanism to protect cells from overstimulation and is referred to as receptor desensitisation (Rose et al., 2004, Bennett et al., 2011).

The fate of the receptor after internalisation may affect the strength, type and duration of signal that is generated. Internalisation of GPCRs is mediated by either clathrin-mediated endocytosis or caveolae-dependent pathways. Clathrin-mediated endocytosis involves the recruitment of adaptin 2 (AP-2) and β-arrestin; adaptor molecules that function to link the receptor to a lattice of clathrin that facilitates internalisation. Ligand-induced phosphorylation of serine and threonine residues in the intracellular loops and on the *C*-terminus of the chemokine receptor is thought to facilitate this process, with β-arrestin having a stronger association to phosphorylated receptors. The recruitment of clathrin and the formation of clathrin-coated pits ensues from the association of the receptor with AP-2 and β-arrestin, leading to the 'pinching off' of the receptor associated membrane. Dynamin, a large GTPase, is a key regulator of the 'pinching off' process which leads to the formation of clathrin-coated vesicles within the cell. The clathrin-coated vesicle then fuses with endosomal vesicles leading to either receptor recycling to the cell surface following dephosphorylation, or targeting of the receptor to the lysosomal compartment, where it will undergo degradation (Neel et al., 2005, Claing et al., 2002). Clathrin-independent internalisation is most often mediated by caveolae. Caveolae are cholesterol-rich, highly organised membrane invaginations that contain a specific set of proteins called caveolins which self-assemble as high mass oligomers to form a cytoplasmic coat on the membrane invaginations. Once the membrane invagination is 'pinched off', similarly involving the action of dynamin, the intracellular caveosome compartment fuses with endosomal vesicles and the receptor can either be recycled or targeted for degradation as described previously (Borroni et al., 2010, Neel et al., 2005).

Particular chemokine receptors have been shown to use both clathrin- and caveolae-mediated internalisation pathways, while others may preferentially use only one (Signoret et al., 2005, Borroni et al., 2010, Comerford et al., 2006). The cell type in which the chemokine receptor is expressed is often critical as to which method of internalisation is favoured; therefore care must be taken when analysing such mechanisms, and indeed, chemokine receptor signalling in general, with use of transfectant cells, as these cells may not be representative of the regulation of such chemokine receptors *in vivo*, as different cell types express varying levels of intracellular modulators of receptor function. (Borroni et al., 2010, Neel et al., 2005).

1.3 Chemokines and chemokine receptors in disease

Leukocytes are typically and fundamentally involved in disease biology. Since the primary function of chemokines, and their receptors, is to direct the recruitment and infiltration of leukocytes, it is not unexpected that chemokines are implicated in many diseases and pathologies, including chronic inflammatory and autoimmune diseases, pathogenic infections and cancers (Gerard and Rollins, 2001), some of which will be discussed further.

1.3.1 Human immunodeficiency virus (HIV)

The intense escalation of research in the chemokine field over the past 15-20 years is largely attributable to the discovery that certain chemokines can specifically block human immunodeficiency virus type 1 (HIV-1) infection. This is because particular chemokine receptors, together with CD4, are required by HIV-1, HIV-2 and simian immunodeficiency virus (SIV) for cellular entry (Berger et al., 1999). HIV was identified in 1983, as a retroviral pathogen, with infection eventually leading to acquired immunodeficiency syndrome (AIDS) (Barresinoussi et al., 1983, Costin, 2007). The virus infects CD4 expressing cells, initially $CD4^+$ macrophages, and advanced HIV-mediated disease is characterised clinically by a fall in the number of circulating $CD4^+$ T cells (Gallo and Montagnier, 2003).

The model of HIV-1 binding and entry into a host cell is a two-step process involving a 120kDa glycoprotein (gp120) subunit expressed on the viral envelope. Firstly gp120 binds

to CD4 expressed on the host cell. This results in rearrangement of the viral envelope and the exposure of a conserved chemokine receptor binding site. Secondly the engagement of the chemokine receptor by gp120 generates further changes in conformation, ultimately allowing viral entry into the cell (Dimitrov, 1997, Wilen et al., 2012). For the vast majority of HIV-1 and HIV-2 strains, entry is facilitated by either CCR5 (R5 strains) or CXCR4 (X4 strains), or by both (R5X4 strains); however a number of other chemokine receptors, including CCR2b, CCR3, CCR8, CX_3CR1 and D6, have been shown to assist the entry of particular strains of HIV-1 *in vitro* (Neil et al., 2005, Gorry et al., 2007).

The idea of targeting CCR5 as a treatment for HIV-1 was particularly attractive because of the observation that CCR5 ligands such as CCL3, CCL4 and CCL5 can all competitively block the binding and subsequent entry of most R5 strains into cells (Cocchi et al., 1995). Similarly, high serum levels of CCR5 ligands are associated with slower disease progression (Ullum et al., 1998). Also, individuals homozygous for a genetic polymorphism in the CCR5 gene, resulting in complete ablation of CCR5 expression, are highly resistant to HIV-1 infection (Liu et al., 1996). This mutation is a 32-base-pair deletion; referred to as CCR5 Δ32, and 1% of the Caucasian population are homozygous for this mutation. Individuals heterozygous for the CCR5 Δ32 mutation can still be infected with HIV-1, however their rate of progression to AIDS is predictably slower than WT individuals (De Roda Husman et al., 1997). This crucial discovery further encouraged efforts to therapeutically block CCR5-mediated entry of HIV-1 and in 2005 a small molecular antagonist, termed Maraviroc, was approved by the FDA as an antagonistic blocker of CCR5. Maraviroc is capable of preventing the interaction between gp120 and CCR5, and therefore the membrane fusion events necessary for viral entry (Dorr et al., 2005, Fatkenheuer et al., 2005). See **section 1.3.5** for more detail.

Other genetic variations are also associated with disease caused by HIV-1, including variation in copy number of CCL3L1, resulting from duplicated non-allelic variations of the gene which encodes CCL3. Individuals with low CCL3L1 copy numbers seem to be more susceptible to HIV-1 infection, and this has been attributed to the fact that CCL3L1 is a potent ligand for CCR5 (Gonzalez et al., 2005).

HIV-1 tropism refers to the ability of different strains of virus to infect different cell types and HIV-1 isolates were originally classified in this way as being either M-tropic, T-tropic or dual tropic. Simplistically, M-tropic strains are able to infect macrophages and $CD4^+$ lymphocytes and primarily use CCR5 as a means of entry, whereas T-tropic strains infect

$CD4^+$ T cells but not macrophages and preferentially use CXCR4. Dual tropic strains are capable of infecting both cellular targets and can use either co-receptor for cell entry (Moore et al., 2004, Unutmaz and Littman, 1997, Gray et al., 2005). At present HIV-1 strains are classified according to the co-receptor they exploit rather than the cell type they infect, for example the R5 strains preferentially target CCR5, whereas the X4 strains principally utilize CXCR4, and the R5/X4 strains can employ both (Berkowitz et al., 1998, Berger et al., 1998, Soulie et al., 2012). During the early stages of infection, R5 viral strains predominate, whereas X4 strains are not frequently transmitted and often fail to replicate (Gray et al., 2005, Regoes and Bonhoeffer, 2005). The underlying mechanisms for this are yet to be clarified, but most likely involve a combination of both host and viral factors that give an advantage to R5 strains during the initial stages of infection. Interestingly, HIV-1 strains have been shown to phenotypically switch from R5 to X4 during the course of infection, and this is very important in the progression from HIV to AIDS (Hartley et al., 2005, Regoes and Bonhoeffer, 2005, Shankarappa et al., 1999). X4 strains start to appear after about 5 years in 40-50% of infected individuals and this is associated with rapid $CD4^+$ lymphocyte decline. This effect is thought to be attributed to the high levels of expression of CXCR4 on lymphocyte precursors in the thymus and on circulating $CD4^+$ lymphocytes, increasing the infection potential of the X4 strain (Connor et al., 1997, Glushakova et al., 1998).

The chemokine receptor binding domains utilized by gp120 are similar but not identical to those employed by chemokines and involve conserved sites on the chemokine receptor *N*-terminus and extracellular loops 2 and 3 (ECL2 and ECL3 respectively) (Atchison et al., 1996, Alkhatib et al., 1997). Certain extracellular mutations on CCR5 have been shown to ablate chemokine binding but only partially reduce HIV-1 entry (Blanpain et al., 1999b). Similarly, studies involving monoclonal antibodies directed at either the *N*-terminus or ECL2 of CCR5 demonstrated that gp120 and chemokines have different binding site preferences (Lee et al., 1999). As touched upon previously, tyrosine sulphation of the *N*-terminus of CCR5, and, to a lesser extent, CXCR4, is important for the binding of many HIV-1 isolates (Farzan et al., 1999). Intriguingly, the removal of a single *N*-linked glycosylation site located on the CXCR4 *N*-terminus allowed it to bind both R5 and X4 strains (Chabot et al., 2000). These observations demonstrate the importance of post-translational modifications to chemokine receptor biology and in this case, viral pathogenicity.

1.3.2 Cancer

Cancer is a disease brought about when the normal growth control mechanisms of cells are lost, leading to the formation of a tumour which has escaped detection by the many anti-cancer mechanisms managed by the immune system. The migration of tumour cells to secondary sites, in organs distant from the primary tumour, is termed metastasis and it is this ability of tumour cells to establish themselves, survive and proliferate in new environments, that correlates with poor clinical outcome and often ultimately leads to death of the individual (Hanahan and Weinberg, 2000).

The chemokine network is intricately involved in the many stages of cancer formation, from tumour cell growth and leukocyte infiltration into tumours, to angiogenesis and metastatic behaviour (Balkwill, 2012). Tumours are composed not just of cancerous cells, but also a wealth of different stromal cells such as endothelial cells and fibroblasts, and leukocytes such as macrophages and lymphocytes (Talmadge, 2011). The chemokine expression profile of both tumour cells and stromal cells within the tumour environment, is responsible for the type of leukocytes that infiltrate the tumour, with different tumours often producing different chemokines and therefore attracting different cell infiltrates (Balkwill and Mantovani, 2001). For example an assortment of both CC and CXC chemokines, especially CCL2 and CCL5, were associated with epithelial ovarian cancer tumours and were thought to be responsible for the infiltration of different inflammatory cells, including T lymphocytes and macrophages (Negus et al., 1997). These cells can be stimulated to generate inflammatory cytokines and additional chemokines, contributing to processes such as angiogenesis and protease production, and ultimately ensuring growth and success of the tumour (Scotton et al., 2001a).

Chemokine production can be induced by the expression of oncogenes themselves. *Ras* proteins are GTPases which are often mutated in human cancer, resulting in their constitutive activation (Takashima and Faller, 2013). Downstream signalling pathways induced by oncogenic *Ras* promote the production of chemokines such as CXCL8 and CXCL1. Expression of such chemokines in the tumour environment has been shown to promote neutrophil recruitment and angiogenesis (Sparmann and Bar-Sagi, 2004).

Unlike the cells within benign tumours, which are encapsulated, malignant cells are able to invade the tissues surrounding the primary tumour, which can ultimately lead to metastasis

to secondary sites; a process crucial in disease progression (Joyce and Pollard, 2009). The chemokine receptor expression profiles of various malignant cancer types have highlighted CXCR4 and CCR7 as contributing to the process of metastasis (Scotton et al., 2001b, Mashino et al., 2002, Muller et al., 2001). For example in breast cancer, hypoxia-induced CXCR4 expression allows malignant cells to metastasize towards sites of CXCL12 production, typically the lymph nodes, lungs, liver and bone marrow (Muller et al., 2001). Similarly transfection of CXCR4 into a melanoma cell line was shown to increase its migration and establishment in the lungs (Murakami et al., 2002). Most if not all haematological malignancies begin in the stem cell compartment, and since the CXCL12/CXCR4 axis is a crucial regulator of many types of stem cells, it is not surprising that organs which constitutively express CXCL12 are common sites of metastasis (Kucia et al., 2005, Ratajczak et al., 2006).

Likewise CCR7 expression on the malignant cells of various types of cancer, including squamous cell, colorectal and gastric carcinomas, are associated with metastasis to the lymph nodes; a site of constitutive CCL19 and CCL21 production (Ding et al., 2003, Gunther et al., 2005, Mashino et al., 2002). The exact up-regulation mechanisms of certain chemokine receptors by tumour cells have yet to be clarified, however it is thought that common features associated with most tumours such as a highly hypoxic environment and the constitutive expression of transcription factors such as nuclear factor kappa B (NFκB), may act as triggers or regulators of chemokine receptor expression (Schioppa et al., 2003, Helbig et al., 2003).

1.3.3 Psoriasis

Psoriasis is a chronic inflammatory skin disease and can exist in many forms with plaque psoriasis, known medically as psoriasis vulgaris, being the most common form and affecting 80% of psoriasis patients. Psoriasis vulgaris is the most prevalent T-lymphocyte mediated disease in humans and is characterised by red and scaly plaques which are sharply demarcated on areas such as the elbows, knees and scalp (Perera et al., 2012).

Psoriasis is a multifactorial disease involving a number of different cells and immune regulators. The activation of T lymphocytes by Langerhan’s cells, which are APCs resident in the skin, results in the release of inflammatory cytokines, such as tumour necrosis factor

alpha (TNF-α) and interferon gamma (IFNγ); ultimately leading to the disregulation of keratinocyte proliferation (Krueger, 2002).

Since the chemokine system is intricately involved in the migration of immune cells including T-cells, during both homeostasis and inflammation, many chemokines and their receptors have been implicated as important contributers to the inflammatory processes which persist in psoriasis and indeed in many other chronic inflammatory diseases (Nickoloff et al., 2007).

A number of different chemokines and chemokine receptors have been linked to the recruitment of T-cells to the skin; a process which is crucial to the pathophysiology of psoriasis. For example CCL20 and CCR6 are both markedly upregulated in psoriasis, with keratinocytes expressing high levels of CCL20 to attract CCR6 expressing Th17 cells (Homey et al., 2000). Likewise, skin infiltrating T-lymphocytes which express CCR10 are characteristic of psoriasis and other dermatological disorders, and one of its ligands, CCL27 is commonly expressed by keratinocytes, indicating that the CCR10/CCL27 network is significant in skin inflammation mediated by T-cells (Homey et al., 2002).

CCL2 and its receptor, CCR2 are also substantially involved in the pathogenesis of psoriasis. Studies show that CCL2 is expressed by basal keratinocytes upon stimulation with TNF-α and IFN-γ in psoriatic lesions (Vestergaard et al., 2004). This results in the infiltration of macrophages into the dermal-epidermal junction of the skin, which is a hallmark of psoriatic disease (Gillitzer et al., 1993). Over-expression of CCL2 on keratinocytes in a murine model has also been implicated in the recruitment of dendritic and Langerhan's cells to the skin; an event thought to be critical in the establishment of psoriasis in humans (Nakamura et al., 1995). In addition, CCR2 is up-regulated on the peripheral blood monocytes of psoriatic patients, and CCR2 positive cells are also found within psoriatic plaques (Vestergaard et al., 2004). Interestingly D6; an atypical chemokine receptor that scavenges CC-chemokines including CCL2 (**section 1.5**) is up-regulated in the un-involved skin of psoriatic patients. The up-regulation of D6 in such areas is thought to act as a protective mechanism to scavenge inflammatory chemokines and prevent the infiltration of leukocytes (Singh et al., 2012).

1.3.4 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by synovial inflammation, increased cell proliferation and neovascularisation. These processes involve a complexity of immune regulators and lead to damage of bone and cartilage, ultimately resulting in the destruction of the joints (Firestein, 2003). Synovial fluid from joints affected by RA contains a mixed inflammatory cell infiltrate, involving the action of many chemokines and chemokine receptors (Szekanecz et al., 2010), some examples of which will be briefly discussed.

The type II collagen-induced arthritis (CIA) model in the arthritis-susceptible DBA/1j mouse strain is the most commonly used immunization-based model of RA and a lot of what is currently known about the disease has come from research using these mice (Anthony and Haqqi, 1999, Luross and Williams, 2001, Schurgers et al., 2011).

The complexity of RA can be demonstrated by analysis of previous studies on the role of CCL2 and its receptor CCR2 in the pathology of this disease. CCR2 positive mononuclear cells are found in the inflamed joints and the synovial fluid of RA patients and also in rodent models of RA (Bruhl et al., 2001, Haas et al., 2005). Correspondingly, CCL2 is present in the synovial fluid and sites of bone remodelling of RA patients as well as in the inflamed joints of CIA mice (Volejnikova et al., 1997, Koch et al., 1992, Thornton et al., 1999). Neutralising CCL2, with use of CCL2 analogues or monoclonal antibodies, has been shown to prevent disease initiation and progression in different animal models (Gong et al., 1997, Ogata et al., 1997). Taking these findings into account, it is reasonable to predict that blocking CCR2 may have a similar effect. In fact the opposite is true, with disease progression and severity highly augmented in CCR2-null mice compared with controls (Quinones et al., 2004). On the contrary, blocking CCR2 with antibodies during the early stages (0 to 15 days) of CIA significantly reduced the severity of arthritis, whereas blocking at later stages (21 to 36 days) distinctly increased the severity of symptoms, in concordance with the CCR2-KO mouse studies (Bruhl et al., 2004). This discrepancy between the CCR2 KO mouse data and early stage antibody-induced blocking of CCR2 is thought to be due to the phenotype of the KO mouse before disease has been initiated rather than the effect that blocking CCR2 has on disease pathology. These studies highlight the importance of CCR2 in the down-regulation of the inflammatory response in

later stages of CIA, and also the potential pitfalls associated with use of knock-out mice when studying the initiation and progression of a disease.

More recently CCR1 has come to the forefront as an important mediator of RA. Both CCR1 and CCR2 are expressed on monocytes, macrophages and T-cells which infiltrate into the synovial fluid of RA patients, however a study using a CCR1 antagonist demonstrated that monocyte infiltration was mediated by CCR1 and not CCR2 (Dairaghi et al., 2011). Furthermore potent CCR1 ligands activated by proteolytic processing are found in the synovial fluid of RA patients (Berahovich et al., 2005), and monocytes isolated from RA patients showed preferential activation of CCR1, compared with CCR2 and CCR5 in order to migrate towards the synovial compartment (Lebre et al., 2011). CCR1 therefore remains a primary target in the therapeutic treatment of RA (Szekanecz et al., 2011).

Activated T cells with the ability to infiltrate into inflammatory sites express high levels of CXCR3 and CCR5. These cells are found in the synovial fluid of RA patients and are associated with high levels of IFN-γ, implying a Th1 phenotype and implicating CXCR3 and CCR5 expression in RA pathogenesis (Qin et al., 1998). Blocking CCR5 and CXCR3 using a non-peptide agonist resulted in a reduction in clinical symptoms of CIA (Yang et al., 2002, Gao et al., 2003). Controversy exists regarding individuals with the Δ32-CCR5 homozygous genotype and whether or not a lack of CCR5 expression confers protection against the development of RA. Indeed some studies fully support this notion (Gomez-Reino et al., 1999, Prahalad, 2006, Lee et al., 2013b) and others find no correlation (Lindner et al., 2007, Garred et al., 1998, John et al., 2003). Preventing the action of CCR5 *in vivo* has also had conflicting results, with studies using CCR5 peptide blockers on mice and rhesus monkeys showing a decrease in CIA-induced pathology (PlaterZyberk et al., 1997, Vierboom et al., 2005), whereas CCR5-KO CIA-induced mice showed similar symptoms to WT mice (Bao et al., 2005).

More recently Th17 cells expressing CCR6 have been shown to be preferentially recruited to inflamed joints in both RA patients and in a T-cell mediated murine model of RA. The production of CCL20 by synoviocytes in inflamed joints recruit such T-cells and this can be blocked by a monoclonal antibody against CCR6, resulting in a substantial decrease in arthritis progression in mice and implicating CCR6 expression in disease pathology (Hirota et al., 2007). Moreover a polymorphism in the CCR6 gene has been associated with rheumatoid arthritis susceptibility (Kochi et al., 2010). Individuals with this polymorphism have enhanced expression of CCR6, and an increased susceptibility to rheumatoid arthritis.

1.3.5 Pharmaceutical targeting of the chemokine system

Chemokines and their receptors are integrally involved in driving inflammation, therefore it is extremely attractive to target the chemokine system when designing therapeutics for treatment of inflammatory or auto-immune diseases. GPCRs are known to be very 'druggable' molecules, with ~30% of all FDA-approved small molecule drugs thought to target GPCRs (Hopkins and Groom, 2002). However, despite many promising *in vitro* studies and clinical trials using different strategies to target the chemokine system, only two drugs which block the action of chemokine receptors have been approved by the FDA to date. One of these is Maraviroc; a CCR5 inhibitor which prevents HIV infection (see **section 1.3.1** for details on HIV) (Dorr et al., 2005, Fatkenheuer et al., 2005), and the other is AMD3100 (also known as Mobozil and Plerixafor); a CXCR4 inhibitor which was originally developed for use as another anti-HIV drug (Hendrix et al., 2004). Problems with dosage and effectiveness ceased its development as an anti-retroviral drug, however its ability to rapidly mobilize haematopoetic stem cells made it attractive for use on patients requiring transplants, e.g. during cancer therapy (Broxmeyer et al., 2005).

Although the approval and effective use of such antagonistic drugs generates hope for future success in this field, other clinical trials using this approach for the treatment of autoimmune diseases such as rheumatoid arthritis and multiple sclerosis have been disappointing (Horuk, 2009). This has been attributed to the multifaceted complexity of such immune diseases, in which an assortment of immunological factors, including chemokines and their receptors, are implicated in the development and progression of the disease. Chemokine receptor promiscuity may also contribute to the difficulty in developing therapies as more than one chemokine receptor is likely to be implicated in the disease pathophysiology; therefore it is more difficult to inhibit the activation of all such receptors (Mantovani, 1999, Ribeiro and Horuk, 2007).

More recently it has been proposed that the concept of redundancy is inappropriate, and that it may be more accurate to describe the chemokine system as being under both temporal and spacial control *in vivo*. Indeed, it has been demonstrated that binding of different chemokines to a given receptor can elicit distinct signalling responses, and also that heterodimerization of chemokine receptors can have considerable implications for signal generation after ligand binding. In this way, structural features of chemokines and

their receptors, the cell type they are expressed by, and timing of expression, all help to determine different biological outcomes in separate tissues. (O'Hayre et al., 2010, Schall and Proudfoot, 2011, Proudfoot, 2002).

Shortcomings of previous clinical trials with small molecular antagonists against chemokine receptors have highlighted incorrect dosage as a contributing factor to the failure of such compounds to reduce inflammation. It is widely known that small molecular compounds have a tendency to bind to serum proteins and lipids in the blood, which can dramatically decrease drug availability. In cases such as this; where dosage of an inhibitor is not sufficient to impede the activity of all the receptors it is targeting, a very small amount of residual chemokine activity can still be enough to set up a positive feedback loop that drives inflammation. Therefore future clinical trials that target the correct receptor at the right time with the optimal dose may be more successful (Schall and Proudfoot, 2011).

1.5 Atypical chemokine receptors

In addition to the ‘classic’ chemokine receptors described throughout this chapter, there exists a small family of ‘atypical’ chemokine receptors (ACKRs) that are also able to bind chemokine ligands with high affinity. However, ACKRs do not generate a conventional, G-protein coupled signal after ligand binding, nor mediate a typical cellular response, such as cell migration; which is a hallmark of the other chemokine receptors (Ulvmar et al., 2011, Nibbs and Graham, 2013). However recent studies have suggested that ACKRs may be able to use alternative signalling pathways, for example through β-arrestins, in order to exert their functional effects (Rajagopal et al., 2010, Borroni et al., 2013). Therefore signalling induced by these ‘silent’ atypical receptors, might in fact have important consequences for their function.

ACKRs have a similar structure to other chemokine GPCRs, with an extracellular *N*-terminus, seven helical domains spanning the cell membrane, and an intracellular *C*-terminus. The inability to signal through classical means is thought to be brought about by alterations in the DRYLAIV amino acid motif which is found in the second intracellular loop of signalling chemokine receptors (Graham et al., 2012). Since this motif is required for G protein-coupling, ACKRs are incapable of initiating the key event required for the cascade of downstream signalling processes that occur in GPCRs and ultimately lead to

processes such as cell migration. However, other than this change in the DRYLAIV motif, ACKRs are only loosely related to each other and do not share many other structural or functional similarities. At present there are four ‘atypical’ chemokine receptors that fit this description; **DARC** (Duffy antigen receptor for chemokines), **CCR11**, **CXCR7** and **D6**. Because signalling is a requirement for a molecule to be classed as a receptor, these proteins (with the exception of CXCR7 for historical reasons) have not been appointed names under the systematic nomenclature assigned to the ‘classic’ chemokine receptors. Instead the name ‘Atypical chemokine receptor’ has recently been formalized in the new Nomenclature Committee of the International Union of Pharmacology and therefore DARC is now known as **ACKR1**, D6 as **ACKR2**, CXCR7 as **ACKR3** and CCR11 as **ACKR4** (Bachelerie et al., 2014). Despite this, for reasons of consistency, the former names will be used in this thesis.

Regardless of the ACKRs inability to signal conventionally after ligation, it has been shown that ACKRs can influence immune and inflammatory responses in other ways. This includes acting as decoy and scavenger receptors (Mantovani et al., 2006).

1.5.1 DARC

DARC was originally identified as a molecule located on the surface of red blood cells (RBCs) through which the malaria parasites *Plasmodium vivax* and *Plasmodium knowlesi* can enter (Horuk et al., 1993, Miller et al., 1975). DARC was later found to be able to bind both CC and CXC inflammatory chemokines (Gardner et al., 2004) **see Table 1-3**, and has unique features that set it apart from both the ‘classical’ and ‘atypical’ receptors. For example, the gene encoding this protein is situated in a separate chromosomal location different from the other receptors, and its sequence bears little resemblance to the other receptors (Rot, 2005).

DARC is expressed on RBCs and endothelial cells, as well as in tissues such as the brain, lungs and kidney (Graham, 2009, Mantovani et al., 2006). Since RBCs are considered incapable of endocytosis, chemokines associated with DARC on RBCs stay on the cell surface, and have the potential to be displaced by other chemokines, or different molecules such as heparin and activated coagulation factors (Schnabel et al., 2010). In contrast, DARC expressed on nucleated cells can internalise bound chemokines; a characteristic associated with the other ACKRs (Nibbs et al., 2003).

The idea that DARC expressed on RBCs acts as a negative regulator or ‘chemokine sink’ to mop up excess inflammatory chemokines in the blood has long been postulated. Accordingly, DARC null mice exhibited exaggerated inflammatory responses upon exposure to the bacterial toxin lipopolysaccharide (LPS) (Dawson et al., 2000). DARC is also thought to play a role in the inhibition of tumourogenesis in prostate and breast cancer (Wang et al., 2006, Shen et al., 2006) and this may relate to the ability of DARC to bind pro-angiogenic CXC chemokines.

Conversely, DARC may also be important in maintaining chemokine levels in the blood. Chemokine concentrations are rapidly depleted when injected into DARC-null mice, however concentrations of chemokine can be preserved at a measurable quantity in wildtype. Despite this, the purpose of sustaining inflammatory chemokines in the blood is still unclear (Jilma-Stohlawetz et al., 2001, Fukuma et al., 2003).

Remarkably, the expression of DARC on RBCs has been selected against in over 95% of West Africans in areas where malaria is prevalent, although these individuals still express DARC in non-erythroid sites (Zimmerman et al., 2013). This lack of DARC expression was thought to provide resistance to *Plasmodium vivax*, although recent studies have shown that the relationship between *P. vivax* and DARC is more complex and DARC-null populations have been identified that can be infected by the parasite (Menard et al., 2010). Various suggestions have been put forward as to why such a huge selection pressure exits despite the ability of *P.vivax* to infect DARC-null individuals. It may be the case that *P. vivax* has recently evolved alternative RBC invasion pathways that do not rely on DARC. Equally, an inability of DARC-null individuals to infect the *anopheles* vector with *P.vivax* parasites may also be an important component, and would help to explain the resistance to *P.vivax*-induced malaria of this population as a whole. Surprisingly, unlike previous studies investigating CCR5-null individuals, investigation into the consequences of having DARC-null RBCs with regards to the immune response and inflammation have not provided any convincing results relating to the function of DARC.

Recent studies investigating the function of DARC expressed on endothelial cells highlighted its importance for supporting chemokine activity. Both *in vitro* and *in vivo* experiments confirmed DARC-mediated binding and unidirectional transport of chemokines from the baso-lateral to the apical side of venular endothelial cells, establishing DARC as a transcytosis receptor, functioning to support optimal chemokine-

induced leukocyte migration (Pruenster et al., 2009). This is in contrast to the earlier assumptions that DARC acts as a decoy receptor.

1.5.2 CCR11

The atypical chemokine receptor CCR11 (also known as CCRL1 and CCX-CKR) is thought to be widely expressed in several tissues including the skin, intestine, heart, lung and lymph nodes and is able to bind three homeostatic chemokines; CCL19, CCL21 and CCL25, see **Table 1-3** (Ulvmar et al., 2011). Again, signal transduction through typical G-protein mechanisms does not seem to occur after ligand binding, however a recent study suggests that CCR11 can couple to a certain class of G-protein (G_s), and this interaction induces an increase in cAMP levels in response to chemokine stimulation (Watts et al., 2013). However, this interaction may not be physiologically relevant because such a response was only observed upon treatment with pertussis toxin, which inhibits the higher affinity G_i protein-CCR11 interaction and therefore allows coupling to G_s.

Studies *in vitro* have revealed CCR11 to act as a 'decoy' or 'scavenger' receptor, with an ability to internalise bound chemokines and target them for lysosomal degradation, however whether CCR11-internalisation requires the recruitment of β-arrestin remains contentious (Comerford et al., 2006, Watts et al., 2013). A recent *in vivo* study has corroborated CCR11's scavenging function by demonstrating that CCR11-deficient mice have increased levels of CCL21 in the blood, and increased levels of CCL21 and CCL19 in lymph nodes. CCR11 knock-outs also exhibited enhanced pathology in the murine experimental autoimmune encephalitis (EAE) model, which is a model of CNS autoimmunity. Surprisingly this increase in disease symptoms was not due to more efficient priming in the draining lymph node, but seemed to be linked to enhanced Th17 responses. CCR11-deficient mice with EAE also had increased levels of CCL21 in the CNS, suggesting a possible role for CCR11 in the brain (Comerford et al., 2010).

CCR11-deficient mice have also been reported to exhibit a higher incidence of spontaneous autoimmunity; similar to Sjogren's syndrome in humans. These abnormalities in self-tolerance have been attributed to defects in thymic development (Bunting et al., 2013).

CCR11 expression has been suggested to affect a variety of diseases, one of which is breast cancer, whereby a significant correlation between over-expression of CCR11 and more

favourable clinical outcome was reported, suggesting that increased CCR11 expression inhibits cancer cell progression (Feng et al., 2009). Conversely up-regulation of CCR11 was measured in transcripts from the epithelial cells of patients with pulmonary sarcoidosis, however the way in which CCR11 modulates the inflammatory response in this disease is not yet known (Kriegova et al., 2006)

These studies clearly indicate that CCR11 influences adaptive immune responses, but the way in which it does this must be further investigated to gain a more thorough understanding of CCR11 function.

1.5.3 CXCR7

CXCR7, like the other ACKRs mentioned, does not mount classical G protein-mediated signalling responses upon activation by its ligands CXCL11 and CXCL12, see **Table 1-3**. In contrast, recent studies have demonstrated that when cells are transiently transfected with CXCR7, the addition of ligand activates MAP kinases through β-arrestins, suggesting that CXCR7 is an example of an endogenous seven transmembrane receptor that signals through β-arrestins instead of G-proteins (Rajagopal et al., 2010).

Unlike most ‘classical’ chemokine receptors, CXCR7 activation does not promote cell migration, but is thought to play roles involved in cell survival and tumour progression (Burns et al., 2006). CXCR7 also differs from the other ‘atypical’ receptors in that it is primarily expressed during multiple steps of development, and has a clear lack of expression in adult tissues (Naumann et al., 2010). In addition to CXCR4, CXCR7 binds the primordial chemokine CXCL12, and studies in mice and zebra fish have confirmed a particular importance for CXCR7 in cardiac and vascular development, with CXCR7-null mice dying shortly after birth due to defective ventricle and valve formation in the heart (Sierro et al., 2007, Gerrits et al., 2008). Furthermore, CXCR7 is involved in the regulation of primordial germ cell migration through its ability to internalize CXCL12 and therefore control CXCL12 concentrations during development, enhancing the sensitive organisation of this process (Valentin et al., 2007).

Interestingly, although adult tissues generally do not express CXCR7, it is widely expressed in a multiplicity of tumour cells, including tumours of the breast and lung, and seems to provide cells with a growth and survival advantage and increased adhesion

properties (Miao et al., 2007). Studies on active CXCR7 in breast cancer cells have highlighted its ability to act as a scavenger receptor, mediating the internalisation and lysosomal degradation of CXCL12. It seems CXCR7 is constitutively internalised and recycled to the cell surface in these cells, even in the absence of ligand (Luker et al., 2010). It is not clear how CXCR7-mediated scavenging of CXCL12 would promote tumour growth or metastasis, or if CXCR7 has other distinct roles within the tumour environment. Despite the poorly defined mechanisms, an important role for CXCR7 expression in cancer survival and proliferation is clear, and thus opens up the possibility of developing antagonistic therapeutics against CXCR7 in order to limit the survival of tumour cells (Burns et al., 2006).

1.6 The atypical chemokine receptor: D6

1.6.1 D6 identification and characterisation

D6 (gene name *Ccbp2*), is one of the most widely studied 'atypical' chemokine receptors and was identified in 1997 by two separate groups using various cloning strategies (Nibbs et al., 1997, Bonini et al., 1997). The gene encoding D6 is positioned within a major chemokine receptor-coding chromosomal locus in both the mouse (chromosome 9) and human (chromosome 3), and its closest homologues are other chemokine receptors, suggesting that it has evolved from within this family of proteins (Nomiyama et al., 2013).

1.6.2 D6 structure and biochemistry

D6 is a 7-transmembrane spanning receptor which has a fairly similar structural homology to classical G-protein-coupled chemokine receptors, see **Figure 1-7.** The canonical DRYLAIV motif associated with G protein coupling exists as DKYLEIV in D6, and this is the case with all mammalian forms of D6, indicating that the altered DRYLAIV motif is not simply a loss of function mutation, but a conserved feature of the D6 protein with functional importance (Graham, 2009). Studies investigating the structure of the D6 protein using heterologous transfectants have identified a protein of ~49kD which has various post-translational modifications, including a phosphorylated *C*-terminal (see section **1.6.3**) and *N*-linked glycosylation on the *N*-terminal. Glycosylation of the *N*-terminal was postulated to enhance ligand binding, however a non-glycosylated mutant of D6 still binds its ligands with high affinity, therefore the functional significance of D6 *N*-

terminal glycosylation still remains to be determined (Blackburn et al., 2004). Imaging of D6-expressing heterologous transfectant cells demonstrated an unusual distribution of the protein, with > 95% of D6 being present in intracellular vesicles, and only low levels of D6 being seen on the cell surface (Weber et al., 2004, Blackburn et al., 2004).

Similar to the other atypical receptors described, D6 crucially does not display classical G protein-coupled signalling upon ligand binding, but rather internalises the bound chemokine and targets it for degradation by lysosomes (Fra et al., 2003). This has led to its classification as a scavenger or decoy receptor for chemokines. Its ability to scavenge chemokines far exceeds that of CCR11 and is linked to its ability to constitutively travel to the cell surface and rapidly internalize back into the cell. This cyclic trafficking is catalytic, in that D6 can recycle many times to and from the cell surface without being degraded (Weber et al., 2004). This process is also ligand-independent, and unlike many signalling chemokine receptors, the receptor is not desensitized in the presence of a high chemokine concentration, but rather increases D6 expression on the cell surface (Comerford and Nibbs, 2005, Bonecchi et al., 2008). Internalisation of D6 is associated with β-arrestin re-localisation (McCulloch et al., 2008, Galliera et al., 2004) and is mediated in Rab5-positive vesicles through clathrin-coated pits. Following internalisation, D6 is targeted to early endosomes before Rab4 and Rab11-dependent recycling pathways are incorporated to traffic this internalised D6 back to the plasma membrane (Bonecchi et al., 2008).

The *C*-terminal tail of D6 is interesting in that it bears little resemblance to other chemokine receptors. Controversy exists regarding the biochemical features of this region and their consequences for receptor internalisation and recycling. On one hand the serine cluster in the *C*-terminal region of D6 was shown to be constitutively phosphorylated in both the presence and absence of ligand. It was initially thought that this phosphorylation drives the recycling of D6 to and from the cell surface; however this unexpectedly did not seem to be essential for D6 internalization and the scavenging of chemokine ligands. On the other hand, when the last 58 amino acids of the *C*-terminal tail were deleted, D6 could be internalized but could not recycle back to the cell surface, indicating that the *C*-terminus is necessary for the recycling function of D6 (McCulloch et al., 2008). Conversely, in a separate study, D6 was shown to undergo internalisation in a β-arrestin-dependent and receptor-phosphorylation independent manner, with a complete absence of either constitutive or ligand-induced phosphorylation (Galliera et al., 2004). A recent study has reported the ability of D6 to activate a β-arrestin2-dependent signalling pathway which ultimately leads to the phosphorylation of cofilin, and subsequent cytoskeletal

reorganisation (Borroni et al., 2013). This signalling pathway has been postulated to increase the abundance of D6 on the cell surface upon chemokine stimulation and also aid the chemokine scavenging function of D6.

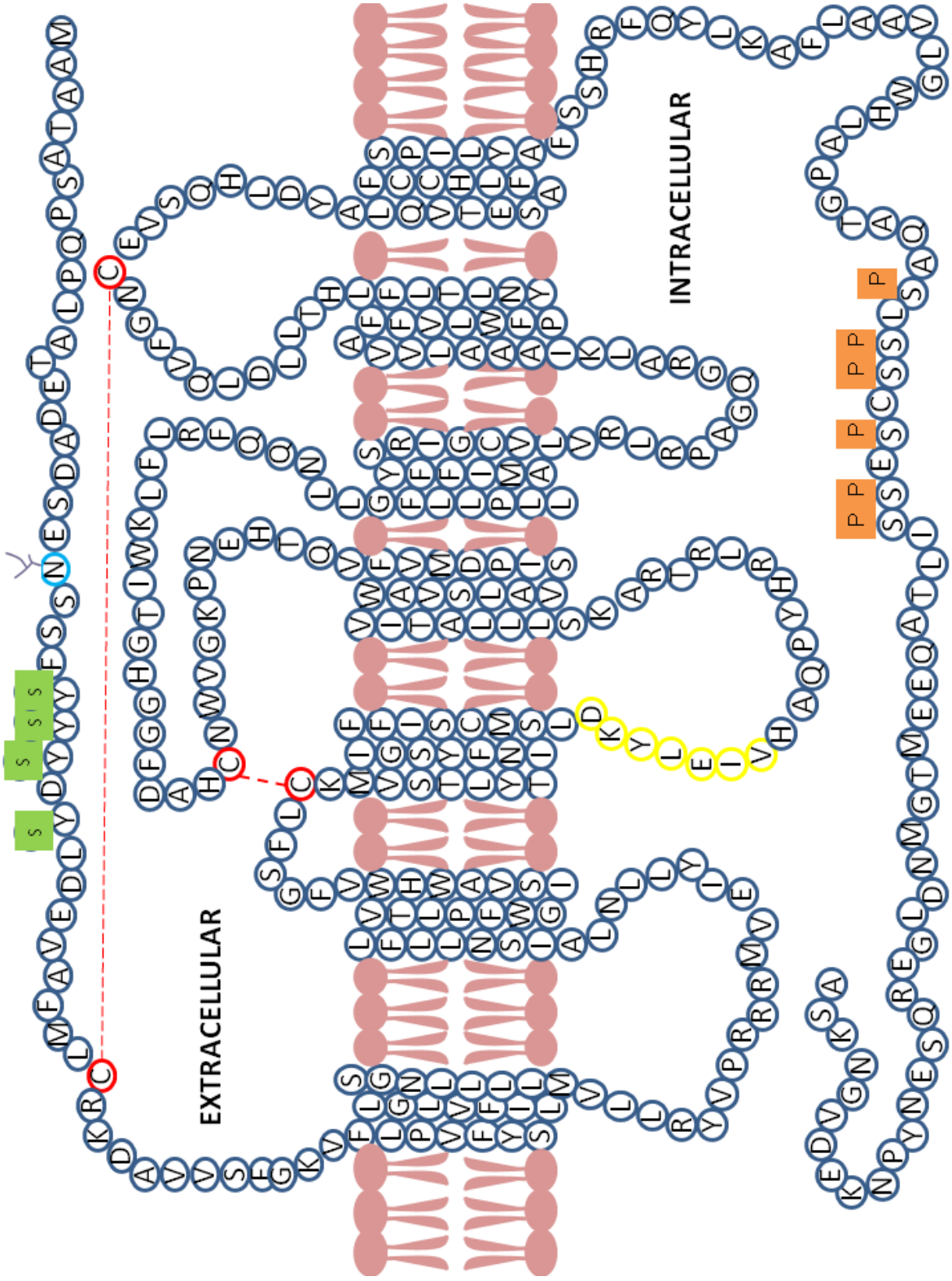


Figure 1-8: D6 within the plasma membrane
2-dimensional diagrammatic representation of D6 showing the predicted arrangement of the transmembrane domains, the intra- and extracellular loops, and the *N*-terminus and *C*-terminus. Amino acids are represented by their coding letters. Possible post-translational modifications are shown. Phosphorylation of serine and threonine residues is represented by an orange P, sulphation of tyrosine residues is represented by a green S and N-linked glycosylation is represented by a sugar molecule on the first asparagine residue (N) on the *N*-terminus. Possible disulphide bonds between cysteine residues are represented by red dashed lines, with the cysteine residues thought to be involved are circled in red. The residues making up the DKYLEIV motif on the 2nd extracellular loop are circled in yellow.

1.6.4 D6 ligands

D6 is highly promiscuous in that it is able to bind at least 14 different chemokines from the CC subfamily; all of which are inflammatory, see **Table 1-3**. However despite this broad specificity, structural observations indicate that in order for D6 to efficiently bind and target ligands for degradation, they must have a proline residue in position 2 of their protein sequence. This has been illustrated in studies of variants of CCL3 and CCL22, where the P2 residue is either changed to another amino acid or is missing (Bonecchi et al., 2004, Nibbs et al., 1999). More recently, studies on D6 binding capability to CCL14 have highlighted three CCL14 isoforms that exert different responses upon binding to D6. Full length CCL14 (1-74) is physiologically inactive and can be cleaved under inflammatory conditions to the biologically active form CCL14 (9-74), a potent agonist for CCR1, CCR3 and CCR5. Interestingly, the truncated *N*-terminus of CCL14 (9-74) is recognised and further processed by CD26 to generate CCL14 (11-74), which is biologically inactive. All CCL14 variants are capable of binding to D6, however only CCL14 (9-74), which has a proline residue in position 2 of its protein sequence, induces increased cell-surface expression of D6 and is properly degraded after binding (Savino et al., 2009). Interestingly, cleavage of chemokines by CD26 has differential effects on their potency depending on the chemokine, and the functional significance of such interactions is very complex (Wolf et al., 2008). In the context of D6, such findings demonstrate that chemokine binding alone does not always induce adaptive up-regulation, or efficient targeting of ligands for lysosomal degradation. Instead the chemokine-scavenging function of D6 is regulated by the chemokine sequence itself, and possibly by CD26, which may help to fine-tune a more effective inflammatory response or have consequences for the resolution of inflammation.

Atypical Receptor	Ligands
DARC	CCL1, 2, 5, 7, 8, 11, 13, 14, 16, 17, 18; CXCL5, 6, 8, 9, 10, 11, 13
CCR11	CCL19, 21, 25
CXCR7	CXCL11, 12
D6	CCL2, 3, 4, 5, 7, 8, 11, 12, 13, 14, 17, 22, 23, 24

Table 1-3: Chemokine ligands for the atypical chemokine receptors

1.6.5 D6 expression

D6 is expressed predominantly on lymphatic endothelial cells (LECs) in ‘barrier’ sites of the body such as the skin, gut and lung, and is also expressed on LECs in lymph nodes.

Expression of D6 on LECs can be up-regulated by pro-inflammatory mediators including IL-6 and IFN-γ, implicating D6 in both inflammatory and tumour contexts (McKimmie et al., 2013, Nibbs et al., 2001). D6 is also highly expressed on syncytiotrophoblasts in the placenta (Madigan et al., 2010) and on subsets of leukocytes (McKimmie et al., 2008, Hansell et al., 2011). Both *in vitro* and *in vivo* studies of D6 have highlighted its significance in the removal of chemokines from inflamed sites, which is thought to be crucial for the resolution phase of inflammation (Graham, 2009). Several hypotheses concerning D6 expression on leukocytes during inflammation have been proposed. Because leukocytes can move directly to sites of chemokine expression or deposition, it was thought that D6-expressing leukocytes are ideally suited to a role in the targeted removal of inflammatory CC-chemokines from inflamed tissue. However, surprisingly it has been found that D6 expression is low on 'classical' inflammatory leukocytes such as macrophages and neutrophils, but high on DCs and B cells (McKimmie et al., 2008, Hansell et al., 2011). This suggests additional roles for D6 in the coordination of the immune response. The possible roles for D6 expression, particularly on LECs will be discussed further in **section 1.6.5.6.**

1.6.5 Pathophysiological role of D6

D6 expression has been knocked out in mice to examine D6 function *in vivo.* D6-null mice develop normally and have no obvious physiological abnormalities under resting conditions; however the immune system is affected by this knockout with D6-deficient mice displaying an exaggerated inflammatory response to various types of inflammatory stimuli. These key observations, along with *in vitro* data generated from transfected cells, led to the proposal that D6 acts as a 'scavenger' or 'decoy' receptor to mop up superfluous inflammatory chemokines and target them for degradation, thereby providing protection against excessive inflammatory responses and ensuring the normal resolution of inflammation (Graham and Locati, 2013). D6 function has also been studied with emphasis on different tissues and organs such as the skin, lung, gut and placenta, and in diseases such as cancer. Key findings are discussed below.

1.6.5.1 D6 and the skin

Compared to WT mice, D6-null mice display an exaggerated inflammatory response to stimuli such as treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or the

injection of immunopotentiators such as Freund's complete adjuvant (Jamieson et al., 2005, Martinez de la Torre et al., 2005). In both models, D6-null mice demonstrate an impaired ability to remove inflammatory CC chemokines from inflamed skin, resulting in increased inflammatory cell infiltrates and a less effective and extended resolution phase. Interestingly in the TPA model, whereby phorbol ester was topically applied to the skin, D6-null mice developed a psoriasis-like pathology. This was characterised by the recruitment of T-cells and the accumulation of dermal mast cells in the inflamed skin, leading to hyperproliferation of the epidermis and altered differentiation and angiogenesis throughout the dermis (Jamieson et al., 2005).

Notably, in this model, neutrophil accumulation in both WT and D6-deficient skin is similar, although more recent studies using the same model demonstrated that neutrophil localisation is altered in the D6-deficient mice, with an increase in neutrophil positioning at the dermal/epidermal junction. This abnormal neutrophil positioning is thought to result in destruction of the dermal/epidermal barrier and epidermal shedding in D6-deficient mice. Further characterisation of this phenotype revealed that both WT and D6-deficient neutrophils express CCR1 and migrate towards CCL3; however the D6-deficient neutrophils displayed a significantly more marked migration. This study suggests a novel function for D6 expression on hematopoetic cells to act in a cell-autonoumous manner to limit the movement of these cells towards inflammatory ligands. Curiously this effect was overcome by exposure to very high levels of chemokine, suggesting that under conditions of extreme inflammation, the control of neutrophil migration by D6 can be overcome (Rot et al., 2013).

Expression of D6 has also been associated with impaired lesion development in human psoriasis. Strikingly D6 expression in uninvolved psoriatic skin is eight times higher than that seen in healthy control skin, however this expression level drops at sites within the lesion or at the lesion border. D6 expression in uninvolved psoriatic skin is thought to maintain 'normal' skin histology, despite the presence of low-grade inflammation in such areas. Remarkably induction of minor tissue insults, by tape-stripping of uninvolved psoriatic skin, reduced D6 expression in such areas compared with untouched uninvolved psoriatic skin. This relates to the 'Koebner phenomenon' observed with some psoriatic patients whereby plaques develop in areas that have suffered mild trauma, and further suggest a role for D6 expression in the prevention of plaque formation (Singh et al., 2012). Elevated D6 expression in the peripheral blood leukocytes of patients with psoriasis and systemic sclerosis; an autoimmune disease characterised by thickening of the skin, has also

been reported. In the case of systemic sclerosis, D6 expression by leukocytes correlated negatively with levels of circulating inflammatory chemokines, suggesting that D6 on leukocytes scavenges inflammatory chemokines present in the blood of patients with this disease (Codullo et al., 2011). Recent studies on leukocytes from the blood of RA patients also demonstrated elevated D6 expression, suggesting this to be a common theme on leukocytes in a wide range of chronic inflammatory diseases (Baldwin et al., 2013).

1.6.5.2 D6 and the placenta

In the placenta D6 confers protection against inflammation associated miscarriage, with D6 knockout mice showing an increased vulnerability to miscarriage when inflammatory agents such as LPS were administered. This is thought to be associated with higher levels of inflammatory CC chemokines (compared to WT mice) and therefore an increased infiltration of leukocytes into the placenta (Garlanda et al., 2008). Additionally, studies whereby either $D6^{+/+}$, $D6^{+/-}$ or $D6^{-/-}$ embryos were transferred into fully allogeneic recipients demonstrated a higher incidence of resorption of foetuses with the $D6^{-/-}$ genotype, indicating a critical role for fetal D6 in limiting resorption (Madigan et al., 2010).

1.6.5.3 D6 and the lung and heart

Lung inflammation also seems to be affected in D6 null mice, with exaggerated inflammatory responses in models of pulmonary airway disease and *Mycobacterium tuberculosis* infection (Whitehead et al., 2007, Di Liberto et al., 2008). D6-deficient mice infected with *M. tuberculosis* display a striking phenotype, with a rise in the numbers of infiltrating macrophages, DCs and both CD4 and CD8 T lymphocytes migrating to inflamed lung tissues. This is thought to be associated with increased numbers of inflammatory CC chemokines in the bronchiolar lavage and serum. Augmented inflammatory cytokine production, presumably as a secondary effect of increased inflammatory leukocyte infiltration, resulted in liver and kidney damage, ultimately leading to increased fatality. Such results suggest that *M. tuberculosis* lung infection in D6-deficient mice creates a cascade of inflammation-associated events which dramatically enhances susceptibility to tuberculosis-induced death in these mice (Di Liberto et al., 2008).

More recently increased D6 expression has been identified on alveolar macrophages in patients with chronic obstructive pulmonary disorder and is positively correlated with markers of immune activation, however its role during this disease needs further clarification (Bazzan et al., 2013).

A role for D6 expression in the heart has also been proposed, particularly during myocardial infarction (MI). D6 was shown to be expressed in human and murine infarcted myocardium and is thought to control levels of inflammatory CC-chemokines after MI. D6-deficient mice displayed increased pathogenic inflammation, cardiac rupture and adverse left ventricular remodelling after MI (Cochain et al., 2012).

1.6.5.4 D6 and the gut

In humans, D6 is expressed both in healthy control patient samples from the gut, and those from patients with inflammatory bowel disease and colon cancer (Vetrano et al., 2010). Similarly in mice, D6 is expressed on B cells and stromal cells of the resting colon and is up-regulated during colitis (Bordon et al., 2009). The role of D6 in the gut is still unclear, with contradictory studies in the literature. On one hand, an enhanced susceptibility to colitis and to colitis-associated cancer has been shown in D6-deficient mice. From such results it was postulated that D6 expression in the gut may prevent irritable bowel disease (IBD) and IBD-associated cancer in humans (Collins et al., 2010, Vetrano et al., 2010). On the other hand, an earlier study reported a reduced susceptibility to colitis in D6-deficient mice that were treated using the same colitis-inducing model. This reduced susceptibility was associated with increased numbers of IL-17A secreting gamma-delta T cells in the lamina propria, resulting in enhanced IL-17A activity. Interestingly, treatment with antibodies against IL-17A worsened clinical symptoms of colitis in D6-deficient mice, suggesting a role for D6 in contributing to colitis (Bordon et al., 2009).

1.6.5.5 D6 and cancer

The close association between inflammation and cancer has led to the hypothesis that a molecule like D6, capable of scavenging inflammatory chemokines may also play a protective role in the inhibition of inflammation-induced cancer. A study investigating this demonstrated that D6 knockout mice are more likely to develop cancer after being treated with a mutagen and inflammatory agent to induce skin tumours, compared with WT mice. Likewise, a mouse strain that does not normally exhibit any susceptibility to such treatment

has been shown to develop cancer when D6 is knocked out (Nibbs et al., 2007). These findings highlight the significant role of inflammatory chemokines in the development and progression of cancer, and the potential importance of D6 acting as a 'decoy' receptor in order to sequester such chemokines, and as a consequence, prevent tumour formation (Nibbs et al., 2007). Similarly, clinical studies support such findings, showing that D6 expression is positively correlated with disease-survival in a variety of different cancers, including breast, gastric and cervical cancers (Wu et al., 2008, Zeng et al., 2011, Hou et al., 2013, Zhu et al., 2013).

1.6.5.6 D6 function on LEC's

Taking into account that D6-expressing trophoblasts in the placenta have been shown to protect against excess inflammation during pregnancy (Garlanda et al., 2008); it was proposed that LEC-D6 may act in the same way to regulate chemokine concentration in surrounding tissues and protect against an extreme inflammatory response. It was also suggested that LEC-D6 acted as a 'gate-keeper' to prevent inflammatory CC-chemokine drainage to lymph nodes (Locati et al., 2005). At first glance both hypotheses seem plausible, however closer examination of LEC physiology does not support these ideas: Firstly, lymphatic vessels are separated by large distances (100-500μm), therefore chemokines residing within tissue spaces are unlikely to come into contact with LEC-D6 and be degraded, as the lymphatic vasculature is too sparse for such a function. Secondly, lymphatic vessels are permeable meaning that a 'gatekeeper' role is unlikely as proteins and particulate matter, including inflammatory chemokines, are known to be able to flow freely into the lymphatic vasculature. Finally, D6 ligands have been detected in draining lymph nodes and seem to have a functional significance whereby they can drain from inflamed peripheral tissues and appear presented on the luminal surface of high endothelial venules in draining lymph nodes (LNs); a phenomenon described as 'remote control'. Such characteristics of the lymphatic system make the role of LEC-D6 difficult to define (McKimmie and Graham, 2006).

It is well documented that initiation of adaptive immune responses at inflamed sites involves the maturation of antigen presenting cells (APCs) such as dendritic cells (DCs), during which time they down-regulate inflammatory chemokine receptors and up-regulate the expression of CCR7. This changes their status from an immature dendritic cell (iDC) to

a mature dendritic cell and enables them to migrate towards lymphatic vessels, where LECs selectively present the CCR7 ligands; CCL19 and CCL21 (Forster et al., 2008). This interaction is responsible for moving APCs from the damaged or infected site to the draining LNs where a substantial immune response can be generated. Despite this established mechanism, it is still unclear how GAGs on LEC surfaces selectively present only CCR7 ligands. Evidently, inflammatory chemokines are also produced at very high concentrations during inflammatory responses; therefore accumulation of such chemokines at this site would also be expected. However, very little inflammatory leukocyte or iDC accumulation is apparent on LEC surfaces, thus alluding to a mechanism whereby LEC surfaces are ‘swept clean’ of inflammatory chemokines (Graham and McKimmie, 2006).

Observations using D6-deficient mice have shown that in the absence of D6, LEC surfaces in inflamed tissues are characterised by the accumulation of large numbers of inflammatory leukocytes including iDCs. This leads to congestion of the lymphatic system, with implications for fluid drainage and APC migration, resulting in an extended period of inflammation (Lee et al., 2011, McKimmie et al., 2013). It appears, therefore, that D6 expressed on LECs has a distinct role in controlling cellular migration to draining lymph nodes, most likely by acting as a scavenger to keep an inflammatory chemokine-free surface on the LECs. In this way, D6 acts as a key regulator of lymphatic function, and importantly plays a role in the integration of innate and adaptive immune responses (Lee et al., 2013a), see **Figure 1-9**.

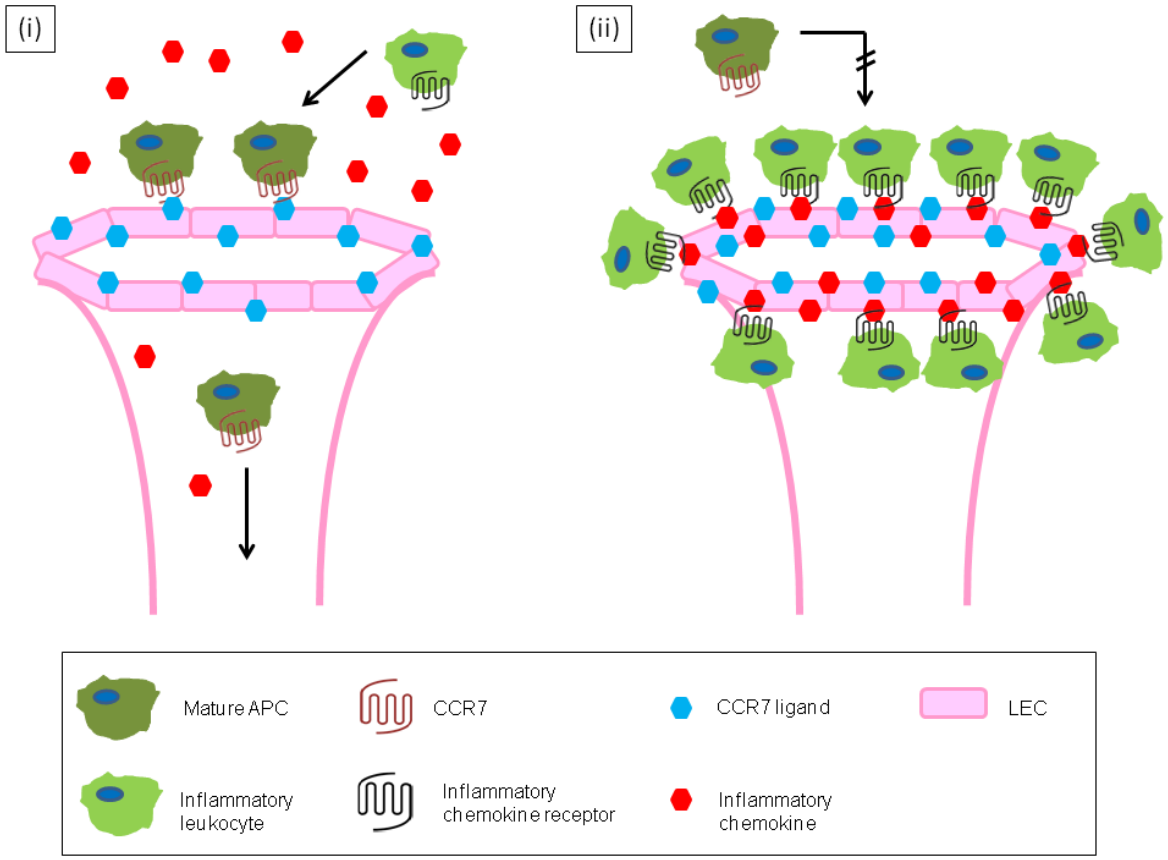


Figure 1-9: D6-deficient mice show disrupted antigen presentation
During inflammation, antigen presentation involves the maturation of APCs, whereby the lymph-node homing chemokine receptor CCR7 is up-regulated and inflammatory chemokine receptors are down-regulated. **(i)** In WT mice CCR7 ligands (CCL19 and CCL21) are presented on the surfaces of LECs, facilitating the interaction between CCR7 and its ligands and ensuring APC migration from inflamed tissues into lymph nodes. Notably the selective presentation of CCR7 ligands takes place against a background of inflammatory CC chemokines. **(ii)** In D6-deficient mice the process of antigen presentation is dis-regulated. Lack of D6 leaves LECs unable to scavenge inflammatory CC chemokines, resulting in inappropriate presentation of inflammatory chemokines, thus attracting inflammatory leukocytes to the lymphatic vessel surface. The resulting congested lymphatic vessel cannot drain fluid or mature APCs as effectively, resulting in decreased efficiency of antigen presentation and an extended period of inflammation. Adapted from (Lee et al., 2013a).

1.7 Thesis aims and objectives

As detailed in this introduction, previous *in vitro* and *in vivo* studies support the hypothesis that D6 functions to minimise the inflammatory response by acting as an inflammatory CC chemokine scavenger. Inflammatory chemokines are often thought of as the initial driving force of various chronic inflammatory and auto-immune diseases, therefore modulation of inflammatory chemokines may provide a novel approach to treatment of such conditions. The *N*-terminus of D6 is thought to be a major component of the binding site for inflammatory chemokine ligands. A synthetic peptide analogue of the D6 *N*-terminus (D6-N) may have the ability to target a broad spectrum of inflammatory chemokines, and consequently inhibit cognate receptor binding. Thus D6-N may have the potential to be used therapeutically as a non-immunogenic, broad-based chemokine scavenger that would be beneficial in the treatment of chronic inflammatory conditions.

This thesis aims to determine the biochemical properties of both synthetic and naturally derived molecules of D6-N in order to define its therapeutic usefulness. The importance of post-translational sulphation of the D6 *N*-terminus and the role of GAG molecules in chemokine presentation to D6 will also be investigated.

In **Chapter 3** the role of GAGs in chemokine presentation will be investigated, with specific emphasis on its requirement for D6-mediated chemokine internalisation and scavenging.

In **Chapter 4** a chemically synthesized D6-N peptide will be analysed for its biochemical properties and its ability to bind D6 ligands.

Chapter 5 the natural processing of the D6 *N*-terminus is examined, with attempts to characterise an *N*-terminal cleavage product. Protease-mediated cleavage of the D6 *N*-terminus is also addressed.

In **Chapter 6** the functional role of post-translational sulphation of the *N*-terminus of D6 is explored.

Chapter 2

Materials and Methods

2.1 General solutions and consumables

2.1.1 Composition of chemical solutions

The composition of chemical solutions that were used in experiments detailed in this thesis is given in **Table 2-1**. All laboratory chemicals were supplied by Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated.

Solution	Component	Concentration
1 x PBS	NaCl KCl Na_2HPO_4 KH_2PO_4	137mM 2.7mM 10mM 1.76mM
PBST (0.05%)	PBS Tween 20 (Polyoxyethylene sorbitan monolaurate)	1x 0.05% (v/v)
5-10% Milk PBST	PBS Tween 20 Skimmed milk powder (Marvel)*	1x 0.05% (v/v) 5-10% (w/v)
Fixative	Ethanol Acetic acid Ultrapure water	40% (v/v) 10% (v/v) 50% (v/v)
Fluorescence-activated cell sorting (FACS) buffer	PBS Foetal calf serum Ethylenediaminetetraacetic acid (EDTA) Sodium-Azide	1x 3% (v/v) 2mM 0.01% (v/v)
'Freezing down' buffer	Foetal calf serum Dimethylsulphoxide (DMSO)	90% (v/v) 10% (v/v)
Binding buffer	RPMI FCS L-glutamine Penicillin Streptomycin 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	1x 10% (v/v) 4mM 100U/ml 100mg/ml 5mM
Tris-acetate-EDTA (TAE) Buffer	Tris-acetate EDTA	40mM 0.05M

Table 2-1: Composition of solutions
* Marvel; Chivers, Dublin, Republic of Ireland

2.1.2 Plastic lab-ware

Plastic lab-ware and other consumables were obtained from a variety of suppliers. Manufacturers and suppliers of equipment, reagents and commercially produced kits are detailed in the main body of the text.

Thin walled 0.2ml and 0.5ml PCR tubes
ABgene (Vancouver, Canada)

BD Falcon™ conical tubes, 15ml and 50ml
Polystyrene 2054 5ml round-bottomed FACS tubes
BD Plastipak™ syringes (1ml, 2ml, 5ml, 10ml, 20ml, and 50ml)
Becton-Dickinson Labware (Le Pont de Claix, France)

Tissue culture plates (6-well, 12-well, 24-well and 96-well)
Tissue culture flasks with filter cap (25cm^2, 75cm^2 and 175cm^2)
Corning Inc (Poole, UK)

Black 96-well tissue culture plates
Greiner Bio One (Stonehouse, UK)

Microcentrifuge tubes (1.5ml and 2ml)
Eppendorf (Hamburg, Germany)

2.0ml cryo-tubes
Nunc International (Thermo-Fisher Scientific, Roskilde, Denmark)

2.1.3 Bacterial culture media

The composition of the bacterial culture media and agar is listed in **Table 2-2**.

Medium	Component	Concentration
Luria-Bertani (LB) Broth	Tryptone Yeast extract NaCl Sterilize by autoclaving	1% (w/v) 0.5% (w/v) 1% (w/v)
LB agar plates	Agar LB-broth When cool add ampicillin	15 g/L 1x 50μg/ml

Table 2-2: Composition of bacterial culture media

2.2 Cell culture methods

2.2.1 Cell line maintenance

The many cell lines utilised in this thesis, as well as their routine culture conditions, are detailed below. All procedures involving cell culture were carried out using sterile techniques i.e. all manipulations were performed in a laminar flow hood with HEPA filtration along with use of pre-sterilised equipment and sterile reagents. All surfaces and equipment were sprayed with 70% ethanol prior to work. Centrifuge steps for cell culture were performed at 300 x g for 5 minutes using a Biofuge primo centrifuge (Thermo Scientific) unless otherwise stated. All cultures were incubated at 37°C / 5% CO_2 / 95% humidity.

Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's minimal essential medium (DMEM) (Sigma Aldrich, Poole, Dorset, UK) plus 10% FCS, 4mM glutamine and streptomycin and penicillin (all Invitrogen, Paisley, UK).

Chinese hamster ovary (CHO) cells (both wildtype and GAG deficient, denoted K1 and 745 respectively) were a kind gift from Professor Simi Ali (Newcastle University). Cells were maintained in RPMI-1640 (Sigma), 10% FCS, 4mM glutamine and streptomycin and penicillin (all Invitrogen).

The human acute monocytic leukaemia cell line (THP-1) was maintained in RPMI-1640 (Sigma), 10% FCS, 4mM glutamine and streptomycin and penicillin (all Invitrogen).

2.2.2 Thawing of cell lines from frozen stocks

Cell lines were grown up from stocks stored in liquid nitrogen. Recovery of cells from liquid nitrogen was performed by rapid thawing in a 37°C water bath. Thawed cells were washed in 5ml of the appropriate culture medium (warmed to 37°C) by gently adding medium, 500µl at a time, followed by centrifugation (200 x g for 5 minutes). The supernatant was discarded and the cell pellet re-suspended in 5ml of fresh warmed culture medium before transfer to an appropriate tissue culture flask.

2.2.3 Cell counting

Live cells were counted using a Neubauer Haemocytometer (Hawksley, Sussex, UK). Dead cells were excluded using trypan blue (Sigma): 2µl of trypan blue was added to 8µl of cell suspension, incubated at room temperature for 5-15 minutes and then the sample was loaded into the haemocytometer chamber. The number of live cells in the 4 x 4 grid was counted, with dead cells being easily distinguishable from live cells because they were stained blue. This number was then multiplied by the dilution factor and then by 10^4 to give the number of cells per ml of suspension.

2.2.4 Maintenance of cell lines in culture

Cell lines were routinely passaged when they reached 70-80% confluency. Methods differed depending on whether cells grew as adherent or suspension cultures. In order to minimise acquisition of new mutations, low passage cells were used whenever possible. Cultures were visually inspected daily using an inverted microscope to assess confluency and rule out infection.

2.2.5 Passage of adherent cell cultures

Media were aspirated and adherent cells were washed with 5-10 ml of the appropriate warmed culture media. Cells were removed by either mechanical scraping or the media was replaced with 0.05% trypsin (w/v) (Invitrogen) (1ml for 25cm² flask, 3ml for 75cm²

and 5ml for 175cm²) warmed to 37°C. After incubation for 2-5 minutes at 37°C the cells were examined using an inverted microscope to ensure adequate detachment. Trypsinisation was stopped by addition of 3-7ml of warmed media and the cells were re-suspended by pipetting. The resulting suspension was centrifuged and supernatant discarded. Cells were re-suspended in an appropriate volume of fresh culture media and transferred to a new flask. In general, cells were split between 1:5 and 1:10.

2.2.6 Passage of suspension cell cultures

Suspension cultures were centrifuged and re-suspended in 3-10 ml of fresh culture medium and a proportion of this was transferred to a sterile tissue culture flask depending on the split required, as detailed for adherent cells above.

2.2.7 Freezing down of cell lines

Frozen stocks of early passage cells were established for all cell lines. Cells were washed in PBS, spun down and re-suspended at a concentration of approximately $1x10^7$ cells/ml, in 'freezing down' buffer (see **Table 2-1**). 1.6ml aliquots were transferred to 2ml cryo-vials and placed in a freezing vessel (Nalgene, Hereford) containing room temperature isopropanol. The container was placed at -80°C overnight to allow gradual cooling (1°C per minute), then transferred to liquid nitrogen tanks the following day.

2.3 Plasmid manipulation

2.3.1 Generation of HA-tagged human D6 (HA-D6)

Previously in the laboratory a haemagglutinin (HA) epitope-tagged plasmid encoding human D6 was generated: Nucleotides encoding *N*-terminal HA, protein sequence: MYPYDVPDYAG, were introduced into human D6 cDNA by PCR to generate HA-D6. Products were verified by sequencing (MWG Operon, London, UK) and cloned into pcDNA3.1 (MWG Operon).

2.3.2 Site-directed mutagenesis

The previously described HA-D6 pcDNA3.1 plasmid (see **2.3.1**) was manipulated using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Stratagene, Agilent Technologies, La Jolla, CA, USA) and primers encoding tyrosine to phenylalanine mutations were designed and used to generate point mutations in the D6 sequence. All primers were from IDT, Interleuvenlaan, Belgium, and were designed with the primer design guidelines detailed in the kit instruction manual. Design considerations were as follows and were adhered to as much as possible; (i) forward and reverse primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid (ii) primers should be between 25 and 45 bases in length with a melting temperature of ≥78°C (iii) the desired mutation should be in the middle of the primer with ~10-15 bases of correct sequence on either side (iv) primers should have a minimum GC content of 40% and should terminate in one or more C or G bases (v) Primers must be HPLC purified. Primer sequences for each mutant are detailed in **Table 2-3** and cycling parameters are detailed later in this section.

The mutant strand synthesis reaction was performed using guidelines from the QuikChange Lightning Site-Directed Mutagenesis kit protocol. This involves primers annealing to the plasmid DNA and being extended during a thermal cycling reaction using a high fidelity DNA polymerase. A series of sample reactions were set up using various amounts of dsDNA template: 10ng, 50ng and 100ng. The cycling parameters used are outlined below.

After the mutant strand synthesis reaction, the parental (i.e., non-mutated) supercoiled dsDNA was digested by the restriction enzyme *Dpn I.* This enzyme only recognises methylated DNA therefore degrades only the parental plasmid because almost all *E.coli* strains that are used to produce plasmid preparations *Dam* methylate DNA.

Mutant Name	Primer Sequences
Mutant 1	5-'ATGCCGATTCTGAGAATAGCAGCTTCTTTTTCTTTGACTTCCTGGATGAAGTGG-3' 3'-TACGGCTAAGACTCTTATCGTCGAAGAAAAAGAAACTGAAGGACCTACTTCACC-5'
Mutant 2	5'-ATGCCGATTCTGAGAATAGCAGCTTCTTTTACTATGACTACCTGGATGAAGTGG-3' 3'-TACGGCTAAGACTCTTATCGTCGAAGAAAATGATACTGATGGACCTACTTCACC-5'
Mutant 3	5'-ATGCCGATTCTGAGAATAGCAGCTTCTATTACTTTGACTACCTGGATGAAGTGG-3' 3'-TACGGCTAAGACTCTTATCGTCGAAGATAATGAAACTGATGGACCTACTTCACC-5'
Mutant 4	5'-ATGCCGATTCTGAGAATAGCAGCTTCTATTACTATGACTTCCTGGATGAAGTGG-3' 3'-TACGGCTAAGACTCTTATCGTCGAAGATAATGATACTGAAGGACCTACTTCACC-5'
Mutant 5	5'-ATGCCGATTCTGAGAATAGCAGCTTCTATTTCTATGACTACCTGGATGAAGTGG-3' 3'-TACGGCTAAGACTCTTATCGTCGAAGATAAAGATACTGATGGACCTACTTCACC-5'
Mutant 6	5'-ATGCCGATTCTGAGAATAGCAGCTTCTATTTCTTTGACTTCCTGGATGAAGTGG-3' 3'-TACGGCTAAGACTCTTATCGTCGAAGATAAAGAAACTGAAGGACCTACTTCACC-5'
Mutant 7	5'-ATGCCGATTCTGAGAATAGCAGCTTCTTTTACTTTGACTTCCTGGATGAAGTGG-3' 3'-TACGGCTAAGACTCTTATCGTCGAAGAAAATGAAACTGAAGGACCTACTTCACC-5'
Mutant 8	5'-ATGCCGATTCTGAGAATAGCAGCTTCTTTTCTATGACTTCCTGGATGAAGTGG-3' 3'-TACGGCTAAGACTCTTATCGTCGAAGAAAAAGATACTGAAGGACCTACTTCACC-5'
Mutant 9	5'-ATGCCGATTCTGAGAATAGCAGCTTCTTTTCTTTGACTACCTGGATGAAGTGG-3' 3'-TACGGCTAAGACTCTTATCGTCGAAGAAAAAGAAACTGATGGACCTACTTCACC-5'

Table 2-3: Primer sequences used to generate each mutant.
Primers are the total complement of each other, bases to be changed are underlined and base pair changes from the original plasmid are coloured in red.

Segment	Cycles	Temperature (°C)	Time
1	1	95	2 minutes
2	18	95	20 seconds
		60	10 seconds
		68	2 minutes 45 seconds*
3	1	68	5 minutes

* Note the extension time was worked out by having 30 seconds/Kb of plasmid, therefore for a 5.4Kb plasmid, 2 minutes 45 seconds was deemed to be correct.

2.3.3 Bacterial Transformation with plasmid DNA

Chemically competent XL10-Gold cells were stored at -80°C and single use aliquots thawed on ice prior to use. Cells were transferred to pre-chilled 1.5ml microtubes and 2µl β-mercaptoethanol was added to cells and incubated for 2 minutes on ice. The mutagenized plasmid DNA to be transformed was added to the ultracompetent cells and gently mixed. Cells were left on ice for 30mins before being heat-shocked in a thermomixer set to 42°C

for 30 seconds. Tubes were placed on ice for 2 minutes before addition of 0.5ml LB Broth (without antibiotic) and incubation on the thermoshaker at 37°C for 1 hour with shaking at 300 rpm. 100µl of each transformation reaction was plated and spread onto pre-made LB agar plates containing 50µg/ml ampicillin (Sigma). Plates were inverted and incubated at 37°C for >16 hours. During all work using microorganisms, sterile conditions were maintained.

2.3.4 Plasmid cloning, purification and sequencing

Colonies were picked from plates using sterile pipette tips and placed into 'Universal' tubes with 5ml LB broth supplemented with 50µg/ml ampicillin. Tubes were incubated overnight at 37°C with constant shaking. The following day the cells were pelleted by centrifugation at high speed for 5 minutes using a standard benchtop centrifuge. A QIAprep mini kit (Qiagen, Crawley, UK) was used to purify plasmids from overnight cultures of XL10-Gold cells according to manufacturer's instructions. This method relies on the alkaline lysis of bacterial cells followed by adsorption of DNA to a silica membrane in high salt conditions. This is followed by washing and finally elution of plasmid DNA. Resulting plasmid preparations were sequenced across the multiple cloning site (MCS) in both directions using both T7 and BGH reverse primers (MWG Operon). Sequences were verified using online programs BLAST (http://blast.ncbi.nlm.nih.gov/) and ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/).

2.4 Transfection of plasmids into mammalian cell lines

2.4.1 Transfection of adherent cells

Plasmids were stably transfected into HEK 293 cells and both CHO K1 and CHO 745 cells using the Effectene® transfection reagent kit (Qiagen). The night before transfection, cells were seeded into 6-well plates at a concentration of 5×10^6 cells per well. The plasmids to be used in the transfection reaction were diluted to a concentration of 0.4µg per 100µl of DNA-condensation buffer with 3.2µl enhancer. This was incubated for 5 minutes at room temperature before addition of 10µl effectene transfection reagent. The DNA: effectene complex was incubated for 10 minutes at room temperature to allow transfection

complexes to form. After incubation, 600µl of complete growth media was added to the DNA: effectene mixture and this was subsequently added to the appropriate wells in a drop-wise manner. Cells were incubated with the transfection complexes under their normal growth conditions for 24 hours, after which cells were passaged 1:10 into complete growth media containing the selective agent G418 (Promega, Southampton, UK). Stable colonies were grown up and maintained in complete growth media containing 0.8mg/ml G418.

2.4.2 Obtaining clonal populations of transfected cells

The D6 (clone 4A5) antibody was biotinylated using the EZ-Link® Micro-PEO_4-Biotinylation Kit (Pierce, Rockford, USA). High D6 expressing cells were separated from low and non-expressing cells by adding the biotinylated D6 (clone 4A5) antibody to cells suspended in FACS buffer and mixing with Streptavidin MicroBeads (Miltenyi Biotec, Gladbach, Germany). Cells were 'run through' MACS separation columns (Miltenyi Biotec) attached to a MidiMACS™ separator (Miltenyi Biotec) and eluates collected. To obtain clonal populations of high D6-expressing cells, cells were seeded at a very low density and colonies isolated by ring cloning using borosilicate glass cloning rings (SciQuip, Shropshire, UK). Populations as low as two or three cells were isolated and grown up to confluency before their D6 expression was assessed by flow cytometry with the methods described in section **2.4.4.2**. Populations of cells with almost identical levels of D6 expression were chosen for further analysis.

2.4.3 Surface receptor assessments of transfected cells

2.4.3.1 Antibody staining for flow cytometry

Approximately 1 x 10^7 freshly isolated cells were harvested by centrifugation for 5 minutes at 4^{o}C, 300 x g, washed with FACS buffer and re-suspended in ice-cold FACS buffer. Cells were then incubated with primary antibodies or isotype controls in ice-cold FACS buffer for 15 minutes with occasional gentle agitation. Samples were washed twice with 2ml chilled FACS buffer before being incubated with fluorescently-labelled secondary

antibodies in FACS buffer on ice for 15 minutes with occasional gentle agitation. Cells were again washed twice with 2ml chilled FACS buffer and re-suspended in 200µl FACS buffer. Viaprobe (BD Biosciences, San Jose, CA, USA) was added to each cell suspension to identify non-viable cells. Samples were analysed on the MACSquant analyser (Miltenyi Biotec). As a negative control, samples of un-transfected cells were used alongside test samples. The antibodies used are detailed in **Table 2-4** and were all used at concentrations between 1 - 5µg/ml.

Epitope	Primary antibody or isotype	Secondary antibody
D6	mouse anti-human D6 antibody (4A5 clone)	anti-mouse FITC (R&D Systems)*
	mouse IgG2a isotype control (Dako)**	
HA	anti-HA-biotin (Miltenyi)	PE-streptavidin (R&D Systems)
	mouse IgG1- isotype control biotin (R&D Systems)	

Table 2-4: Antibodies for surface receptor assessment of HA-D6 transfected cells.
* R&D systems (Abington, UK), ** Dako (Cambridgeshire, UK)

2.4.3.2 Flow cytometry

Stained cells or cells from binding and competition assays were analysed using a MACsquant™ analyser. Acquisition parameters were established using unstained and untransfected cells. Data were analysed subsequent to acquisition using MACSquantify™ software (Miltenyi Biotec), using unstained samples and un-transfected cells to set gates.

2.5 Cell based assays

2.5.1 Chemokine uptake assay

Adherent cells were trypsinised, or scraped, from culture dishes and re-suspended in a 96-well plate at a concentration of 0.5×10^5 cells per well in 100µl binding buffer. Alexafluor-647 labelled CCL2 (AF-CCL2) (Almac) was added to cell suspensions at varying concentrations ranging from 1nM to 20nM or where otherwise stated. In some cases a 20-fold molar excess (400nM) of unlabelled CCL22 (Peprotech, Rockyhill, NJ, USA) was

also added to cells. Cells were incubated for various time periods ranging from 30 minutes to 2 hours at 37°C in 5% CO_2. Subsequently cells were washed twice in ice cold FACS buffer. Cells were then re-suspended in FACS buffer and viaprobe (BD Biosciences) was added to each cell suspension to identify non-viable cells. Fluorescence intensity of the cells was measured on a MACSquant analyser (Miltenyi Biotec).

2.5.2 Chemokine uptake assay with D6-N* as a competitor

Cells were manipulated as detailed in section **2.5.1**; however differing quantities of D6-N (s) or D6-N (non-s) (Almac) ranging from 0.5µg to 4µg were added to wells at the same time as 60ng per well of AF-CCL2 before incubation for 1 hour at 37°C in 5% CO_2. In similar experiments 60ng AF-CCL2 was incubated with D6-N (s) or D6-N (non-s) in a total volume of 20µl PBS for 15 minutes at room temperature before addition to wells. Cells were subsequently analysed on a MACSquant analyser (Miltenyi Biotech) as detailed in section **2.4.3.2**. Mean fluorescence intensity (MFI) values for the alexa-fluor 647 positive gate were recorded for each sample.
*****Details on the synthesis of the D6-N peptides can be found in section **2.8.1**.

2.5.3 Chemokine degradation assay

Cells were plated out the night before the assay into a 96-well plate to ~80% confluency (2 x 10^4 cells per well) in regular media. The following day, human biotinylated CCL2 (bio-CCL2) (Almac) was added to the media at a concentration of 50µg/ml and cells incubated at 37°C in 5% CO_2. Media was collected at different time points from 0 hours to 30 hours after addition of bio-CCL2 and stored at -20°C before being analysed by Western blotting (section **2.8.6**).

2.5.4 Protease cleavage assays

Cells were plated out the night before the assay into 6-well plates to about 80% confluency (2 x 10^5 cells per well) in regular media. The following day one of either elastase (Sigma), at 10nM, 30nM, or 300nM, cathepsin G (Sigma), at 10nM or 70nM or staphopain A (Sigma) at 0.5µM or 2µM, was added to wells and cells incubated for either 15 minutes

(elastase and staphopain A) or 2 hours (cathepsin G), (unless otherwise stated), at 37°C in 5% CO_2. Afterwards media was taken and stored for further analysis. Cells were scraped and cell lysates were prepared (section **2.8.4)**. Both media and lysate samples were then analysed using Western blotting (section **2.8.6**).

2.5.5 Chemokine uptake assay after staphopain A treatment

Cells were plated out the night before the assay into 6-well plates to about 80% confluency (2 x 10^5 cells per well) in regular media. The following day 2μM staphopain A (Sigma) was added to wells and cells incubated for 15 minutes at 37°C in 5% CO_2. Cells were washed twice with PBS before scraping off and re-suspending in complete media and adding 25nM AF-CCL22. Cells were incubated for 1 hour at 37°C in 5% CO_2 and subsequently washed twice in ice cold FACS buffer. DRAQ7 (BioStatus) was added to each cell suspension to identify non-viable cells. Fluorescence intensity of the cells was measured on a MACSquant analyzer (Miltenyi Biotec).

2.5.6 Chemokine fluorescence assay following staphopain A treatment

Chinese hamster ovary (CHO K1) cells expressing D6 cells were plated out the night before on black 96-well plates to ~80% confluency. The following day, cells were given staphopain A (Sigma) in PBS at concentrations of 0.5μM and 2μM and incubated for 15 minutes at 37°C in 5% CO_2. Cells were washed twice in PBS and put back into cell growth media supplemented with 10% FCS. Human alexa-fluor-647 labelled CCL22 (AF-CCL2) (Almac) was added to the media at a concentration of 20nM and cells incubated at 37°C in 5% CO_2 for 1 hour. Cells were washed 3x with PBS and then analysed on a PHERAstar FS fluorescence plate reader (BMG Labtech, Ortenberg, Germany). Media was transferred to a new black 96-well plate and analysed on the fluorescence plate reader. The raw data generated was calculated using the integrated MARS data analysis software (BMG Labtech).

2.5.7 Sodium chlorate treatment of cells

Cells were grown in complete media supplemented with concentrations of sodium chlorate (Sigma) ranging from 30mM to 150mM for various time periods before performing chemokine binding / uptake assays as detailed in **2.5.1**. Sodium chlorate was used to competitively inhibit the formation of PAPS, the high energy sulphate donor in cellular sulphation reactions.

2.5.8 siRNA and D6 transfection

HEK 293 cells were plated out the night before on 12-well plates to ~80% confluency. The following day cells were transfected with TPST-1 siRNA (5nM), TPST-2 siRNA (5nM), both TPST-1 and TPST-2 (2.5nM each), or negative control siRNA (5nM) using HiPerFect Transfection Reagent and guidelines (All Qiagen). Briefly, on the day of transfection, siRNA was diluted to a concentration of 5nM (or 2.5nM) in culture medium without serum. 6µl of HiPerFect Transfection Reagent was added and mixed by vortexing. Samples were incubated at room temperature for 5-10 minutes to allow the formation of transfection complexes. Complexes were then added drop wise to the cells. 24 hours later cells were transiently transfected with the HA-D6 plasmid described previously (section **2.3.1**) using the Effectene® transfection reagent kit (Qiagen). The following day cells were tested for their ability to uptake AF-CCL22 using chemokine uptake assays (section **2.5.1**). Cell lysates were also prepared (section **2.8.4)**, and analysed by Western blotting (section **2.8.6**).

2.6 Molecular Biology: RNA

While performing all methods involving RNA, RNA degradation by environmental RNases was kept to a minimum by various means. All plasticware and glassware was either supplied guaranteed RNase free or was autoclaved. Sterile filter tips were used throughout and RNase / DNase free water (Ambion) was used in all solutions. Before starting work, all surfaces, pipettes and equipment were sprayed with RNAzap RNase decontamination solution (Ambion).

2.6.1 Isolation of RNA from cells

RNA was isolated from cell lines using an RNeasy® Mini Kit (Qiagen). Cells were pelleted and re-suspended in 350-600µl RLT Buffer and homogenized using a QIAshredder spin column. RNA extraction was performed according to the Qiagen RNeasy® Mini Kit instruction manual. This included the optional on-column DNase digestion step using the Qiagen RNase-Free DNase Set. The RNA concentration of each sample was determined using a ThermoFisher Nanodrop 1000™ spectrophotometer.

2.6.2 cDNA synthesis from RNA

A kit comprising of a two-step Reverse transcription (RT) process was used to make complementary DNA (cDNA). Firstly the 'annealing' step anneals the RT primer to the denatured RNA, and secondly in the 'extension' step the RNA is reverse transcribed to make cDNA. The Precision nanoScript Reverse Transcription kit (Primerdesign, Southampton, UK) was used for all RT reactions according to manufacturer's instructions. Briefly, 300ng of starting RNA for each sample was added to a 0.2ml PCR tube and made up to 10µl with RNAse/DNAse free water and with 1µl random nonamer primermix. Samples were then incubated at 65°C for 5 minutes and immediately cooled in an ice water bath. To each sample, a 10µl mix of the following components was added: 2µl nanoScript 10X Buffer, 1µl of dNTP mix (10nM of each), 2µl of 100mM DTT, 4µl RNAse/DNAse free water, and 1µl nanoScript enzyme. For -RT controls, 1µl of nuclease-free water was added in place of nanoScript enzyme. Samples were incubated at 25ºC for 5 minutes and then 55ºC for 20 minutes. The reaction was then heat inactivated by incubation at 75ºC for 15 minutes. cDNA samples were stored at -20°C until they were used for QPCR.

2.7 Molecular Biology: QPCR by absolute quantification

QPCR by absolute quantification allows the quantification of the total number of gene copies of a gene of interest in a given sample. This technique utilizes SYBR green, an asymmetrical cyanide dye which preferentially binds to double stranded DNA. The resulting DNA-dye complex emits green light and the amount of green light emitted relates to the amount of double stranded DNA present in a given well after a QPCR reaction.

There are many pre-assay steps that need to be taken before a QPCR reaction can be set up. There are also post-assay analysis steps that must be taken to gain meaning from the data. All the steps involved in a QPCR experiment are detailed below.

2.7.1 Primer Design

Two sets of primers specific to the gene of interest need to be designed. These two sets of primers are referred to as *standard* primers and *quantification* primers. Standard primers were designed to be able to amplify a product which is typically 700-800 basepairs (bp) in length. This product was used as the template in order to generate standards (**section 2.7.2**). The quantification primers are used to identify and amplify a smaller (100-200bp) product from the gene of interest to allow quantification. In order to design appropriate primers, genetic sequences were obtained using Ensembl computer software (www.ensembl.org). 'Primer 3' computer software (http://frodo.wi.mit.edu/) was used to design efficient and accurate primer pairs. The attributes required for ideal primer design are given below:

Attribute	Optimal Value	Acceptable Range
GC Content	50%	40-65%
Sequence Length	N/A	18-23base pairs
Melting Temperature (Tm)	60°C	59.5 - 61°C
Maximum Self Complimentarity	N/A	2
Maximum 3' Self Complimentarity	N/A	1
Amplified Product Size: Inner	150 basepairs	<150 basepairs
Outer	750 basepairs	between 700-800 basepairs

Both sets of primers were checked using the Basic Local Alignment Search Tool (BLAST) from NIH PubMed (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm that the primers recognise only the gene of interest. Primer sequences are listed in **Table 2-5**. Primers for D6 and TATA binding protein (TBP) are used regularly in this laboratory and were designed previously. Primers for TPST 1 and TPST 2 were designed for this study.

Primer Name	Primer sequences	Product length (bp)
D6 *quantification*	Forward 5'-AGGAAGGATGCAGTGGTGTC-3' Reverse 5'-CGGAGCAAGACCATGAGAAG-3'	98
D6 *standard*	Forward 5'- TCACCTTGTTTCTGCACTCG -3' Reverse 5'- GTGGAAGTGGAGGGAGATCA -3'	531
TATA Binding Protein (TBP) *quantification*	Forward 5'- TGCTGTTGGTGATTGTTGGT -3' Reverse 5'- AACTGGCTTGTGTGGGAAAG -3'	99
TATA Binding Protein *standard*	Forward 5'- GAGTTGCTTGCTCTGTGCTG -3' Reverse 5'- ATACTGGGAAGGCGGAATGT -3'	274
TPST-1 *quantification*	Forward 5'- GCTGGGGGAGTGTCTCTGT -3' Reverse 5'- TCCGTAGTTAGGTGGGTTGG -3'	186
TPST-1 *standard*	Forward 5'- CTGAACGGTGGATGAGAACA -3' Reverse 5'- TCAAAAGGAGACTGCCCACG -3'	756
TPST-2 *quantification*	Forward 5'- TCGGACCTCTAATCCAAGCA -3' Reverse 5'- TCCATACCCTTCATTCTCTACCC -3'	160
TPST-2 *standard*	Forward 5'- TGGAGGTAGGCAAGGAGAAGT -3' Reverse 5'- GGGTCAATAGGAGAGGCACA -3'	836

Table 2-5: Inner and outer primer sequences used in PCR and QPCR reactions

2.7.2 Generation of Standards

In absolute QPCR, standard amounts of cDNA template, in which the total number of copies of the gene of interest is known, are what allow us to generate a standard curve. Standards for both TPST-1 and TPST-2 were generated by using their specific standard primers in a simple PCR reaction with cDNA generated from HEK cell lines, primary keratinocytes and primary HD LECs. cDNA from keratinocytes and HD LECs was kindly provided by Mark Singh, University of Glasgow, and was generated using similar methods described in **section 2.6**. 2µl of cDNA was added to a pre-made Red PCR master mix (Rovalab, Teltow, Germany) (see **2.7.6** for master mix compositions) along with 1µl of forward/reverse outer primer mix (1µM). Reactions were performed on a Veriti thermal cycler (Applied Biosystems, Paisley, UK). The PCR cycling parameters used are described below:

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

Standards for D6 and TBP have been generated previously in the lab using similar methods (McKimmie et al., 2008).

2.7.3 Gel Electrophoresis

Products of the reaction were run out on a 2% agarose gel made by mixing 2g agarose (Sigma) with 100ml of TAE buffer and heating in the microwave until dissolved. Upon partial cooling, 0.5µg/ml ethidium bromide (Sigma) was added to allow visualisation of DNA under UV light. The gel was cast in a tray using sample-combs to create wells to insert samples. Once set, the gel was placed in a horizontal electrophoresis tank. Combs were removed and the tank was filled with TAE buffer until just covering the surface of the gel. Samples were loaded to the gel alongside a type IV hyperladder (Biolegend, San Diego, CA, USA), used to gauge the size of the PCR product. Electrophoresis was performed at 90V until the band of visible loading dye reached near the end of the gel. The gel was imaged under UV light using an AlphaImager (Alpha Innotech, ProteinSimple, Santa Clara, California).

2.7.4 Gel extraction

The gel was exposed to the minimum duration of UV light at reduced wavelength (304nm) to prevent damage of DNA. Gel fragments containing bands that corresponded to the PCR products were cut out with a clean scalpel blade and placed in a microcentrifuge tube. The DNA in the gel fragments was obtained by DNA extraction using a QIAquick gel extraction kit (Qiagen). This kit was used according to manufacturer's instructions and utilises spin column technology and a silica membrane column to which DNA can bind.

2.7.5 Standard Verification

The product from gel extraction was used as a template for making standards for QPCR. The above set of protocols produces a concentrated solution of DNA, which after a set of serial dilutions, gives a useable set of standards that can be accurately quantified.
In order to quantify the absolute copy number in each of the serial dilution the optical density (O.D.) had to be measured using a nanodrop (ThermoFisher). Quantification primers were verified by PCR to test their ability to recognise the newly produced standards.

2.7.6 Polymerase Chain Reaction (PCR)

PCR was performed using Red PCR Mastermix (Rovalab); a commercially available pre-mixed master mix made up of 1.1x buffer, $MgCl_2$, 1mM dNTPs and Taq DNA polymerase. This was supplied as 45µl pre-aliquoted PCR tubes, to which 2.5µl template and 1.25µl of each quantificaion primer (10µM), was added. Reactions were performed on a Veriti thermal cycler (Applied Biosystems, Paisley, UK) and cycling parameters are described below:

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

Products were run on a gel as described previously and imaged under UV light. A strong band of ~100-200bp in length is expected with no other bands or primer dimers. This result is indicative of well designed primers.

2.7.7 QPCR Assay

Following verification of the quantification primers, the QPCR was set up to assess the expression of a given gene in a sample. The assay was performed on an Applied

Biosystems 7900HT Fast Real-Time system. All sample assays were performed in triplicate and normalised to TATA binding protein transcript levels, which were also assayed in triplicate. A 'no template control' (NTC) was also run for each primer pair in which nuclease free water is added in place of the cDNA sample. All master mix and plate preparations were carried out on ice.

Master mix was prepared as follows:

Ingredient	Volume (µl/well)
SYBR© Green Fast Mix	2.5
Nuclease Free Water	2
Primer Mix (equal volumes of forward and reverse primer)	0.1

To perform the assay in triplicate, 3x template mix (1.5µl) and 3x master mix (13.8µl) were mixed thoroughly in the wells of a 'set-up' plate and 5µl was transferred to each of the 3 wells on a 384 well thin-walled PCR plate. Once all the samples were loaded in this way, the plate was sealed with a protective plastic film (Applied Biosystems), spun down briefly in a refrigerated centrifuge and run on a 7900HT thermal cycler (Applied Biosystems). The machine was run on the programme as follows:

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

2.7.8 Analysis of QPCR Data

Cycle threshold (CT) is a measure of the cycle of PCR in which the product becomes detectable to the machine reader. A higher expressing sample will have a lower CT value. Before data analysis, key checks were performed to make sure the data could be counted as valid. Firstly the R^2 value was checked. This value relates to the quality of the standard curve and depends upon how similar replicates are to each other and how each standard's mean CT value relates to each other. The closer the R^2 value is to 1, the higher quality the

standard curve and therefore the more reliable the sample data will be using the standard curve as a guide for quantification. Secondly, non-template control (NTC) wells, where template is not added to wells, were checked to eliminate the possibility of contamination. The CT values for these wells should be very high or undetectable as replication should be nil.

Following the key data checks, all the sample data was exported into a Microsoft Excel spreadsheet. Data for genes of interest were normalised against TATA binding protein, which showed little variation in expression across different samples.

2.8 Molecular biology: Protein

2.8.1 Synthesis of D6-N peptide

Peptides comprising the first 35 amino acids of the *N*-terminus of the D6 protein (D6-N) have been chemically synthesized (Almac Scotland, Edinburgh, UK). Two versions of D6-N were generated, with either sulphated or non-sulphated tyrosine residues. These will be denoted as D6-N (s) and D6-N (non-s) respectively. D6-N (s) was synthesized in two batches, with differing degrees of sulphation recorded between each batch.
A hexa-histidine tag was incorporated onto the end of both D6-N (s) and D6-N (non-s). Peptide sequences are detailed below:

Peptide	Amino Acid Sequence
D6-N (s)	MAATASPQPLATEDADSENSSFY(SUL*)Y(SUL*)Y(SUL*)DY(SUL*)LDEVAFML HHHHHH
D6-N (non-s)	MAATASPQPLATEDADSENSSFYYYDYLDEVAFMLHHHHHH

sul* indicates a potential sulphate group. It is impossible to determine exactly which and how many tyrosine residues have been sulphated in a single peptide, however information from mass spectrometry of each batch indicates the abundance of different species of sulphated tyrosine, i.e. mono, di, tri or tetra-sulphated species.

2.8.2 Chemokine - D6-N binding assay using nickel beads

To assess the ability of D6-N peptide to bind to chemokines; 10µg of either D6-N (s) or D6-N (non-s) peptide was incubated with 2µg/ml recombinant murine CCL2, human

CCL22 or murine CCL19 (all Peprotech), or biotinylated human CCL2 (bio-CCL2), biotinylated human CCL3 (bio-CCL3) or biotinylated human CCL19 (bio-CCL19) (all Almac) in PBS / 5mM imidazole (Sigma) for 10mins at room temperature. 50µl PureProteome™ Nickel Magnetic Beads (Millipore, Temecula, CA, USA) were added to the tube and vortexed. Nickel magnetic beads were used because they can bind to the hexa-histidine tag present on both D6-N peptides. The D6-N - chemokine mixture was incubated for 30mins at room temperature with regular re-suspension. Controls without addition of chemokine were run alongside the experimental tubes. Tubes were spun down in a centrifuge at 18407 x g for 2mins. Beads sink to the bottom of the tube and form a pellet. Supernatant was taken off and stored and beads were washed 2x in PBS / 5mM imidazole. Elution buffer (PBS / 400mM imidazole) was added to the beads and incubated for 2mins at room temperature. This high concentration of imidazole is necessary for recovery of captured HIS-tagged D6-N, and any chemokine binding to D6-N. Tubes were spun down again at 14000rpm for 2mins and bead eluate was taken for further analysis.

2.8.4 Preparation of protein cell lysates

Lysis of Adherent cells

After removal of medium from culture dishes, cells were washed once with PBS then pre-cooled (4°C) lysis buffer (150mM NaCl, 1% Triton® X-100, 50mM Tris HCl, pH 8.0; Miltenyi) with added protease inhibitors (Pierce) was added to cells. The lysate was scraped from the culture dish using a cell scraper and transferred to a 1.5ml microcentrifuge tube. Samples were mixed by vortexing and incubated on ice for 30 minutes with occasional mixing. Samples were then centrifuged for 10 minutes at 10,000 x g at 4°C to sediment the cell debris. The supernatants were transferred to fresh tubes and either used immediately or stored at -80°C until use.

Lysis of suspension cells

Media containing cells was transferred to a tube and centrifuged for 5 minutes at 300 x g. Cell pellets were washed by re-suspending in PBS and re-centrifuged to form a cell pellet. Pre-cooled (4°C) lysis buffer (150mM NaCl, 1% Triton® X-100, 50mM Tris HCl, pH 8.0; Miltenyi) with added protease inhibitors (Pierce) was added to cell pellets. Tubes were vortexed and incubated on ice for 30 minutes with occasional mixing. Samples were then centrifuged for 10 minutes at 10,000 x g at 4°C to sediment the cell debris. The

supernatants were transferred to fresh tubes and either used immediately or stored at -80°C until use.

2.8.5 Sodium Dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

Samples were mixed 1:4 with NuPAGE® LDS sample buffer (Invitrogen) and either heated to 95°C for 5 mins or, when performing D6 assays, incubated at room temperature for 10 mins. Samples were mixed 1:10 with NuPAGE® Sample Reducing Agent (Invitrogen). 10-20µl of each sample was loaded onto a pre-cast NuPAGE Novex 4-12% Bis-Tris gel, (Invitrogen), in a vertical electrophoresis tank (Xcell Surelock, Invitrogen) filled with NuPAGE MES SDS running buffer (Invitrogen). Novex® Sharp Pre-Stained Protein standard (Invitrogen) was run alongside samples for size determination. Electrophoresis was performed for 1-2 hours at 150V until protein standard bands had separated enough.

2.8.6 Western blotting

Gels were transferred onto a nitrocellulose membrane using an iBlot® system (Invitrogen) according to manufacturer's instruction. Following transfer, the membrane was washed in PBS Tween (PBST) and blocked in 5% Milk PBST for either 1-2 hours at room temperature or overnight at 4°C. After blocking, the membrane was washed briefly in PBST and incubated with primary antibody overnight at 4°C. The membrane was subsequently washed 4x for 5 minutes each in PBST and incubated with secondary antibody for 1 hour at room temperature. Blots were developed via a chemiluminescence reaction (SuperSignal WestPico kit; Pierce) before being placed between acetate sheets and exposed to X-ray film (Kodak, Carestream Health Inc, New York, U.S.A) in a dark room for varying time periods. Film was then developed in a developing machine (XOmat, Konica-Minolta, Bainbury, UK). A list of all primary and secondary antibodies and suppliers are detailed in **Table 2-6**.

Primary antibody	Isotype	Supplier	Concentration used	Secondary antibody	Supplier
Anti-HA tag antibody	mouse IgG3	Abcam[1]	1µg/ml	Anti-mouse IgG Horseradish Peroxidase	Amersham[2]
Anti D6 (clone 4A5) (human)	mouse IgG	In-house	Hybridoma supertatant diluted (1:5)		
Anti-Sulphotyrosine (multiple species)	mouse IgG2a	Millipore	1 µg/ml		
Anti-CCL19 (murine)	goat IgG	R&D systems	0.1µg/ml	Rabbit anti-Goat IgG (H+L) Conjugate (Zymax™ Grade)	Invitrogen
Anti-CCL2 (murine)	goat IgG	R&D systems	0.1µg/ml		
Anti-CCBP2 (human)	rabbit	Prestige antibodies (Sigma)	1.6 µg/ml	Anti-rabbit IgG, HRP-linked Antibody	Cell Signalling[3]
Anti-CXCR2 (mouse, rat, human)	rabbit	Abcam	1.1 µg/ml		
Anti-TPST1 (human)	rabbit	Abcam	1 µg/ml		
Anti-TPST2 (human)	rabbit	Abcam	1 µg/ml		
Anti-beta tubulin	rabbit	Cell Signalling[3]	2 µg/ml		

Table 2-6: Western blotting primary and secondary antibodies
[1] Abcam, Cambridge, UK.
[2] Amersham, GE Healthcare, Buckinghamshire, UK.
[3] Cell Signalling Technology, Danvers, MA, USA

2.8.7 Estimation of protein loading

Equal starting amounts of cells were used for protein extraction wherever possible. Equal protein loading was either checked by staining the protein on the membrane with Ponceau S reagent (Sigma), or confirmed by stripping the membrane and re-probing with a housekeeping gene (e.g. beta-tubulin). For Ponceau S staining the membrane was covered with stain for 5 minutes with regular shaking at room temperature before being washed with distilled water until background was removed and protein bands could be distinguished clearly. To strip blots, membranes were immersed in Restore Western Blot

stripping buffer (Pierce) for 15-20 minutes at room temperature. Blots were then washed in PBST before being re-blocked and re-probed with different primary and secondary antibodies as described previously.

2.8.8 Protein band staining

After performing SDS PAGE as described previously, gels were rinsed in ultrapure water and stained in 20ml SimplyBlue™ SafeStain (Invitrogen) for 1 hour at room temperature. Gels were then washed repeatedly for 1 hour periods with ultrapure water until background is reduced and bands become visible. The SimplyBlue™ SafeStain is a type of coomassie dye which binds to protein, chiefly arginine residues, although it also binds weakly to other amino acid residues.

2.8.9 Silver Staining

Subsequent to SDS PAGE, gels were stained using the SilverQuest™ Silver Staining kit (Invitrogen) according to manufacturer's instruction. Before starting, reagents provided in the kit were used to prepare the following solutions for staining; sensitizing solution, staining solution and developing solution. All incubations were performed on a rotary shaker at room temperature. Following gel electrophoresis, the gel was fixed in 100ml of fixative (**Table 2-1**) for 20 minutes to remove interfering ions and detergent from the gel and help restrict movement out of the gel matrix. The gel was then washed in 30% ethanol for 10 minutes and incubated in 100ml of sensitizing solution for 10 minutes. Sensitizing solution is used to increase the sensitivity and contrast of the stain. The gel was then washed again for 10 minutes with 30% ethanol, and for 10 minutes with ultrapure water to remove excess sensitizer and rehydrate the gel before staining. The gel was then stained for 15 minutes with stainer solution which binds silver ions to the protein and forms a latent image. After staining, the gel was washed with ultrapure water for 20-60 seconds before being incubated with 100ml of developing solution for 4-8 minutes or until bands start to appear and the desired band intensity is achieved. Developing solution works by reducing the silver ions to metallic silver resulting in development of the protein bands. Once the appropriate staining intensity was reached, 10ml of stopper was immediately added directly to the gel still immersed in the developing solution. The stopper solution complexes with any free silver still present to prevent further reduction. Finally the gel was washed again in ultrapure water for 10 minutes.

2.8.10 Enzyme-linked immunosorbent assay (ELISA)

Samples were analysed with the Quantikine® Human MDC (CCL22) immunoassay (R&D Systems, Abington, UK) as per manufacturer's instruction. Briefly, a pre-coated plate, to which a monoclonal antibody specific for CCL22 has been immobilised, is provided with the kit. Standards were made up using a serial dilution method and both standards and samples were pipetted into the wells of the pre-coated plate. Standards and samples were measured in triplicate. Any CCL22 present in the standards or samples binds to the antibody. Washing was performed to remove any un-bound molecules and a monoclonal antibody specific for CCL22 is added to the plate. This second antibody is linked to an enzyme, which induces a colour change when a substrate is added. Colour develops in proportion to the amount of CCL22 bound in the initial step, until the reaction is stopped by adding a stop solution. The optical density of the solution in each well was measured using a microplate reader set to 450nm (Sunrise™, Tecan). A best fit curve was constructed by plotting the mean absorbance for each standard on the y-axis against its CCL22 concentration on the x-axis. A line was drawn through the points and the equation of the line was worked out using standard spreadsheet software (Excel, Microsoft). The concentration of CCL22 in each sample was worked out using the equation of the line and substituting for X.

2.8.11 Immunoprecipitation of HA-positive material from media

HEK cells transfected with D6 were seeded at low density in a minimal volume of growth media that could support regular growth and proliferation. Cells were grown in standard conditions (at 37°C in 5% CO_2) and left to proliferate until 100% confluent. Conditioned media from these cells was taken and a μMACS™ Epitope Tag Protein Isolation Kit (Miltenyi) with anti-HA MicroBeads was used to isolate any HA-tagged material from the media. Magnetic labelling was performed by adding 50μl anti-HA MicroBeads to 1ml conditioned media and incubating for 30 minutes on ice with regular gentle agitation. Separation was carried out as per manufacturer's instructions and, subsequent to elution, samples were analysed by SDS-PAGE as described in **section 2.8.5**.

2.8.12 Immunoprecipitation of HA-D6

HA-D6 was immunoprecipitated from transfected HEK D6 cells using the μMACS™ Epitope Tag Protein Isolation Kit (Miltenyi Biotech) with anti-HA MicroBeads. Cell lysates were prepared before magnetic labelling was performed by adding anti-HA MicroBeads to the cell lysates and incubating for 30 minutes on ice with regular gentle agitation. The mixture was run through a μcolumn suspended on a magnet as per manufacturer's instructions. The column was rinsed several times with wash buffer provided in the kit in order to remove any non-specific material. Subsequently, elution buffer (SDS buffer) heated to 95°C was added to the column and samples collected. Samples were then analysed by SDS-PAGE as described in **section 2.8.5**.

2.8.13 Streptavidin bead pull down assay

The purpose of this assay was to find out if the D6-N peptide could be 'pulled-down' on the basis of its interaction with biotinylated chemokines. 2μg of D6-N (s) was incubated with 0.2μg of either bio-CCL2, bio-CCL22 or bio-CCL19 (Almac), in 100μl PBS for 15mins at room temperature. Biotinylated IgG (R&D systems), BSA (Sigma) and unlabelled chemokines (Peprotech) were used as controls in place of biotinylated chemokines. After incubation, samples were mixed with 50μl streptavidin microbeads from the μMACS™ Streptavidin Kit (Miltenyi Biotech) and put through a μcolumn suspended on a magnet. αHA microbeads were used as a control to count out non-specific binding to beads. PBS was washed through the column 4x before elution buffer (NuPAGE® LDS sample buffer (Invitrogen) heated to 95°C) was added to the column and sample collected. This sample is called the 'Target' and refers to anything that is binding to the streptavidin-bound molecule. The column was then taken off the magnet and more elution buffer added before sample collection again. This sample should contain the molecule that was binding to the streptavidin – in this case, the biotinylated chemokine. The process is illustrated in **Figure 4-7** in Chapter 4 of this thesis.

2.8.14 Protein binding assessments using BIAcore

BIAcore binding experiments were performed with the assistance of Dr. Sharon Kelly, University of Glasgow. Surface Plasmon Resonance (SPR) enables the detection of interactants in real time and was used to measure the biochemical affinity of D6-N for

CCL2. A BIAcore 2000TM biosensor system with a Series S Sensor CM5 chip (GE Healthcare, Buckinghamshire, UK) was used for protein binding assessments. Prior to biotinylated chemokine immobilization, the CM5 sensor chip surface was activated with an amine coupling kit which consisted of a mixture of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.1 M N-hydroxysuccinimide (NHS) (GE healthcare) at a flow rate of 5µl/min for 7mins. Neutravidin (Sigma), which was used to immobilise the biotinylated CCL2 on the chip, was added to the chip at 10µl/ml. Ethanolamine was then added in order to block remaining activated carboxymethyl groups. 10µg of Biotinylated CCL2 (Almac) was then added to the chip. The analytes were injected at either 0.1mg/ml or 1mg/ml and regeneration of the chip was performed before each new analyte injection to remove all previously bound analyte. Binding was recorded on a sensorgram which measures response units (RU) against time.

2.9 Statistical analysis

Data were analysed using GraphPad Prism software (San Diego, CA) applying appropriate statistical tests as described in figure legends. Probability values of $p<0.05$ were considered statistically significant.

Chapter 3 – Results

The role of glycosaminoglycans in chemokine presentation to D6

3.1 Introduction

Glycosaminoglycans (GAGs) are long, linear polysaccharide chains that are typically connected to a protein core, thereby forming proteoglycans. They are commonly associated with most cell types, including the surface of endothelial cells and the extracellular matrix (Johnson et al., 2004). In addition to the high binding affinity that chemokines have for their receptors, most chemokines also have a lower-affinity binding interaction with GAGs. It is thought that chemokines become immobilized on the surface of cells via GAGs and that the interaction between chemokines and their receptors may rely on GAGs acting as mediators. This process can involve the presentation of chemokines to their receptors on the same cell (*cis* presentation) or to different cells (*trans* presentation). **Figure 3-1** demonstrates both *cis* and *trans* presentation of chemokines by GAGs.

A previous study investigating the significance of the chemokine-GAG interaction for chemokine activity focussed on the chemokine receptors CCR1 and CCR5. It was shown that GAGs expressed on the surface of cells can enhance the activity of low concentrations of chemokines, presumably by increased chemokine sequestration onto the cell surface (Ali et al., 2000). The aim of this investigation was to focus on the 'atypical' chemokine receptor D6 and examine the role of GAGs in its ability to scavenge inflammatory chemokines.

To facilitate this investigation, a mutant Chinese hamster ovary (CHO) cell line was used which is fully deficient in the synthesis of GAGs (Esko et al., 1985, Graham et al., 1996). This mutant cell line is called CHO 745 and the wild type version of this cell line is referred to as CHO K1.

Key questions were established that were thought to be critical to research in this area:

- Can D6, expressed on CHO 745 cells (CHO 745 hD6), still scavenge inflammatory chemokines and target them for degradation?

- Does D6 expressed on CHO 745 cells (CHO 745 hD6) have reduced functionality compared with D6 expressed on CHO K1 cells (CHO K1 hD6) as a result of the loss of proteoglycan expression?

Taking into account the published literature which suggests that GAGs act as important mediators for chemokine presentation to inflammatory chemokine receptors such as CCR1 and CCR5, it was assumed that this would also be the case for the atypical chemokine receptor D6. Therefore it was hypothesized that chemokines would not be immobilised to the same extent on CHO 745 hD6, compared to CHO K1 hD6, and thus *cis*-presentation, chemokine uptake and degradation by CHO 745 hD6 would be less efficient compared with CHO K1 hD6.

In order to answer similar questions, previous groups working on typical inflammatory chemokine receptors used calcium flux assays and cell migration assays to measure the activity of the chemokine receptors in question. However, as D6 is an atypical chemokine receptor, its activation does not induce a change in calcium levels, nor does it induce cell migration, therefore different experimental approaches were taken and optimised in order to answer the proposed questions. Such experimental approaches and the results are discussed in the following chapter.

Flow cytometry experiments detailed in this chapter measuring uptake of chemokine by D6 assume that a decrease in uptake is indicative of a decrease in internalisation and degradation of chemokine. This is assumption is based on published data.

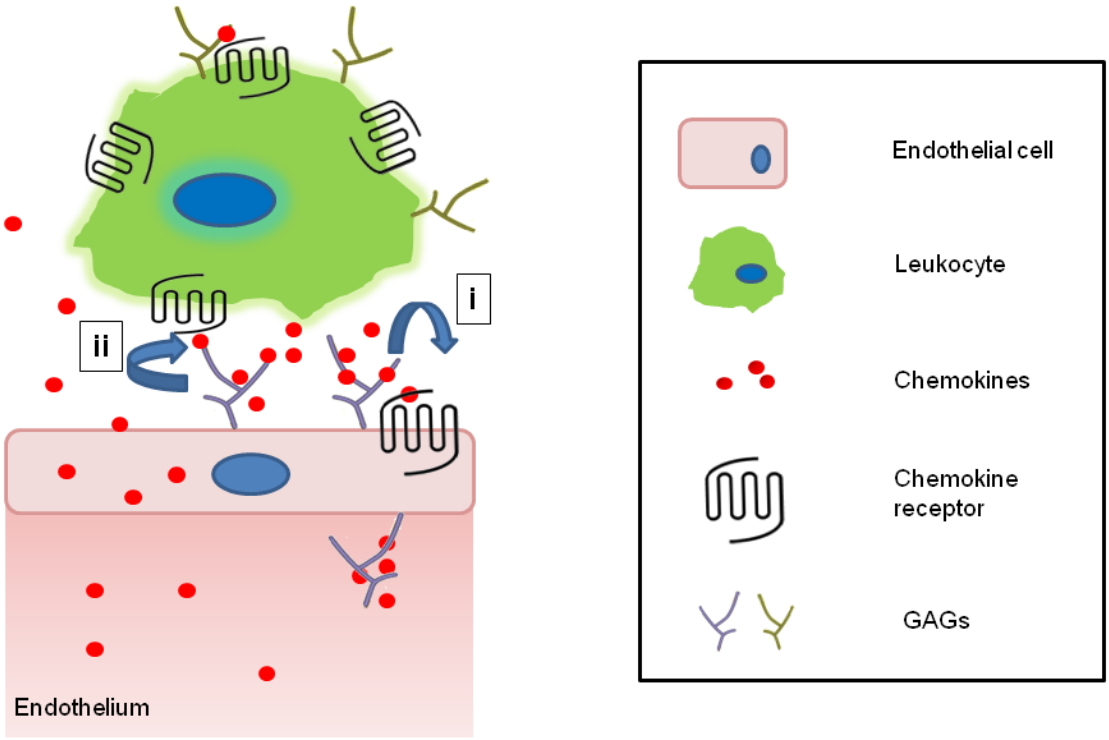


Figure 3-1: The envisaged role of GAGs in chemokine presentation.
(i) *Cis* presentation – presentation of a chemokine to a chemokine receptor by GAGs where all molecules are on the same cell. This method of presentation will be investigated further in this chapter **(ii)** *trans* presentation – presentation of chemokines by GAGs on one cell (endothelial in this case) to chemokine receptors on another cell (leukocyte in this case).

3.2 CHO K1 hD6 and CHO 745 hD6 cell lines

CHO cells are a widely used research tool in molecular biology and are often used for the production of recombinant proteins. The CHO K1 cell line was derived as a sub-clone from the parental CHO cell line, which was initiated from a biopsy of an ovary from an adult Chinese hamster. CHO cells are epithelial-like and grow as an adherent monolayer in culture. The CHO 745 cell line is a mutant generated from the CHO K1 line (Esko et al., 1985). CHO 745 cells are GAG-deficient because they lack the enzyme xylosyl transferase, which is required for the biosynthesis of heparan sulphate and chondroitin sulfate *in vivo*. This makes CHO 745 cells a valuable tool when investigating the importance of GAGs for chemokine binding by D6.

3.2.1 The absence of GAGs reduces chemokine immobilisation by CHO cells

Chemokine binding assays were set up in order to determine if a difference exists between the GAG-mediated chemokine binding of CHO K1 and CHO 745 cell lines. This involved the addition of Alexa-fluor-647 labelled CCL2 (AF-CCL2) to cells and incubation for 1 hour. Concentrations of AF-CCL2 ranging from 0nM to 20nM were added to cells. Cells were then washed twice to remove excess chemokine and subsequently analysed by flow cytometry to detect bound AF-CCL2. Viaprobe, a dead cell discriminator containing 7-amino-actinomycin D (7-AAD), was used to exclude dead cells from the analysis. **Figure 3-2** shows the results of the chemokine binding experiment.

More CHO K1 cells appear fluorescent compared to CHO 745 cells after the addition of 20nM AF-CCL2 (**Figure 3-2 (a)**). This difference in the number of fluorescent cells between CHO K1 and CHO 745 cells was consistent at every concentration of AF-CCL2 tested (**Figure 3-2 (b)**), suggesting that it is a consequence of the absence of GAGs on CHO 745 cells. This result further substantiated the use of CHO 745 cells in this investigation.

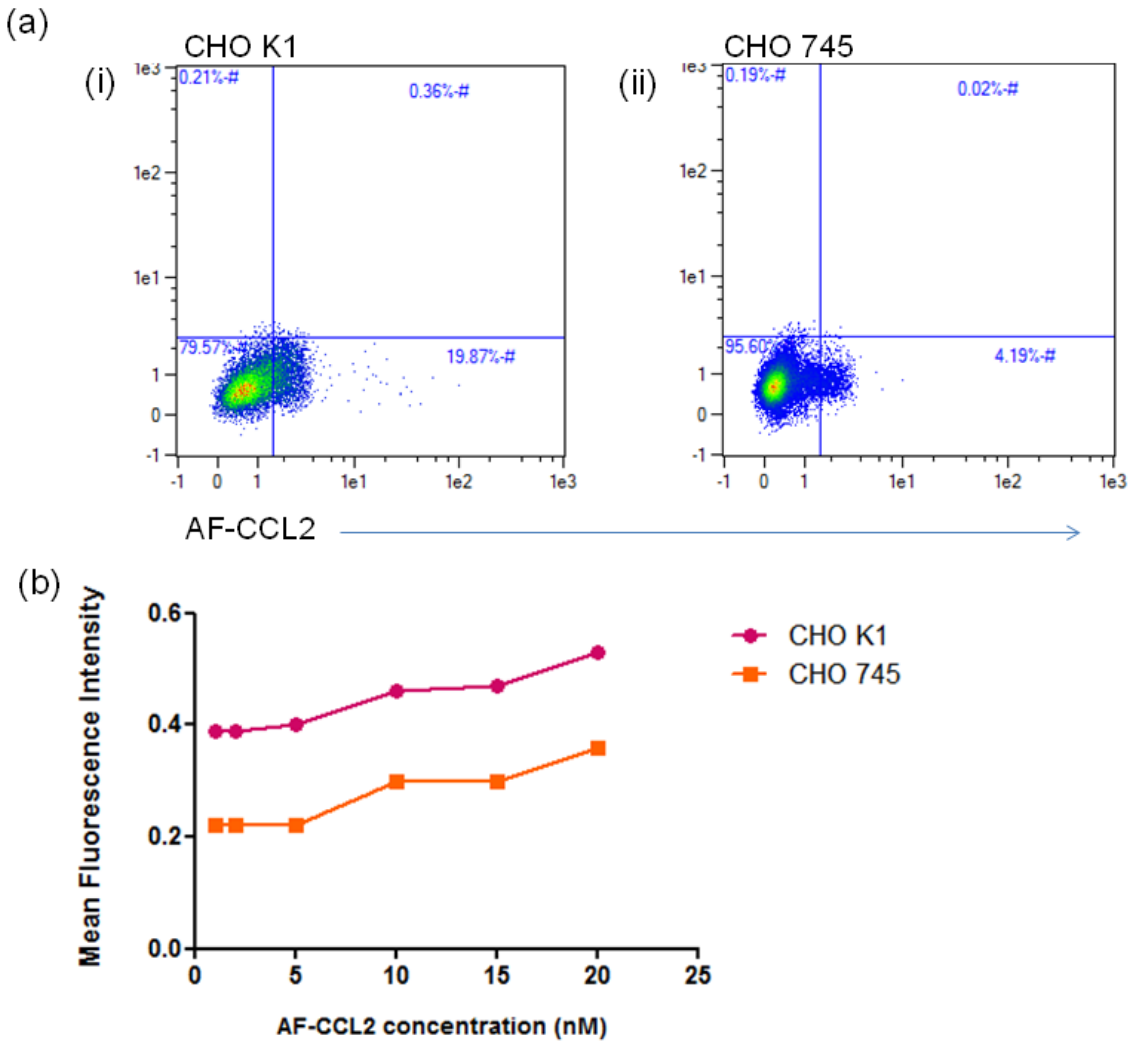


Figure 3-2: CHO K1 and CHO 745 chemokine binding affinity
(a) Flow cytometry profiles of either **(i)** CHO K1 or **(ii)** CHO 745 cells after addition of 20nM AF-CCL2. **(b)** Concentrations of AF-CCL2 ranging from 1nM to 20nM were added to CHO K1 and CHO 745 cells before analysis by flow cytometry. Dead cells were excluded from the analysis with the addition of viaprobe. The MFI values obtained for both cell types at each chemokine concentration are shown on an X-Y line graph.

3.2.2 Stable transfection of CHO K1 and CHO 745 cells

In order to be able to compare the ability of chemokines to bind to D6 on cells expressing GAGs (CHO K1) with GAG-deficient cells (CHO 745), both cell lines were transfected with a plasmid containing the D6 gene with an *N*-terminal HA (haemagglutinin) tag (HA-D6). This plasmid was previously generated in this laboratory by introducing nucleotides encoding an HA tag (amino acid sequence: MYPYDVPDYAG) at the extreme *N*-terminus of human D6 cDNA by PCR.

CHO K1 and CHO 745 cells were stably transfected with pcDNA3.1 HA-D6 using effectene (as described in Chapter 2, section 2.4). Transfected cells were analysed by flow cytometry to assess their levels of D6 expression. Cell membrane D6 levels were detected using the anti-D6 (clone 4A5) antibody and a FITC-conjugated secondary antibody. Flow cytometry profiles for both CHO K1 hD6 and CHO 745 hD6, and also the gating strategy employed to analyse these cells are shown in **Figure 3-3.**

Flow cytometry analysis indicated that both transfected cell lines had very low levels of D6 expression, with ~10% D6 positivity in CHO K1 cells (**Figure 3-3 (a) (iii)**) and only ~2.5% positivity in CHO 745 cells (**Figure 3-3 (b) (iii)**). This was surprising because both cell lines grew normally in media supplemented with G418, indicating that they had been transfected with a G418-resistant plasmid. This suggested that many of the plasmids may not contain the intact HA-D6 gene.

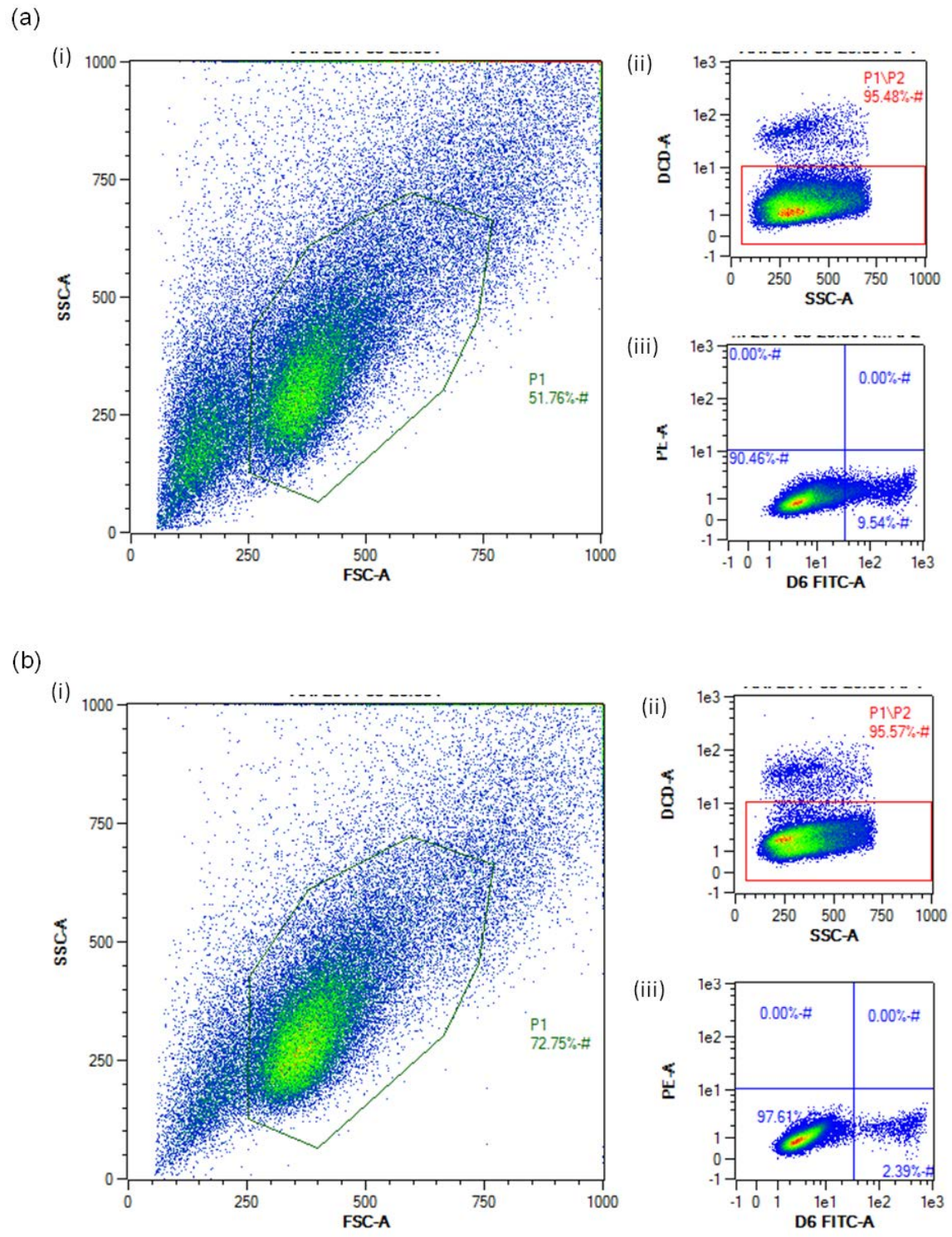


Figure 3-3: D6 expression of CHO cell lines after transfection
Flow cytometry profiles for **(a)** CHO K1 cells and **(b)** CHO 745 cells. **(i)** Forward scatter (FSC) and side scatter (SSC) was used to gate the main population of cells **(ii)** dead cells were excluded from the analysis using a dead cell discriminator (DCD) **(iii)** D6 expression was determined using the mouse anti-human D6 antibody (clone 4A5) and an anti-mouse FITC secondary antibody**.** Cells positive for D6 expression are FITC positive.

3.2.3 Enrichment for D6 positive cells

Due to the low transfection efficiency in both CHO K1 and CHO 745 transfectants, D6 positive cell enrichment was attempted by labelling transfected cells with biotinylated anti-D6 antibody (clone 4A5), then using anti-biotin beads to select D6 positive cells. Prior to this experiment, the D6 antibody (clone 4A5) required biotinylation, which was achieved using a commercially available biotinylation kit (see chapter 2, section 2.4.3). The enrichment process is illustrated in **Figure 3-4**. Enrichment of cells was performed twice (D6 positive cells from the first enrichment were cultured and left to grow to confluency before performing the second enrichment). Aliquots of the cells obtained at each stage (pre-sorted cells, unbound cells and D6 positive cells) were analysed by flow cytometry and the results of both enrichments are shown in **Figure 3-5**.

Enrichment for D6 positive cells by this method was successful and eventually resulted in populations of both CHO K1 hD6 and CHO 745 hD6 with over 70% of cells expressing D6 (**Figure 3-5 (c) (iii)** and **(Figure 3-5 (d) (iii)** respectively). Despite this, both cell lines displayed considerable variation in levels of D6 expression. This is apparent by examining the range of mean fluorescence intensity values for the D6 positive cells. This suggested that further development was required.

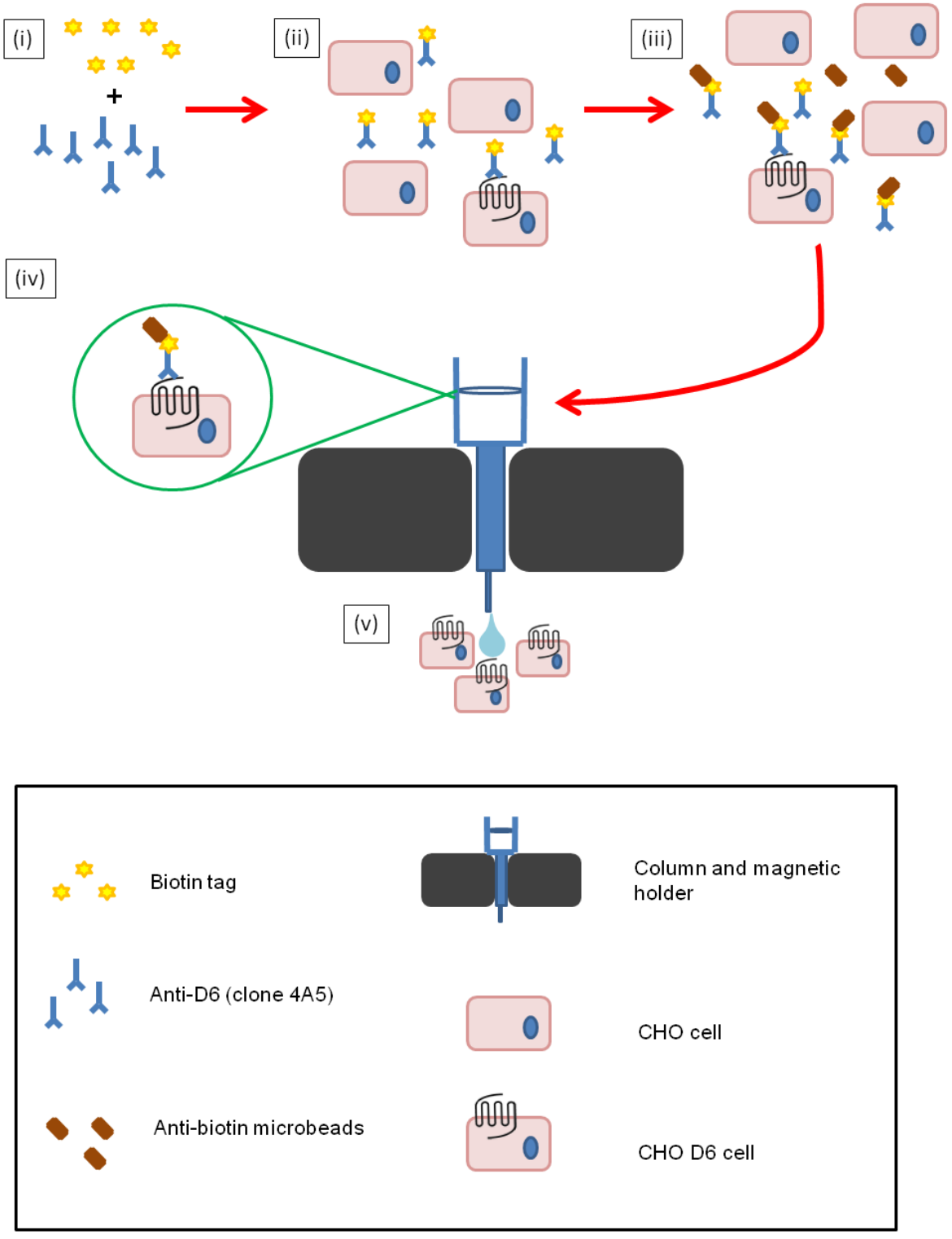


Figure 3-4: Enrichment of D6 expressing cells from CHO K1 hD6 and CHO 745 hD6. **(i)** Anti-D6 (clone 4A5) D6 was biotinylated using a commercially available biotinylation kit **(ii)** Biotinylated anti-D6 (clone 4A5) was added to cells which bound to any cells expressing D6 on its surface **(iii)** magnetic anti-biotin microbeads were added to the mixture which bound to the biotinylated antibody **(iv)** the mixture was passed through a column suspended on a magnet, any cells that bound to a microbead remained inside the column **(v)** after several washes to remove unbound cells (which were kept for flow cytometry analysis), the column was removed from the magnet and contents were obtained. Cell fractions were mixed with an anti-mouse IgG FITC antibody and analysed by flow cytometry.

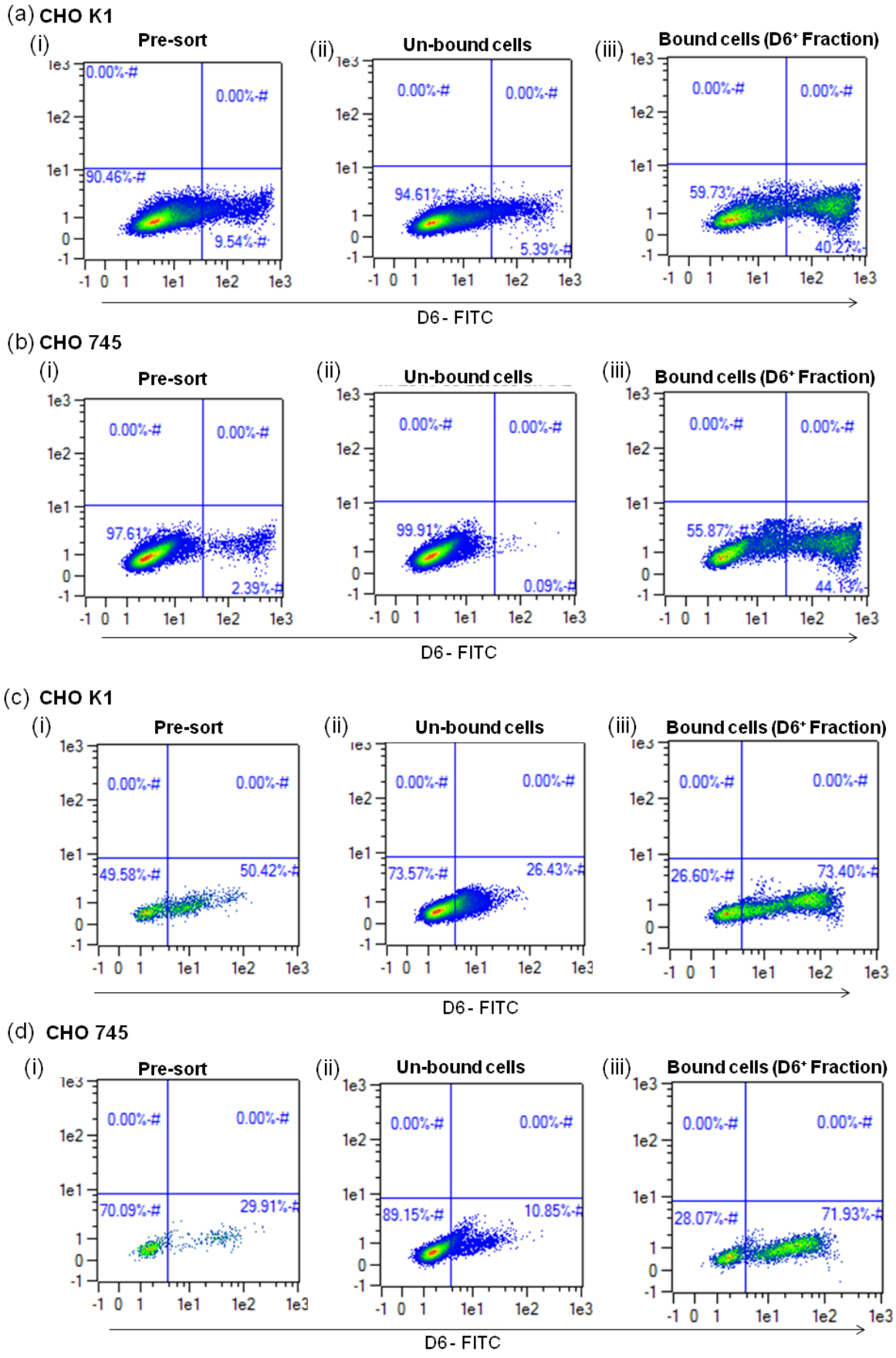


Figure 3-5: Enrichment for D6+ cells.
Cells were assayed by flow cytometry using either anti-D6 (clone 4A5) or a mouse isotype control (not shown) and an anti-mouse IgG FITC secondary antibody. Enrichment 1 for CHO K1 is shown in **(a)** and CHO 745 in **(b)**. Enrichment 2 for CHO K1 is shown in **(c)** and CHO 745 in **(d)**.

3.2.4 Obtaining clonal populations of CHO K1 hD6 and CHO 745 hD6

Given the variable expression levels apparent in Figure 3-5, and to allow appropriate comparison between CHO K1 hD6 and CHO 745 hD6, it was important to carry out further experiments to ensure that the levels of D6 expression for both cell lines were similar. To achieve this, 'ring cloning' was attempted. This method involves the seeding of cells at extremely low density and picking isolated colonies to derive clonal lines of transfected cells (for full details see chapter 2, section 2.4.3). Ten separate colonies were picked from both CHO K1 hD6 and CHO 745 hD6 lines and grown to confluency. 'Ring cloning' is technically challenging and requires multiple rounds of sub-cloning in order to obtain clonal populations of cells. The cell populations which were obtained after the first and second ring cloning experiments are shown in **Figure 3-6**. The populations of CHO K1 hD6 and CHO 745 hD6 obtained after the second round of ring cloning were checked for D6 expression by flow cytometry using both the anti-D6 (clone 4A5) antibody and an anti-HA antibody. The resulting histograms are shown in **Figure 3-7**.

The flow cytometry profiles depicted in **Figure 3-6** indicate that during the first 'ring cloning' process, it appeared that cells from at least two different colonies had been picked and expanded. This is certainly the case for CHO K1 hD6, where the cells still show a substantial range of D6 expression (**Figure 3-6 (a) (ii)**). After the second ring cloning process, there were populations of both CHO K1 hD6 and CHO 745 hD6 where levels of D6 expression was more uniform (**Figure 3-6 (a) (iii)** and **(b) (iii)** respectively). This is indicative that cells from only one colony had been picked to grow up to confluency in these populations. Such populations were selected for further analysis.

Figure 3-7 compares D6 expression between the selected populations of CHO K1 hD6 and CHO 745 hD6 and shows that these populations express D6 at very similar levels, with mean fluorescence intensities (MFIs) of 9.84 and 10.09 for CHO K1 hD6 and CHO 745 hD6 respectively using the anti-D6 (clone 4A5) antibody (**Figure 3-7 (a) (i)** and **(b) (i)**) and MFIs of 107.7 and 110.5 for CHO K1 hD6 and CHO 745 hD6 respectively using an anti-HA antibody (**(Figure 3-7 (a) (ii)** and **(b) (ii)**).

Obtaining clonal populations of CHO K1 and CHO 745 transfectants expressing very similar levels of D6 was a prerequisite for experiments comparing the ability of D6 to uptake chemokine by the two cell lines.

In summary, although ring cloning was a time consuming and challenging technique, it allowed the acquisition of clonal populations of both CHO K1 hD6 and CHO 745 hD6 which express D6 at very similar levels.

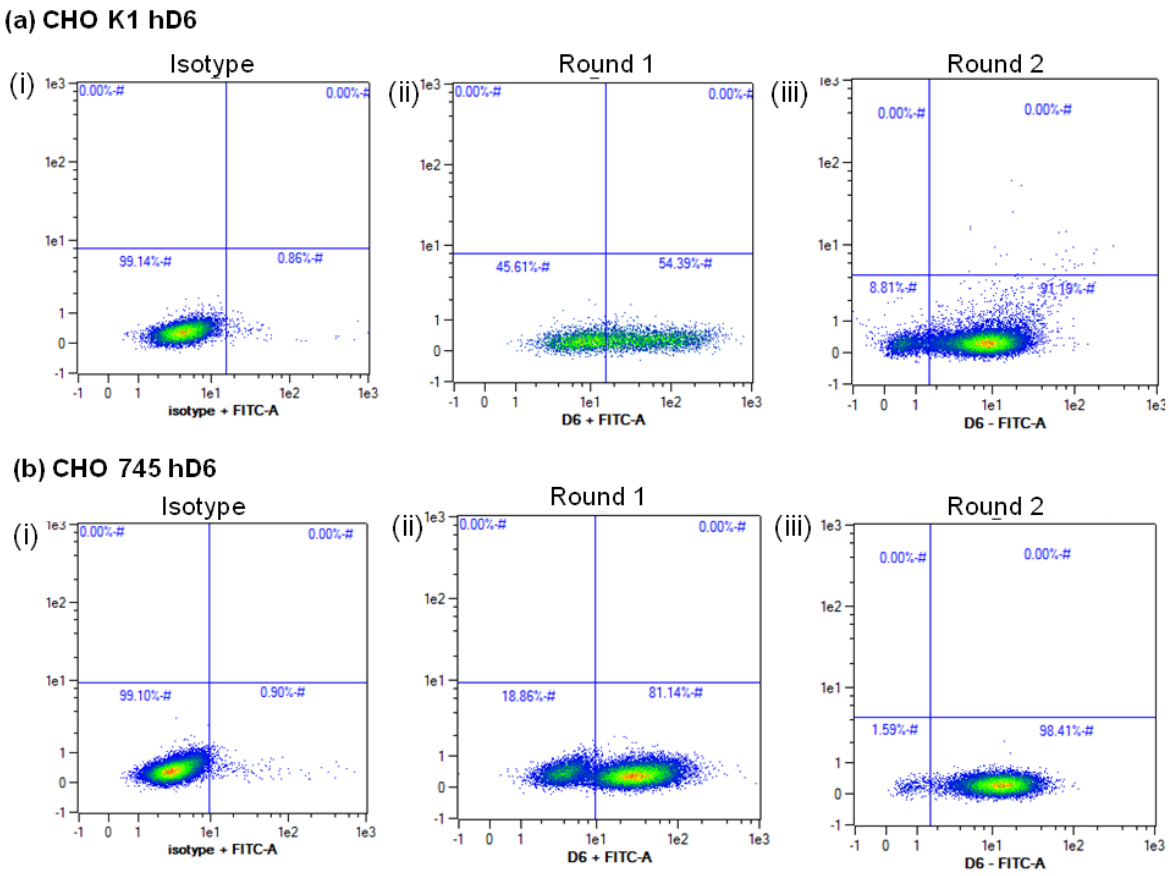


Figure 3-6: D6 expression of CHO cells lines.
(a) CHO K1 hD6 and **(b)** CHO 745 hD6 populations after the first and second ring cloning experiments. Cells were assayed by flow cytometry using either anti-D6 (clone 4A5) or a mouse isotype control (as labelled) and an anti-mouse IgG FITC secondary antibody.

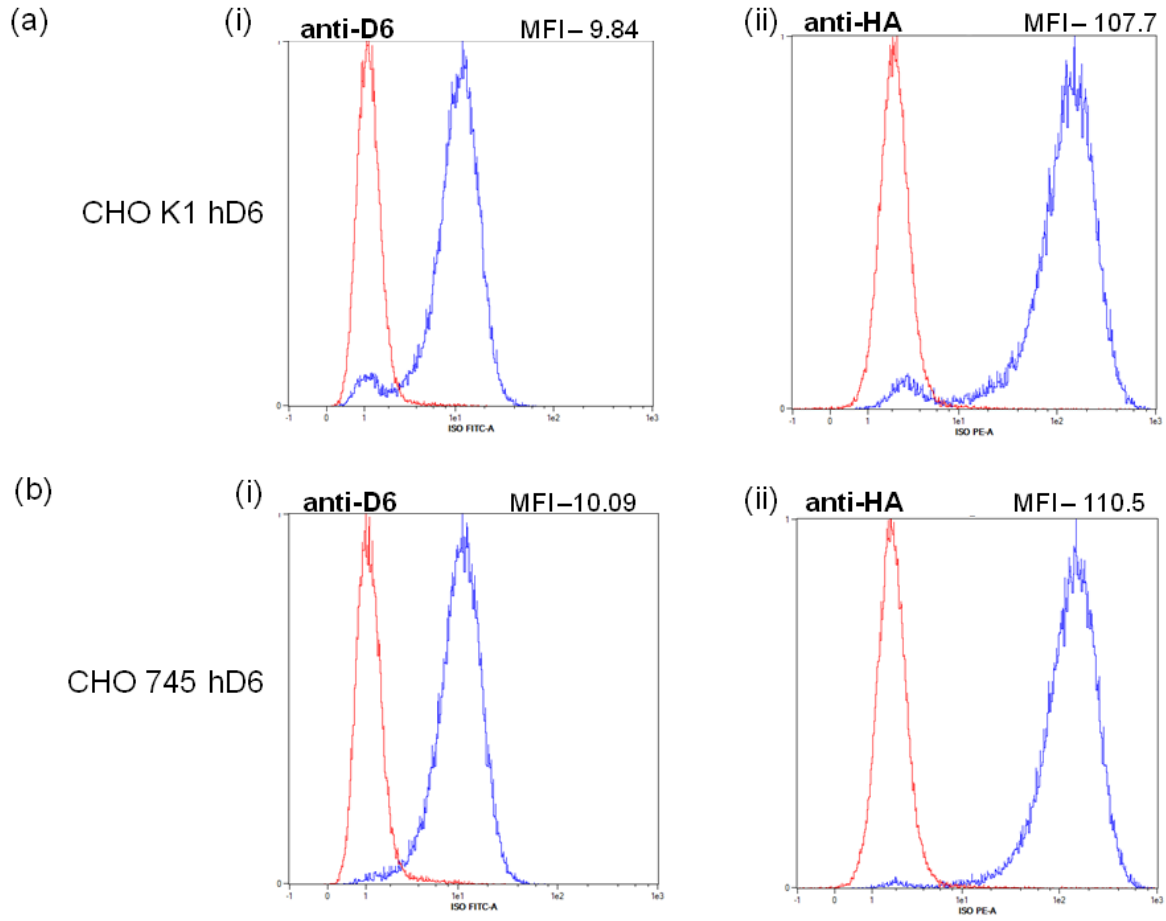


Figure 3-7: CHO K1 hD6 and CHO 745 hD6 express D6 at very similar levels.
(a) CHO K1 hD6 and **(b)** CHO 745 hD6 cells were assayed using **(i)** anti-D6 (clone 4A5) or a mouse isotype control and an anti-mouse IgG FITC secondary antibody. Cells were also assayed using **(ii)** a mouse anti-HA (biotin) antibody or a mouse IgG (biotin) isotype control and a streptavidin conjugated to R-phycoerythin (PE) secondary. Isotype controls are represented in red and D6 expression is represented in blue. The MFIs of D6 expressing cells are indicated above each flow cytometry profile.

3.3 *Cis*-presentation of chemokines by glycosaminoglycans increases D6 activity

Having already established that GAG-based CCL2 binding is lower in CHO 745 cells compared to CHO K1 cells, and with the creation of CHO K1 hD6 and CHO 745 hD6 stably expressing D6 at similar levels, experiments were set out to determine the importance of GAGs for D6-mediated chemokine binding and scavenging.

3.3.1 CHO K1 hD6 and CHO 745 hD6 have different ligand uptake capability

To directly test whether or not the presence of GAGs contributes to chemokine presentation to D6, and therefore to D6 function, chemokine uptake assays were performed on both CHO K1 hD6 and CHO 745 hD6. This involved the addition of Alexa-fluor-647 labelled CCL2 (AF-CCL2) to cells and subsequent incubation at 37°C to allow D6 uptake and internalisation of chemokine. Concentrations of chemokine ranging from 0nM to 15nM were added to cells and incubated at 37°C for 1 hour. Cells were then washed twice to remove excess chemokine and subsequently analysed by flow cytometry to detect internalised AF-CCL2. Viaprobe was used to exclude dead cells from the analysis. Live cells that have taken up chemokine have an increase in MFI compared to cells that have not, and this is shown by a right shift of cells in the flow cytometry plots. **Figure 3-8** shows the results of the chemokine uptake experiment.

Importantly, CHO K1 hD6 displayed a huge increase in MFI compared with un-transfected CHO K1 cells after addition of AF-CCL2 (**Figure 3-8 (a) (i)** and **(ii)).** This confirmed that uptake of CCL2 by CHO K1 hD6 is D6-dependent. The MFI values resulting from the chemokine uptake assay with both CHO K1 hD6 and CHO 745 hD6 are shown in **Figure 3-8 (b)**. MFI values for AF-CCL2 uptake by both CHO K1 hD6 and CHO 745 hD6 increased in a dose-dependent fashion. However initial results suggested that CHO K1 hD6 uptake of AF-CCL2 was increased compared to CHO 745 hD6, and the difference between CHO K1 hD6 and CHO 745 hD6 became more apparent as the concentration of chemokine increased.

In summary, uptake of AF-CCL2 by CHO K1 hD6 and CHO 745 hD6 is D6 dependent. CHO K1 hD6 appeared to display enhanced AF-CCL2 uptake compared to CHO 745 hD6, and the difference between the two cell lines becomes more apparent as the concentration of chemokine added to the cells increases.

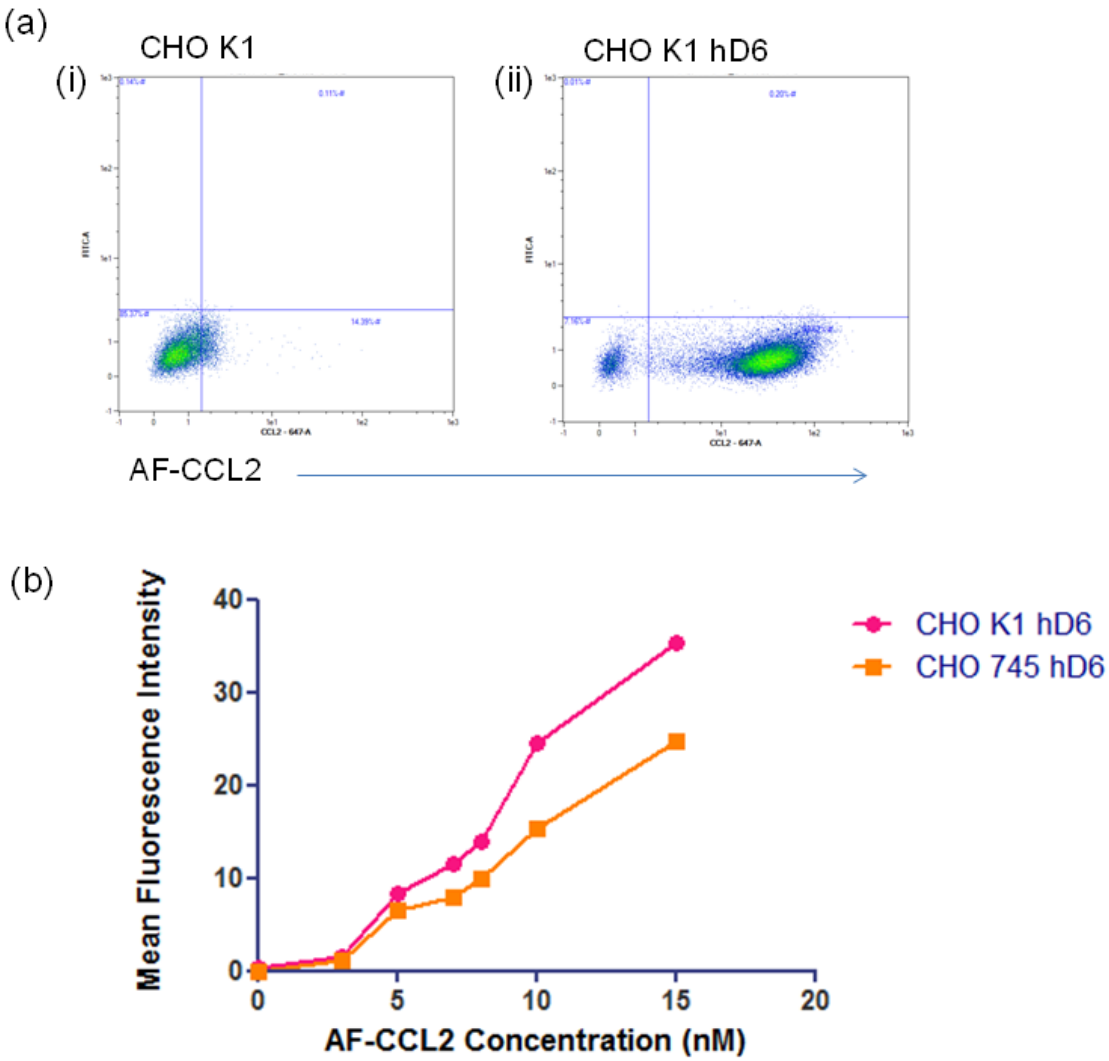


Figure 3-8: Uptake ability of CHO K1 hD6 and CHO 745 hD6 with increasing AF-CCL2 concentration.
(a) Flow cytometry profiles of either **(i)** CHO K1 or **(ii)** CHO K1 hD6 cells after addition of 15nM AF-CCL2. **(b)** Concentrations of AF-CCL2 ranging from 0nM to 15nM were added to CHO K1 hD6 and CHO 745 hD6 cells before analysis by flow cytometry. Dead cells were excluded from the analysis with the addition of viaprobe. The MFI values obtained for both cell types at each chemokine concentration are shown on an X-Y line graph. N=1

3.3.2 CHO 745 hD6 display reduced ligand uptake compared to CHO K1 hD6

The initial experiment highlighted a trend suggesting that the chemokine uptake capabilities of CHO K1 hD6 and CHO 745 hD6 were different, therefore experiments were set up in order to further characterise this. The previous uptake assays revealed that the largest difference in MFI values between CHO K1 hD6 and CHO 745 hD6 occurred at the highest AF-CCL2 concentration used (15nM), for this reason all subsequent chemokine uptake assays were performed with this concentration of AF-CCL2.

To examine different kinetics on the uptake of AF-CCL2 by CHO K1 hD6 and CHO 745 hD6, chemokine uptake assays were performed for 10 minutes or 60 minutes. MFIs for each uptake assay were measured and the results are displayed in **Figure 3-9**. Further assays were performed to determine if and when the uptake of AF-CCL2 plateaus. Chemokine uptake assays were performed as before, however this time incubation at 37°C was for 20 minutes, 60 minutes or 120 minutes. MFIs for each uptake assay were recorded and plotted on a bar chart, which is shown in **Figure 3-10**.

Figure 3-9 indicates that when cells are incubated with AF-CCL2 at 37°C for as little as 10 minutes (**Figure 3-9 (i)**), D6 activity is already significantly different between the two cell lines. This difference increases in significance when cells are incubated for 60 minutes at 37°C (**Figure 3-9 (ii)**). This result suggests that D6 expressed on cells which are GAG-deficient (CHO 745 hD6) cannot take up chemokine as effectively as wildtype cells (CHO K1 hD6). **Figure 3-10** shows that the mean of MFI values for AF-CCL2 uptake by CHO K1 hD6 are similar at 60 minutes and 120 minutes. This suggests that uptake of AF-CCL2 by CHO K1 hD6 has come to a plateau by 60 minutes. In contrast, the MFIs for CHO 745 hD6 are still fairly consistent at 120 minutes suggesting that these cells are only capable of low level ligand uptake at each of the time points analysed.

In summary, CHO K1 hD6 can bind and internalise AF-CCL2 more readily than CHO 745 hD6, and this difference in chemokine uptake is most significant when cells are incubated for 60 minutes with ligand prior to flow cytometry analysis. Uptake of AF-CCL2 by CHO K1 hD6 comes to a plateau after 60 minutes.

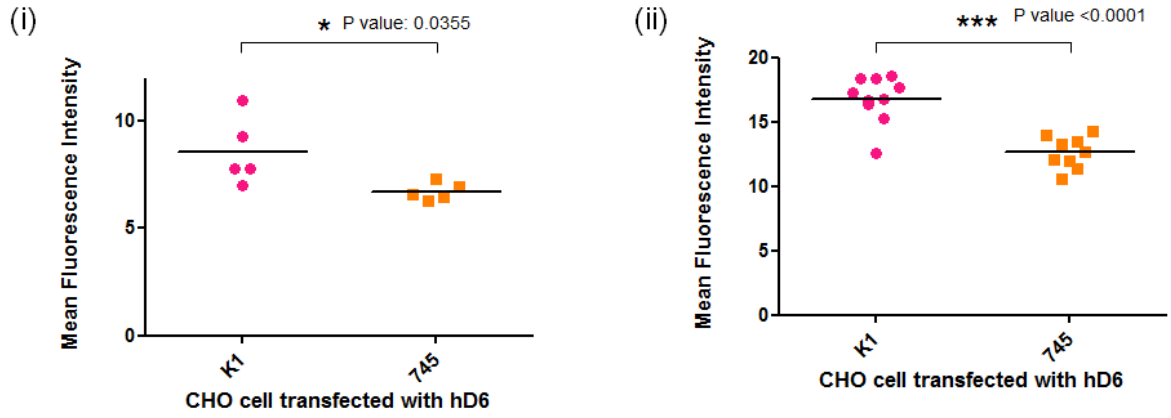


Figure 3-9: Uptake of AF-CCL2 by CHO K1 hD6 and CHO 745 hD6 at different time points. Chemokine uptake assays were performed at **(i)** 10 minutes or **(ii)** 60 minutes. Cells were analysed by flow cytometry and the MFI values for each experiment are plotted. Statistical analysis was performed using two-tailed unpaired t tests. Means were found to be significantly different and P values are indicated. **(i)** N=5, **(ii)** N=10

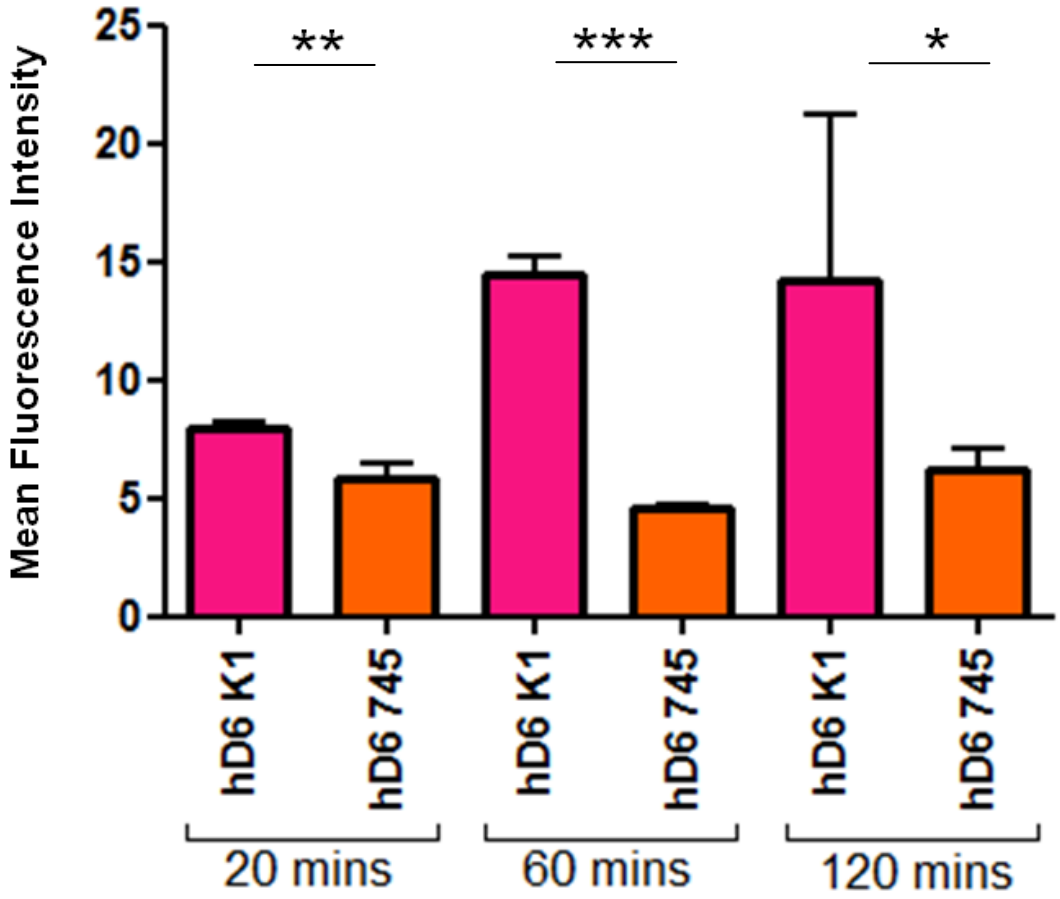


Figure 3-10: Uptake of AF-CCL2 by CHO K1 hD6 is more efficient than CHO 745 hD6 Chemokine uptake assays were performed for 20 minutes, 60 minutes or 120 minutes. Cells were analysed by flow cytometry and the MFI values for each experiment are plotted on a bar chart showing mean with standard deviation indicated. Statistical analysis was performed using two-tailed unpaired t tests. Means were found to be significantly different at each time point. P values and N numbers are as follows: 20 minutes – P value =0.0093, N=10, 60 minutes – P value <0.0001, N=5 120 minutes – P value =0.0419, N=5.

3.3.3 The efficiency of chemokine degradation by D6 is affected by the absence of GAGs

As discussed in the introduction (Chapter 1, section 1.6), D6 not only binds to, and internalises, most inflammatory CC chemokines, but also efficiently targets them for lysosomal degradation, making D6 a highly effective inflammatory chemokine scavenging receptor. Having confirmed that the absence of GAGs decreases the ability of D6 to bind and internalise AF-CCL2, it was necessary to assess whether the absence of GAGs also affected D6-mediated degradation of chemokines. To investigate this, the ability of D6 to remove inflammatory chemokine from its environment was examined.

To test whether the absence of GAGs on the cell surface and the resultant impairment of D6-mediated ligand uptake affects ligand degradation, a fluorescence assay was set up, whereby AF-CCL22 was added to both CHO K1 hD6 and CHO 745 hD6 cells and incubated for either 1 hour or 24 hours. Media was collected and both cells and media were analysed separately for fluorescence intensity. Un-transfected CHO K1 cells were used as a control in these experiments. As a separate control to test degradation of chemokine by un-transfected CHO K1 and CHO 745 cells, biotinylated CCL2 (bio-CCL2) was added to both CHO K1 and CHO 745 cells and incubated for either 1 hour or 24 hours. Media were then collected and the amount of intact bio-CCL2 remaining in the media was determined using Western blotting with streptavidin-HRP. The results of these experiments are presented in **Figure 3-11**.

Figure 3-11 (a) and **(b)** show the fluorescence of CHO K1 hD6, CHO 745 hD6 and CHO K1 after 1 hour and 24 hours respectively. Interestingly the fluorescence of both CHO K1 hD6 and CHO 745 hD6 increases substantially from 1 hour to 24 hours, suggesting that the fluorophore present on AF-CCL22 is not degraded along with the chemokine. In this way the data show uptake of AF-CCL22 over a 24 hour period and indicate that the significant difference in chemokine uptake between CHO K1 hD6 and CHO 745 hD6 continues over a 24 hour period and even increases from ~4-fold to ~5-fold. Note the very low levels of fluorescence exhibited by un-transfected CHO K1, indicating that the increase in fluorescence by CHO K1 hD6 and CHO 745 hD6 is D6-mediated. **Figure 3-11 (c)** shows the fluorescence of the media taken from both CHO K1 hD6 and CHO 745 hD6 after 24 hours. There is significantly more AF-CCL22 in the media from CHO 745 hD6, suggesting D6-mediated uptake and degradation of AF-CCL22 from the media is reduced when GAGs

are absent. A Western blot on media taken from the un-transfected CHO cell lines after 1 hour and 24 hours is shown in **Figure 3-11 (d)** and densitometry analysis of the bands from the 24 hour incubation is shown in **(e)**. The Western blot and densitometry analysis indicates that there is a subtle reduction in the amount of bio-CCL2 left in the media of CHO 745 cells compared to CHO K1 cells after 24 hours.

In summary, D6-mediated uptake and degradation of CC-chemokines is hampered in the absence of GAGs and this effect is not restricted to shorter incubation times.

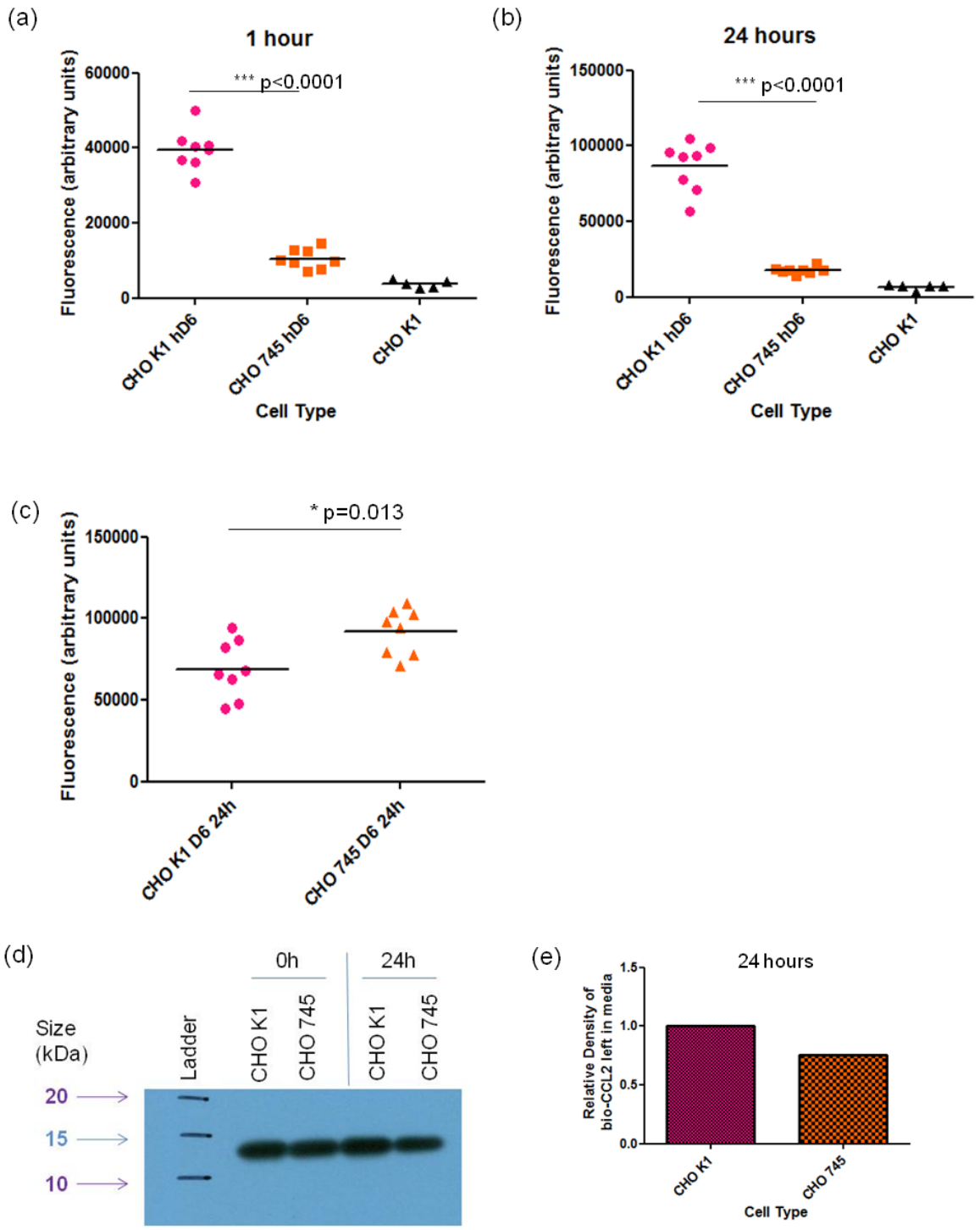


Figure 3-11: D6-mediated degradation of CCL22 is reduced when GAGs are absent.
Dot plot of fluorescence measurements from cells after fluorescence assays using CHO K1 hD6, CHO 745 hD6 and CHO K1 with the addition of AF-CCL22 and incubation from **(a)** 1 hour and **(b)** 24 hours. **(c)** Dot plot of fluorescence measurements from media after fluorescence assays using CHO K1 hD6 and CHO 745 hD6 with the addition of AF-CCL22 and incubation for 24 hours. N=8 for CHO K1 hD6 and CHO 745 hD6, N=5 for CHO K1 ((a) and (b) only). Data were analysed by unpaired, two tailed student T tests. **(d)** Western blot using streptavidin-HRP of Bio-CCL2 left in media of CHO K1 or CHO 745 cells after 0 or 24 hours (indicated). **(e)** Densitometry analysis of the 24h bands from blot the Western blot shown in (d) using image J software.

3.4 Summary of Chapter 3

This chapter describes experiments aimed at investigating the role of GAGs for D6 function and specifically whether the absence of GAGs on the surface of cells affects the ability of D6 to bind, internalise and degrade the inflammatory chemokines CCL2 and CCL22. The inherent difficulty and complexity of trying to block the numerous GAG subtypes present on D6-expressing cells such as lymphatic endothelial cells, led to the decision to use CHO cells as a surrogate for the investigation of GAG impact on D6 function. A well-characterised CHO cell line, deficient in the synthesis of GAGs (CHO 745) was obtained for comparison with WT CHO cells (CHO K1). Transfection of both cell lines with a plasmid containing D6, and subsequent manipulation of D6-expressing cells was required in order to generate clonal populations of CHO K1 and CHO 745 which expressed D6 at very similar levels. Such clones were then used in downstream analysis to investigate the role of GAG presentation of chemokines to D6 and the affect of their absence on (i) uptake and internalisation of chemokine and (ii) D6-mediated degradation of chemokine. The main findings presented in this chapter are the following:

- The efficiency of D6-mediated uptake of CCL2 is reduced in GAG-deficient cells (CHO 745 hD6) compared with WT cells (CHO K1 hD6) *in vitro*. This suggests that *cis*-presentation of ligand by GAGs is important for D6-mediated internalisation and scavenging of inflammatory chemokines.

- The efficiency of D6-mediated uptake of CCL22 and scavenging from the surrounding media also is reduced in GAG-deficient cells (CHO 745 hD6) compared with WT cells (CHO K1 hD6) *in vitro*.

Such findings are consistent with data generated from studies on the significance of GAGs for function of other chemokine receptors and will be discussed further in Chapter 7.

Chapter 4 – Results

An analysis of the biochemistry and binding properties of D6-N

4.1 Introduction

As with the *N*-terminal domains of 'classical' chemokine receptors, the D6 *N*-terminus (D6-N) is likely to act as a binding site for inflammatory chemokine ligands. Previous studies with peptides designed from the sequences of the *N*-termini of classical chemokine receptors, including CCR5, CXCR4, CCR3 and CX_3CR1, have highlighted the potential of such *N*-terminally derived peptides to bind to their cognate ligands (Veldkamp et al., 2006, Ye et al., 2000, Zhu et al., 2011, Mizoue et al., 1999). In the case of CCR5-mediated HIV infection this has been of particular significance, with a tyrosine sulphated peptide based on the *N*-terminus of CCR5 being able to block HIV-1 entry (Farzan et al., 2000). A clear theme from such studies is that chemokine and/or pathogen recognition is frequently dependent on both the presence and the specific position of sulpho-tyrosine residues on both *N*-terminally derived peptides and full length chemokine receptors (Simpson et al., 2009).

In keeping with this, it was hypothesised that a peptide analogue of D6-N may have the ability to bind to a broad spectrum of inflammatory chemokines with high affinity, in a similar way to the full-length D6 molecule. In cases of chronic inflammation, where excessive inflammatory chemokine production is a contributing factor to disease, D6-N could potentially be used therapeutically as a non-immunogenic, broad-based inflammatory chemokine blocker. To achieve this, the peptide should have the ability to bind to, and block the action of, inflammatory CC chemokines, and in doing so prevent excessive inflammatory cell infiltration to sites of damage. **Figure 4-1** illustrates this hypothesis.

To examine the importance of sulphated tyrosines for possible D6-N function, and as detailed in section 2.8.1 of Chapter 2, both sulphated – D6-N (s) and non-sulphated – D6-N (non-s) versions of the D6-N peptide were synthesized and used in experiments to establish the binding potential of these peptides to inflammatory chemokines.
The key questions addressed in this chapter are:

- Does D6-N bind to inflammatory chemokines (D6 ligands)?
- Is sulphation of D6-N essential for binding?
- Can we measure the binding affinity of D6-N for chemokines?
- Can addition of D6-N peptides inhibit inflammatory chemokines from binding to their cognate receptors?

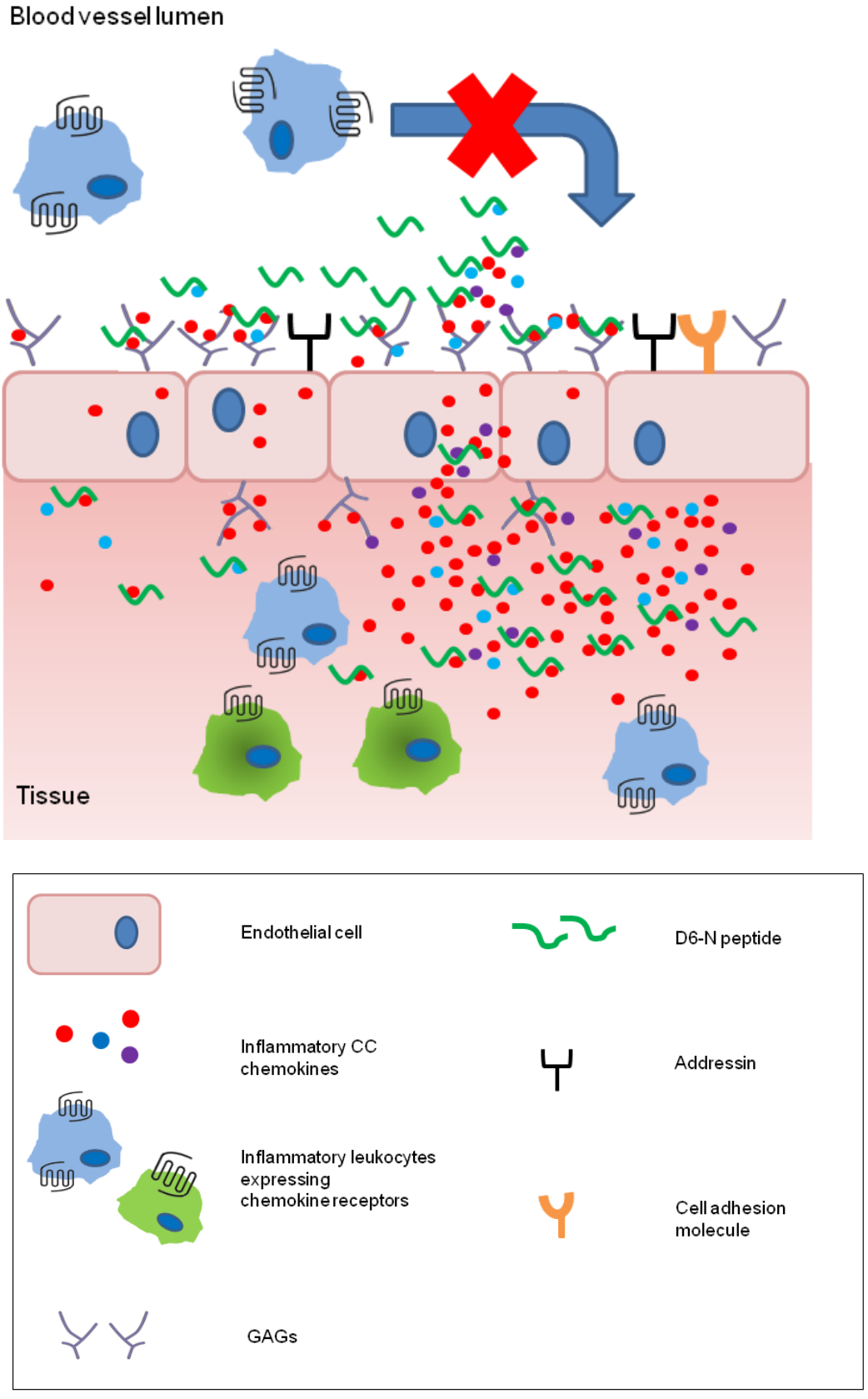


Figure 4-1: The envisaged potential of using D6-N therapeutically.
Chronic inflammation in the tissue results in excessive chemokine production and infiltration of leukocytes from the blood. A therapeutic based on D6-N is capable of binding to and neutralising a wide range of inflammatory chemokines. The chemokines can no longer bind to their cognate receptors expressed on the surface of inflammatory leukocytes, resulting in the inhibition of chemotaxis and the discontinuation of cell infiltration and associated inflammatory responses.

4.2 The D6-N peptide

4.2.1 Synthesis and biochemistry

D6-N (non-s) is a synthetic peptide representing the most *N*-terminal 35 amino acids of D6 plus a hexa-His-tag. In order to make D6-N (s), D6-N (non-s) was tyrosine sulphated post peptide synthesis. The *N*-terminus of D6 contains four tyrosine residues, each of which may or may not be sulphated during the chemical sulphation process. The degree of sulphation of the D6-N peptide is difficult to control or manipulate using this method of sulphation (Seibert and Sakmar, 2008). The molecular weights of both D6-N (s) and D6-N (non-s) can be estimated based on their amino acid composition, with 4.75kDa having been estimated for D6-N (non-s). A differentially sulphated peptide would weigh anything between approximately 4.83kDa and 5.07kDa, as a single sulphate group is 80Da and a maximum of four tyrosine residues may be sulphated. Mass spectrometry analysis of D6-N (s) is shown in **Figure 4-2.**

The data shown in **Figure 4-2** confirm that D6-N (s) is a mixture of differentially sulphated peptides with major peaks at 4749, 4829 and 4908Da, corresponding to non-sulphated, mono-sulphated and di-sulphated peptide species respectively. The Y-axis of the mass spectrometry plot represents the intensity of the signal generated by each peptide species. This signal correlates loosely with the relative abundance of each peptide species. Therefore from the data generated, it seems that the mono-sulphated peptide is the most abundant peptide species in this preparation of D6-N (s). The mass spectrometry data does not give any indication as to the positions of sulphated tyrosines on the peptides. Therefore the peak corresponding to the mono-sulphated peptides may represent a peptide with a sulphate group in any of the four possible positions.

In summary, the chemically synthesised D6-N (s) used in these experiments is a mixture of non-sulphated and differentially sulphated peptides, with mono-sulphated peptides appearing to be the most abundant.

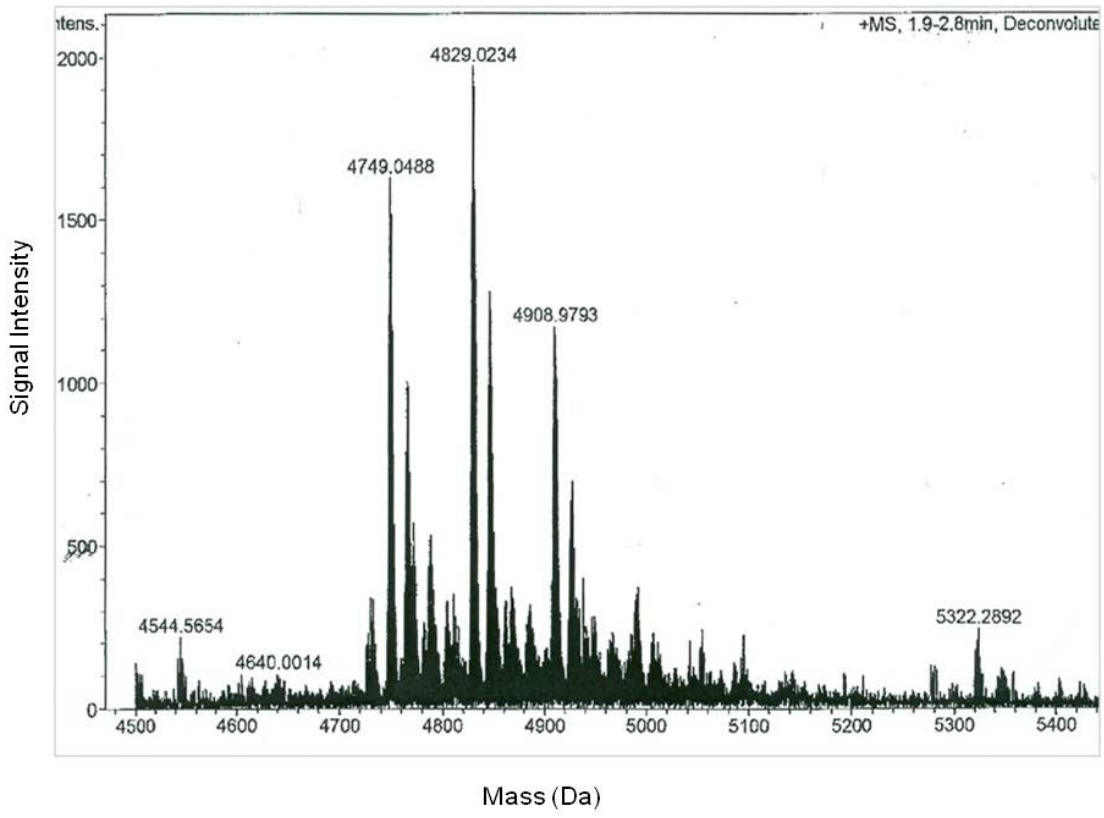


Figure 4-2: D6-N (s) is a mixture of differentially sulphated peptides.
A mass spectrometry plot of D6-N (s) shows it is made up of mostly non-sulphated (4749.05), mono-sulphated (4829.02) and di-sulphated (4908.98) peptides. The Y-axis represents signal intensity, and this is correlated with relative abundance of each peptide species.

4.2.2 Visualisation of D6-N

Being able to visualise both D6-N (s) and D6-N (non-s) was important when exploring the biochemical properties of these novel peptides. We therefore tested available anti-D6 antibodies to see if they could detect the peptides. To this end, peptides were separated by SDS PAGE, and gels were either stained with Coomassie blue dye, or detected by Western Blotting using the anti-D6 (clone 4A5) antibody. The results are shown in **Figure 4-3**.

Coomassie blue staining (**Figure 4-3 (a)**) revealed that both D6-N (s) and D6-N (non-s) form multiple bands on a gradient SDS-PAGE gel. The strongest band for both peptides was at ~8-10kDa (**), although bands at both ~4kDa (***) and ~16kDa (*) were also present. This result suggested that both peptides form dimers and higher order structures as well as existing as monomers when treated with SDS. Interestingly, Western Blotting with the anti-D6 (clone 4A5) antibody revealed that only the sulphated peptide is recognised by this antibody (**Figure 4-3 (b)**). This D6 antibody recognises bands corresponding to D6-N (s), with a strong band at ~8kDa (**), a fainter band at ~16kDa (*) and a very faint band at ~4kDa (***). The 8kDa band is presumably a dimer and the ~16kDa band, a tetramer. Cell lysates from HEK cells transfected with D6 (HEK D6) and un-transfected HEK cells were run alongside samples as a positive and negative control to show the specificity of the anti-D6 (4A5 clone) antibody for the full length D6 protein (49kDa). Comparison of **Figure 4-3 (a)** with **(b)** when 1µg of D6-N (s) was loaded onto the gel shows that Western blotting was as sensitive as Coomassie blue staining for detecting D6-N (s). This suggests that the D6 antibody does not bind D6-N (s) with high affinity.

In summary, D6-N (s) and D6-N (non-s) can be visualised by Coomassie staining revealing that both peptides exist predominantly as dimers at ~8kDa. In addition D6-N (s), but not D6-N (non-s) is detectable by the D6 (clone 4A5) antibody, indicating that this antibody detects a sulphated epitope, and suggesting that the full length D6 protein must be naturally sulphated on its N-terminus.

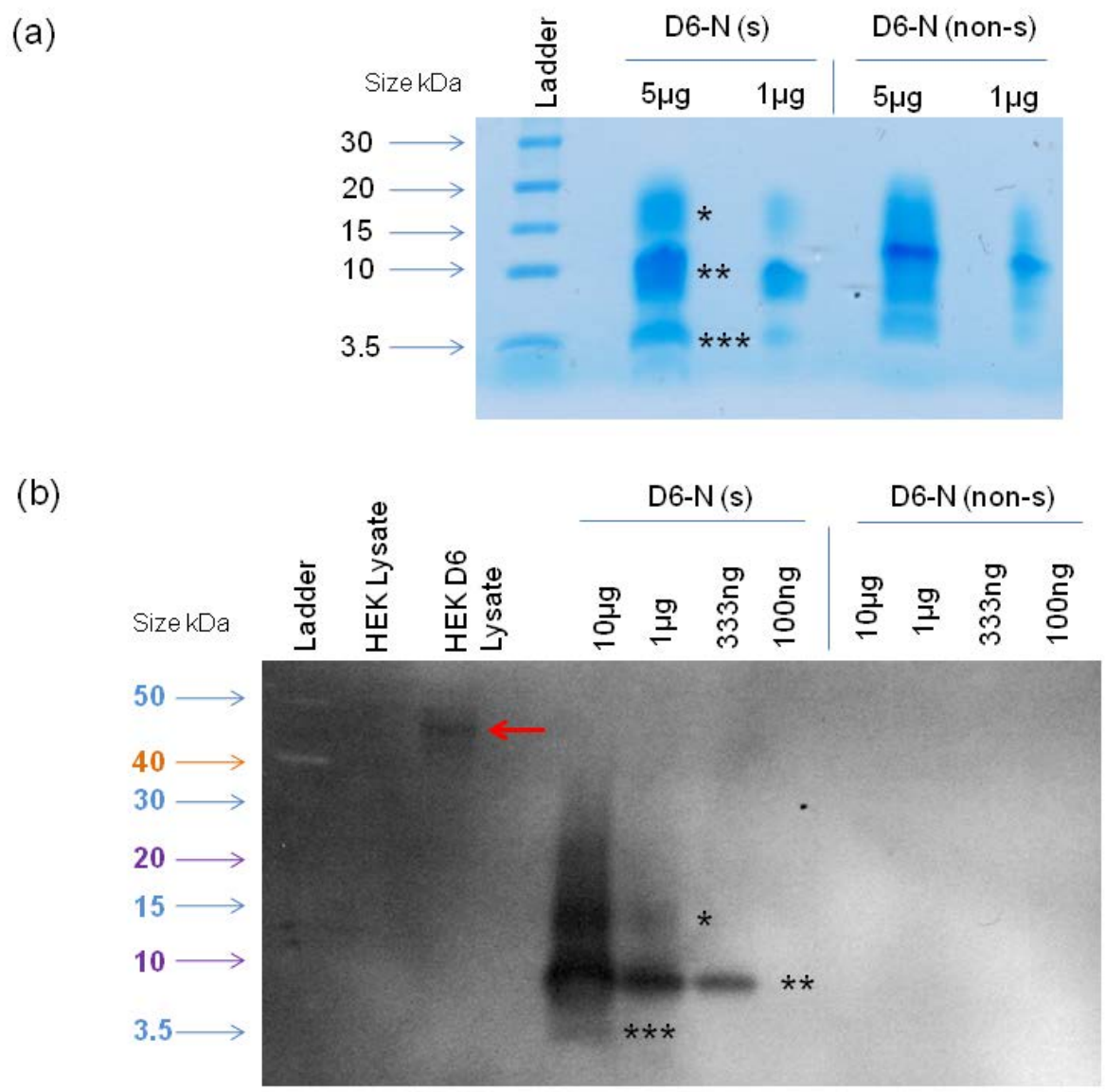


Figure 4-3: Visualisation of D6-N.
(a) D6-N (s) and D6-N (non-s) visualised by Coomassie blue staining. **(b)** Western Blotting the with anti-D6 (clone 4A5) antibody. Bands are marked with asterisks. Cell lysates from HEK 293 cells and HEK cells transfected with D6 (HEK D6) were used as negative and positive controls respectively. The full length D6 protein is highlighted with a red arrow. The blots shown are representative of 3 experiments.

4.2.3 D6-N forms dimers and higher-order aggregates with increasing temperature

It has been shown previously that the full length D6 receptor forms dimers and higher order aggregates when treated with SDS and heated to temperatures exceeding 60°C (Blackburn et al., 2004). The presence of more than one band when D6-N is heated with SDS and run on a gel suggests that D6-N is also prone to multimerisation. To investigate this further, 6 samples of D6-N (s) were mixed with SDS loading buffer and each was incubated at a different temperature for 5 minutes. Samples were then run on a gel and Western Blotted for detection by the anti-D6 (clone 4A5) antibody. The relative density of the bands was calculated using image J software. The data are shown in **Figure 4-4**.

Western Blot analysis, shown in **Figure 4-4 (a)** indicated that the density of the ~16kDa band (*) of D6-N (s) steadily increased as the incubation temperature of D6-N (s) increased, suggesting that the formation of D6-N (s) aggregates occurs, at least partially, as a result of heating. The density of the ~8kDa (***) band also increased slightly when D6-N (s) was heated to high temperatures, suggesting the incidence of D6-N (s) dimer formation increases with increasing temperature. A band of ~12kDa (**) also begins to appear at temperatures exceeding room temperature and increases in density with increasing temperature, suggesting the formation of a trimeric species. Interestingly when D6-N (s) was heated to 95°C then left to cool, the densities of the resulting upper (16kDa) and lower (8kDa) bands resembled those of the sample in which D6-N (s) was incubated at room temperature. This result implies that the heat-induced aggregation of D6-N (s) is reversible. All densitometry analysis is shown in the histogram in **Figure 4-4 (b)**.

This biochemical property of D6-N (s) is important when designing experiments involving the action of this novel peptide because D6-N may behave differently while in the form of a dimer, or higher order aggregate, as opposed to the monomeric form.

In summary, D6-N (s) exists primarily as a dimer at room temperature and forms higher order aggregates when heated. The formation of higher order aggregates is reversible if the temperature is adjusted.

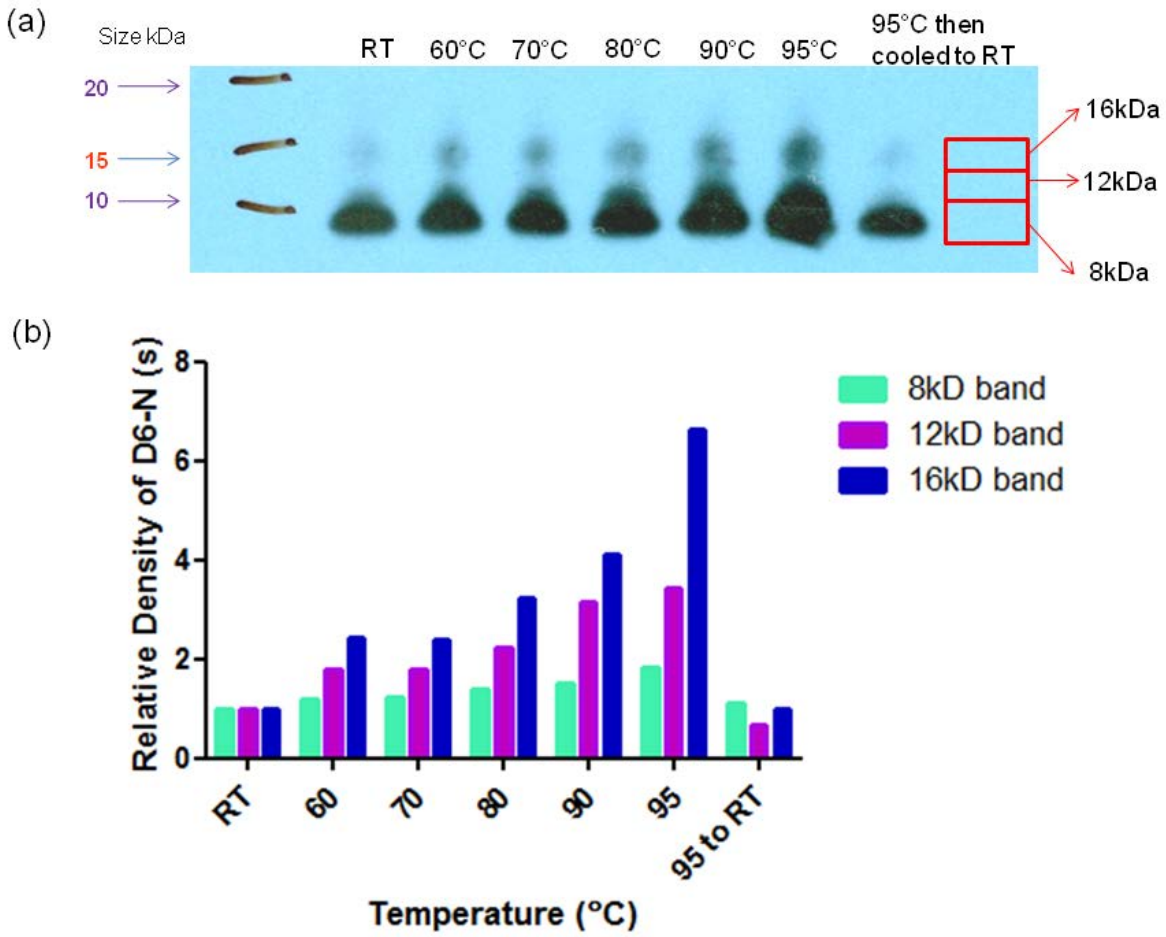


Figure 4-4: D6-N (s) forms dimers and higher order aggregates with increasing temperature. **(a)** Western Blot for D6 using the anti-D6 (clone 4A5) antibody. D6-N (s) was incubated at different temperatures (as indicated) in SDS loading buffer before being run on a gel. Red boxes indicate the boundaries of densitometry measurements **(b)** Relative density plot of bands from blot (a) calculated using image J software. 8kDa, 12kDa and 16kDa bands at RT were set to 1. RT = room temperature, estimated to be ~22-23°C. Representative of 3 experiments.

4.3 D6-N binding to chemokines

A number of different molecular and cellular techniques were utilized to try to establish if either version of the D6-N peptide is capable of binding inflammatory chemokines. The peptide's HIS-tag was pivotal in the design of initial binding experiments because it was thought that this epitope tag would make it easier to track the peptide. HIS-tag epitopes have a high binding affinity for nickel; therefore nickel beads were used in initial experiments. The rationale being that if a chemokine bound to D6-N, the chemokine and the D6-N peptide would remain in complex with nickel beads, resulting in 'pull down' of the chemokine.

4.3.1 D6-N peptide binding experiments utilising the HIS-tag

D6-N (s) and D6-N (non-s) were incubated with CCL2; a high affinity D6 ligand. CCL19 was also used as a negative control as this is not a D6 ligand. Nickel beads were added to the mixture, to bind the poly-HIS tag of the D6-N peptide, before the mixture was centrifuged and supernatant collected. After multiple wash steps, the beads were treated with a concentrated solution of imidazole to elute any bound material. Next supernatant and bead eluate samples were analysed by SDS PAGE with subsequent silver staining or Western Blotting. Results are shown in **Figure 4-5**.

CCL19 was detected in samples by Western blotting using an anti-CCL19 antibody (**Figure 4-5 (a)**). Several attempts to detect CCL2 using two different anti-CCL2 antibodies were unsuccessful; therefore the presence or absence of CCL2 in samples was determined using a silver stain (**Figure 4-5 (b)**). Disappointingly, the results indicated that neither CCL19 nor CCL2 were detectable in supernatants or bead eluates from any of the incubations that were set up. This suggested that the chemokine concentrations used in these experiments were not strong enough to be detected by the techniques utilized. Despite this, silver staining did reveal that D6-N (s) and D6-N (non-s) can be successfully coupled to and eluted from nickel beads, as large amounts of both forms of D6-N were detected in bead eluates, but the peptides were only barely detectable in supernatants (labelled on **Figure 4-5 (b)**).

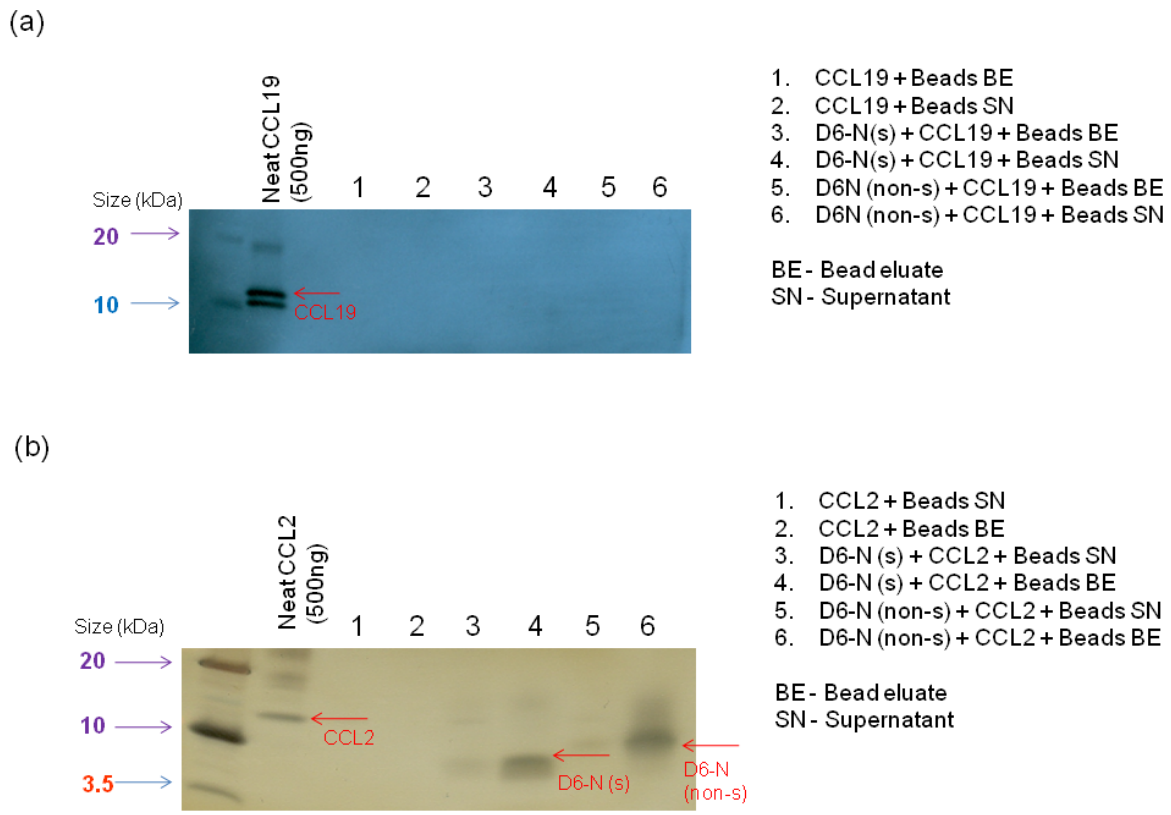


Figure 4-5: CCL19 and CCL2 are not detected in complex with D6-N.
(a) Western Blot using anti-CCL19 on samples (listed) from a nickel bead experiment. CCL19 cannot be detected in supernatants or bead eluates from samples using either D6-N (s) or D6-N (non-s). Neat CCL19 (labelled) was used as a positive control **(b)** Silver stain on samples (listed) from a nickel bead experiment. CCL2 cannot be detected in supernatants or bead eluates from samples using either D6-N (s) or D6-N (non-s). Neat CCL2 (labelled) was used as a positive control. D6-N (s) and D6-N (non-s) are present in bead eluates (labelled). Representative of 3 experiments.

4.3.2 Modification of binding experiments using labelled chemokines

Due to the unsuccessful 'pull down' of chemokines with either form of D6-N, the assay was modified with use of biotinylated chemokines (biotinylated CCL3 and biotinylated CCL22, which are high-affinity D6 ligands, and biotinylated-CCL19), and bead eluates from such incubations were analysed using Western Blots. It was thought that biotinylated chemokines would be more easily detectable on Western Blots, with use of highly sensitive streptavidin-conjugated to HRP, rather than having to use individual antibodies for each of the chemokines. The resultant blot is displayed in **Figure 4-6**.

Figure 4-6 (a) showed that no bands corresponding to biotinylated chemokines were present in any of the bead eluate samples, suggesting that the use of biotinylated ligands did not improve the success of this assay. It was considered that silver staining and Western Blot assays may not be sensitive enough to detect minimal amounts of chemokine in samples. With this in mind, the assay was performed again using considerably higher concentrations of CCL22 (10μg/ml). CCL22 in supernatants and bead eluates was detected using an ELISA for CCL22. The results of the ELISA are shown in **Figure 4-6 (b)** and demonstrate the absence of 'pulled down' CCL22 in these samples.

On consideration, it was realised that these results may be more to do with the experimental process used rather than the binding characteristics of the molecules themselves. For example 5mM imidazole is used while incubating D6-N with chemokine and the nickel beads. This may potentially interfere with the interaction between D6-N and the chemokine, resulting in the chemokine being washed away before the elution step. Bearing in mind the small size of the D6-N peptide (estimated at ~4.75kD); it is also possible that the interaction between D6-N and the nickel bead causes a conformational change of the peptide binding site, thereby preventing it from binding chemokines.

In summary, these results indicate that both versions of D6-N bind to nickel beads but that neither D6-N (s) nor D6-N (non-s) binding of chemokines or biotinylated chemokines was detectable using this assay.

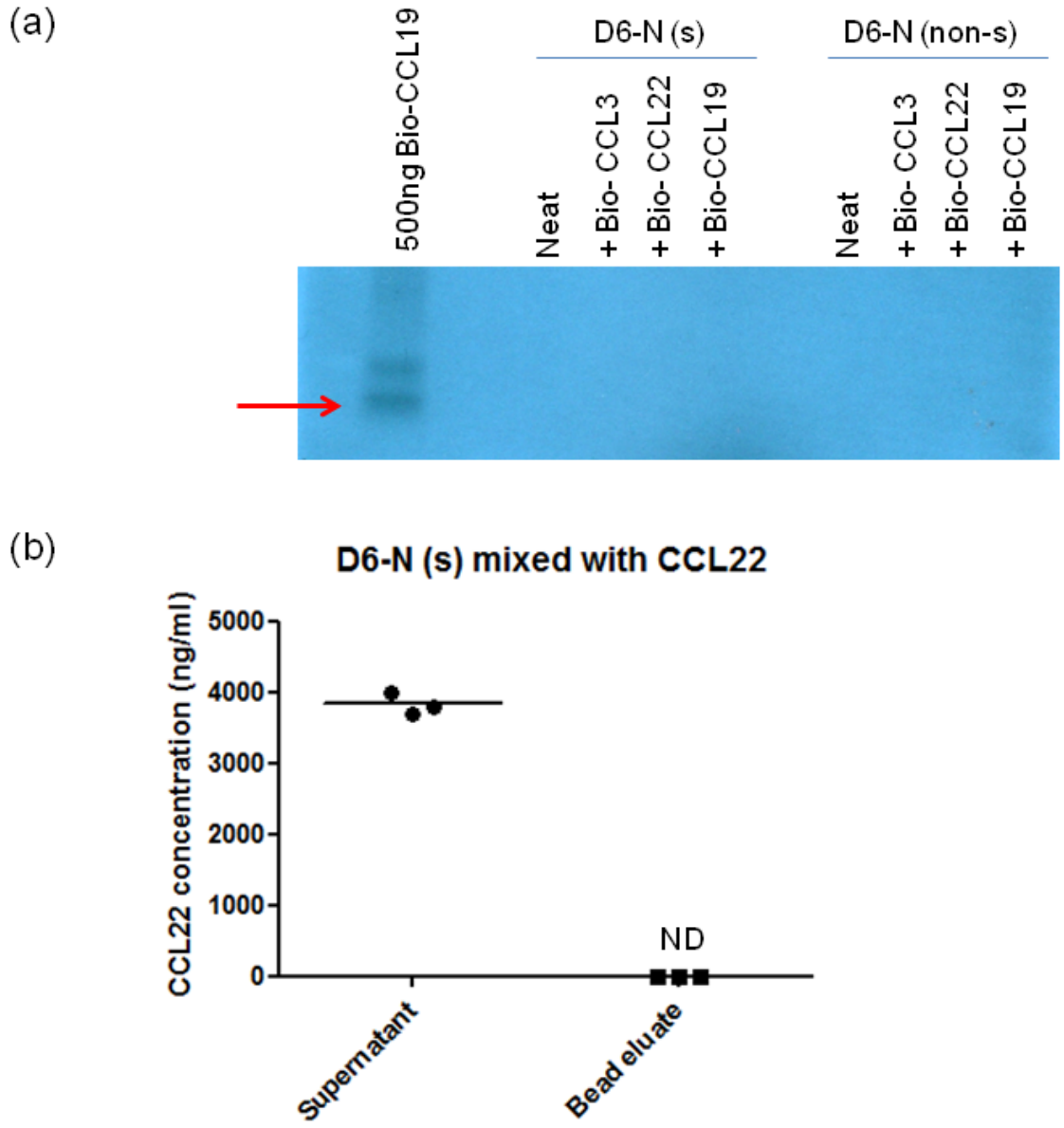


Figure 4-6: Biotinylated chemokines are not 'pulled down' by D6-N attached to nickel beads. **(a)** Western Blot probed with streptavidin-HRP on samples (labelled) from nickel bead experiment with either D6-N (s) or D6-N (non-s) and a panel of biotinylated chemokines. Neat biotinylated CCL19 was used as a positive control and its detection is highlighted by the red arrow. N=1 **(b)** ELISA for CCL22 showing protein levels of CCL22 in both supernatants and bead eluates of samples incubated with D6-N (s). ND = not detected.

4.4 D6-N binding experiments utilising streptavidin beads

Given the failure of the HIS-tag based approach, we next tried a different assay in order to study D6-N's binding properties. As D6-N is a small peptide, its strong attraction to the nickel beads used in previous binding assays may have compromised its ability to bind to chemokines. Therefore it was reasoned that a binding assay in which D6-N is free in solution may be more functionally relevant and allow it to retain its natural conformation.

4.4.1 D6-N (s) binds to CCL22

To test whether keeping D6-N (s) free in solution enhances its ability to bind D6 ligands, an assay was developed that utilised streptavidin beads and biotinylated chemokines. Biotinylated chemokines (bio-CCL2 and bio-CCL22) were incubated with either D6-N (s) or D6-N (non-s) and then streptavidin-linked magnetic beads were added to bind the biotinylated chemokine. The mixture was passed through a magnetic column and washed several times before samples were eluted. The 'target' is eluted first and this sample should contain any molecules that bind to the biotinylated chemokine. The biotinylated chemokine is then eluted by removing the column from the magnet and passing elution buffer through it. The biotinylated chemokine can be thought of as 'bait' in this type of assay. A diagram of the assay is shown in **Figure 4-7**.

After elution, samples were analysed by Western Blotting (**Figure 4-8 (a)**) with antibodies for both D6 (**(i)** and **(ii)**) and streptavidin **(b)** to detect D6-N (s) and biotinylated chemokines respectively. **Figure 4-8 (a)** shows the presence of D6-N (s) in the 'target' sample when D6-N (s) was incubated with bio-CCL22 **(i)** or bio-CCL2 **(ii)**, however D6-N (s) was absent in the 'target' sample on both blots when no chemokine was added. This result suggests that an association exists between D6-N (s) and both the biotinylated chemokines. The Western Blot also again highlights that D6-N (non-s) cannot be detected by the anti-D6 (clone 4A5) antibody (see lane 2 of **Figure 4-8 (a) (i)**), and thus an association between D6-N (non-s) and biotinylated chemokines cannot be determined using this antibody. The streptavidin Western Blot (**Figure 4-8 (b)**) confirms the presence of biotinylated CCL22 on the beads, with most being eluted in the second elution step as expected (described above).

In addition, to ensure that the observed association between D6-N (s) and both biotinylated CCL2 and biotinylated CCL22 was the result of a D6 ligand – D6-N (s) interaction and was not anything to do with the biotin-tag on these chemokines, biotinylated IgG was also used as 'bait'. Subsequent to the binding assay, samples were run on Western Blots (**Figure 4-8 (c)**) using antibodies for D6 **(i)** and streptavidin **(ii)** as outlined previously. **Figure 4-8 (c) (i)** shows that biotinylated IgG had no association with D6-N (s), indicating that D6-N (s) 'pull down' is the consequence of a chemokine specific interaction. **Figure 4-8 (c) (ii)** confirms the presence of biotinylated IgG on the beads, with most being eluted in the second elution step.

In summary, the streptavidin bead binding assay provides evidence of an association between the sulphated D6-N peptide and biotinylated chemokines CCL2 and CCL22. The binding properties of D6-N (non-s) could not be determined because the D6 (clone 4A5) antibody does not detect it. D6-N (s) does not bind biotinylated IgG, suggesting that D6-N (s) preferentially binds to chemokines.

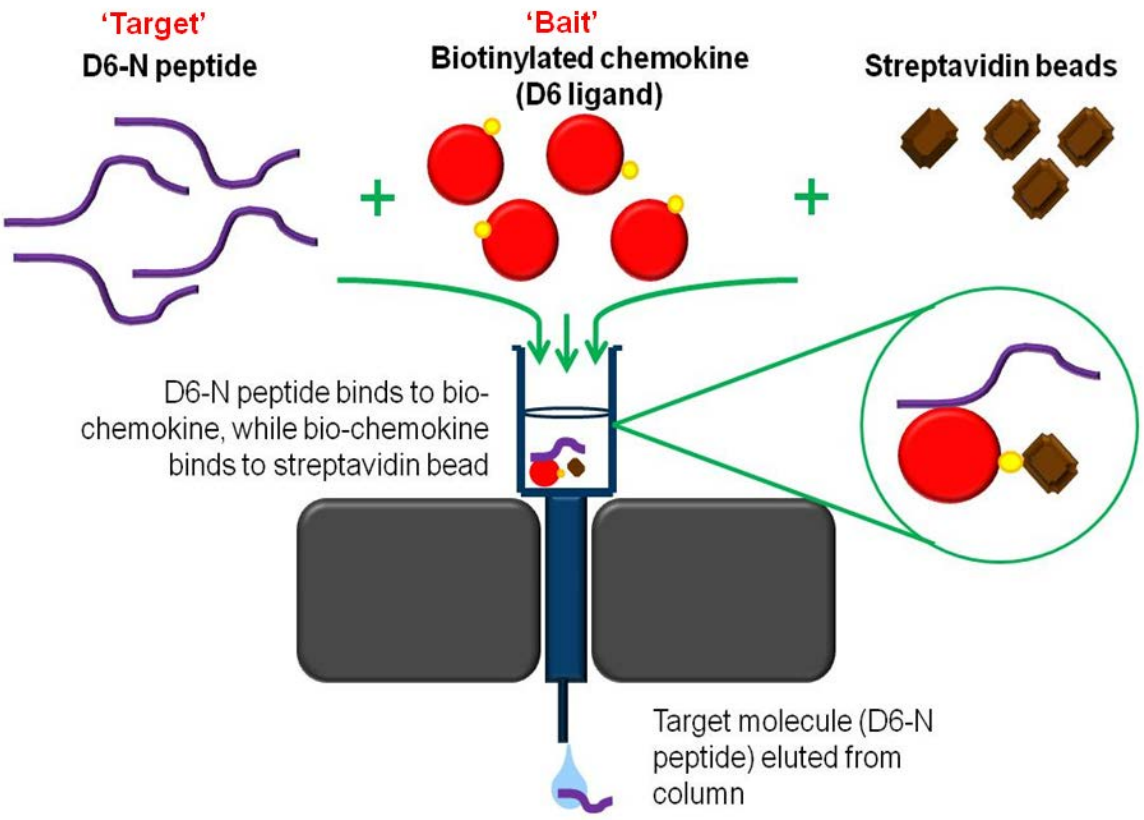
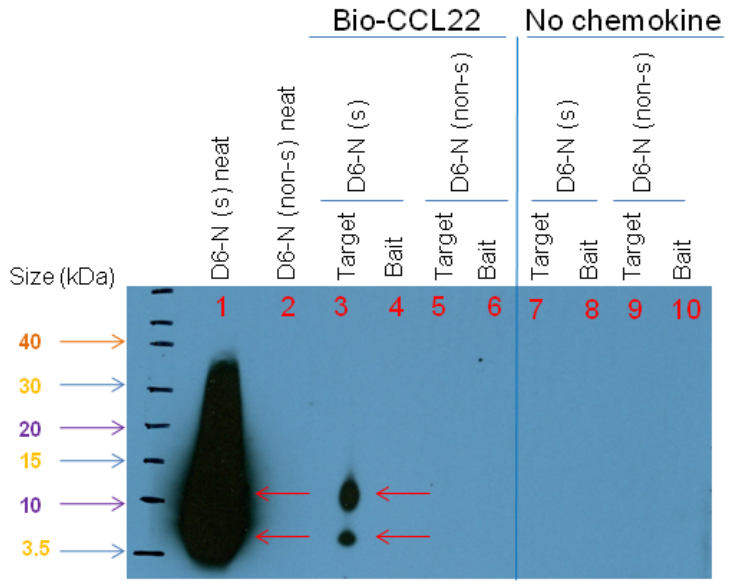
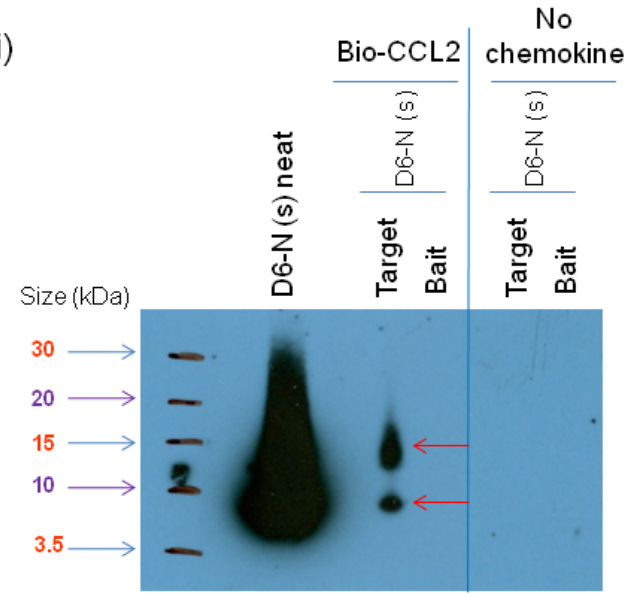


Figure 4-7: Streptavidin bead pull-down assay.
This assay uses biotinylated chemokines as probes to specifically isolate a 'target' molecule – in this case D6-N (s). By magnetic labelling with streptavidin beads, the molecular complex is retained in a column placed in a magnetic field. Washing steps remove non-specifically bound molecules, and afterwards the non-biotinylated 'target' molecules can be eluted with high purity, whereas the magnetically labelled biotinylated probe remains bound to the column. Once the column is removed from the magnetic field, the biotinylated chemokine can be eluted.

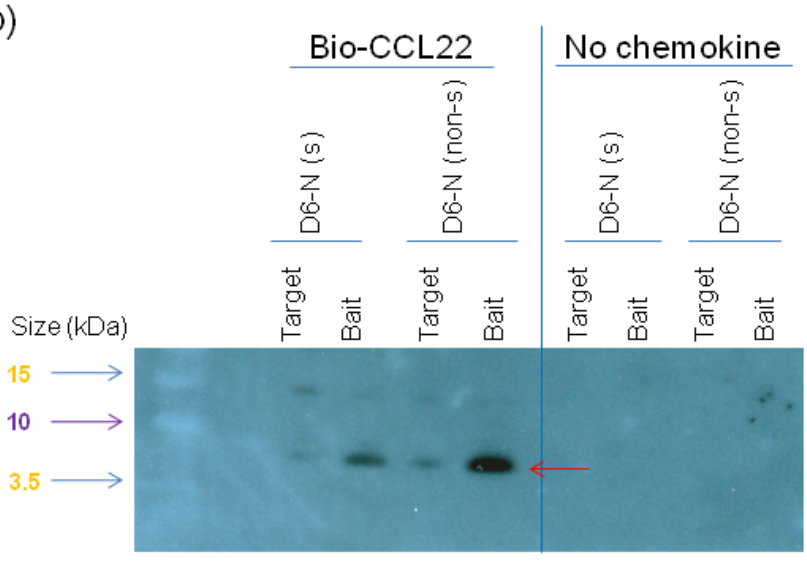
(a) (i)


Bio-CCL22
No chemokine
D6-N (s) neat
D6-N (non-s) neat
D6-N (s)
D6-N (non-s)
Target
Bait
Size (kDa)
1 2 3 4 5 6 7 8 9 10
40
30
20
15
10
3.5

(ii)


Bio-CCL2
No chemokine
D6-N (s) neat
D6-N (s)
Target
Bait
Size (kDa)
30
20
15
10
3.5

(b)


Bio-CCL22
No chemokine
D6-N (s)
D6-N (non-s)
Target
Bait
Size (kDa)
15
10
3.5

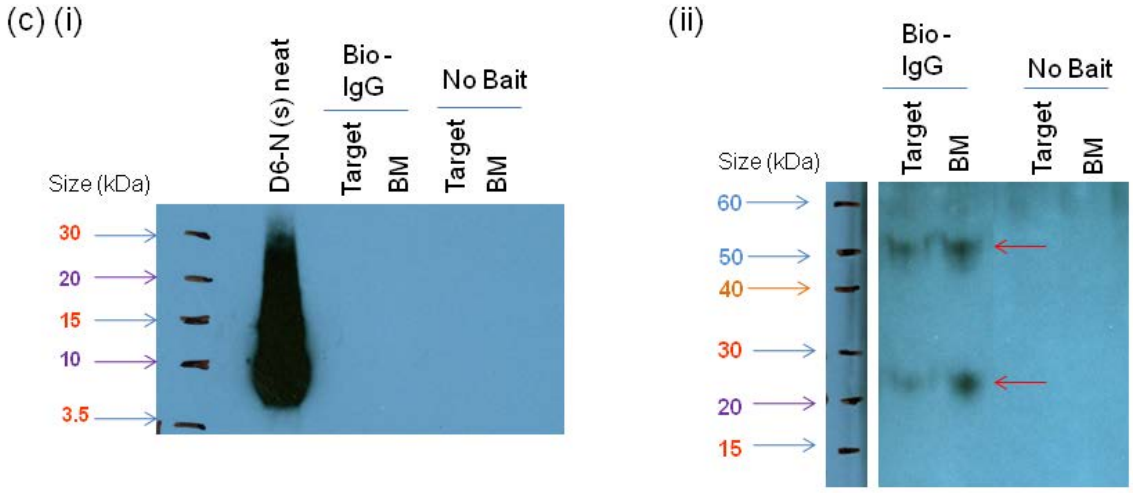


Figure 4-8: D6-N (s) preferentially binds to biotinylated chemokines CCL22 and CCL2. **(a) (i)** and **(ii)** anti-D6 (clone 4A5) Western Blot of samples (labelled) from streptavidin bead binding experiments. D6-N (s) is present in the 'target' sample when bio-CCL22 and bio-CCL2 is incubated with D6-N (s) (red arrows), but is absent when biotinylated chemokine is not incubated with D6-N (s). Neat D6-N (s) and D6-N (non-s) were run as positive controls in **(i)** and neat D6-N (s) was run as a positive control in **(ii)**. **(b)** Western Blot for streptavidin on samples from (a) (i) shows the presence of bio-CCL22 in the samples. **(c) (i)** Western Blot using the anti-D6 (clone 4A5) antibody of samples (labelled) from streptavidin bead binding experiments with bio-IgG. D6-N (s) is not present in either 'target' sample. Neat D6-N (s) was run as a positive control **(ii)** Western Blot using streptavidin HRP on samples (labelled) indicates bio-IgG was present mostly in the second eluate as expected. Bio- IgG is made up of a light chain ~25kDa and a heavy chain ~50kDa, as indicated by red arrows. BM = biotinylated molecule.

4.4.2 D6-N(s) binds CCL2 and CCL22, but not CCL19

In order to fully confirm that the observed 'pull-down' of D6-N (s) was due to a specific interaction with chemokines, and in particular, D6 ligands, the experiment was repeated using unlabelled CCL22 as a competitor for D6-N (s) binding. Unlabelled CCL22 at a 20-fold molar excess was added to a mixture of D6-N (s) and biotinylated chemokine (either CCL2 or CCL19) before the addition of streptavidin microbeads. Biotinylated CCL19 was included in the experiment to assess the ability of D6-N (s) to bind to chemokines which are not D6 ligands. It was hypothesized that most of the D6-N (s) would bind to the large quantities of unlabelled CCL22 in the mixture and this would wash freely through the magnetic column, leaving less D6-N (s) binding to the biotinylated chemokines which are coupled to streptavidin-linked magnetic beads. Therefore it was expected that less D6-N (s) would be eluted in 'target' samples when large quantities of unlabelled CCL22 was also added to the incubations. In samples where only the biotinylated chemokines were used, it was hypothesised that more D6-N (s) would be 'pulled down' by the D6 ligand CCL2 than by the non-D6 ligand CCL19. Western Blots and relative density plots are shown in **Figure 4-9 (a)** and **(b)** respectively.

Two major themes were apparent from the Western Blots on samples from the competition experiment (**Figure 4-9 (a)**). Firstly biotinylated CCL2 'pulls down' more D6-N (s) than biotinylated CCL19 (compare lanes 1 and 3), suggesting that D6-N (s) has a higher affinity for D6 ligands than non-D6 ligands. In effect, the addition of CCL19 is negligible as similar amounts of D6-N (s) were 'pulled down' when no chemokine was added (lane 5, negative control). Secondly, in samples where unlabelled CCL22 was added, instead of competition, the opposite was apparent whereby the more unlabelled CCL22 that was added to the sample, the more D6-N (s) was being 'pulled down'. For example, when unlabelled CCL22 was added at a 20-fold molar excess along with biotinylated CCL19 (lane 4), the amount of D6-N (s) pulled down is almost 18 times the amount that is pulled down by biotinylated CCL19 alone (lane 3), as calculated by the working out the relative densities of the bands using image J software (**Figure 4-9 (b)**).

It was hypothesised that the results observed in lanes 2, 4 and 6 in **Figure 4-9 (a)** were a consequence of adding large quantities of protein to the column, causing such protein to get stuck and retain D6-N (s) in the column until the elution steps. In order to test this, control experiments were set up, including using anti-HA beads to test if chemokines can

bind to any type of bead, and also using large amounts of BSA instead of unlabelled CCL22 to find out if D6-N (s) is being trapped in the column through a non-specific interaction with excess amounts of protein instead of a specific interaction between D6-N (s) and CCL22. The Western Blot on control samples is shown in **Figure 4-9 (c)**.

Figure 4-9 (c) revealed that unlabelled CCL2 (lane 2) and CCL22 (lane 4) added at 0.2µg (the same quantity of biotinylated CCL2 and biotinylated CCL22 added in previous experiments) pulled down similarly low levels of D6-N (s) as the negative control (where D6-N (s) was incubated on its own). This suggested that un-tagged CCL2 and CCL22 are not binding to the streptavidin beads. As before, when 0.2µg of bio-CCL19 was used as 'bait', the amount of D6-N (s) pulled down was similar to the negative control (lane 3). This substantiated the result observed in **Figure 4-9 (a)** (lane 3), and suggests that D6-N (s) has a much higher affinity for the inflammatory chemokines CCL2 and CCL22, than for CCL19. Adding large amounts of BSA (lane 5) pulled down similar amounts of D6-N (s) as the negative control (lane 1). This is in contrast to lane 5 of **Figure 4-8 (a)**, whereby large amounts of unlabelled CCL22 'pulled down' almost 30 times as much D6-N (s) as the negative control. This observation suggests that adding large quantities of protein to the column will result in increased non-specific binding of D6-N (s) to the beads, however D6-N (s) will only remain in the column after washes if there is a specific interaction going on (i.e. between D6-N (s) and CCL22). This result may also be due to the natural electrostaticity of CCL22 and its tendency to form large oligomers, a characteristic that has been observed in radio-ligand binding studies (Viney et al., 2014). Additionally, anti-HA beads 'pulled down' similar levels of D6-N (s) to the negative control ruling out the possibility that there was a problem with non-specificity of the streptavidin beads.

In summary, D6-N (s) binds the inflammatory CC chemokines CCL2 and CCL22 with high affinity, but does not bind to the non-D6 ligand, CCL19, therefore confirming specificity of D6-N for inflammatory CC chemokines. The amount of D6-N (s) 'pulled down' in the binding assay is dependent on the amount of D6 ligand incubated with D6-N (s).

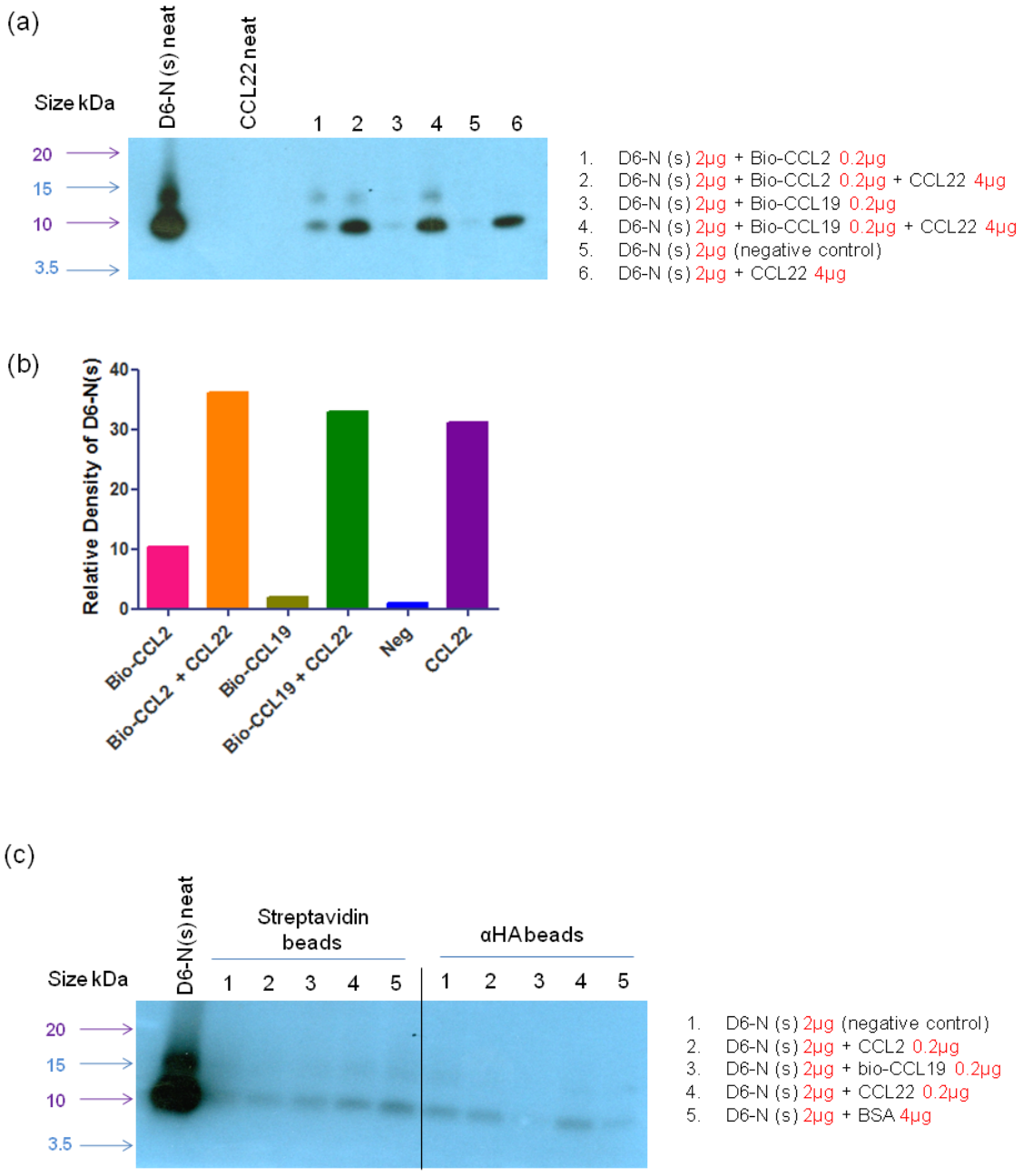


Figure 4-9: D6-N (s) binds to the inflammatory CC chemokines, CCL2 and CCL22 but not the non-D6 ligand CCL19.
(a) anti-D6 (clone 4A5) Western Blot of samples (labelled) from streptavidin bead binding experiments with different mixtures of unlabelled and biotinylated chemokines. D6-N (s) is present in bead eluates when either CCL2 or CCL22 is included, but is absent otherwise. **(b)** Bar chart showing relative densities of D6-N (s) in each lane. Relative densities were calculated using image J software and lane 5 was normalised to 1. **(c)** anti-D6 (clone 4A5) Western Blot of samples (labelled) from control experiments using either streptavidin beads or anti-HA beads. D6-N (s) is not retained in the column by unlabelled CCL2 or CCL22 (lanes 2 and 4), biotinylated CCL19 (lane 3) or large quantities of BSA (lane 5). Streptavidin beads do not non-specifically bind D6-N (s) or unlabelled chemokines. Neat D6-N (s) (200ng) was run as a positive control on both (a) and (c). Neat CCL22 (4µg) was run on (a) to ensure that the anti-D6 (4A5) antibody does not bind non-specifically to chemokines.

4.4.3 D6-N (s) binds with high affinity to CCL2, as determined by Biacore

Surface plasmon resonance (SPR) using Biacore technology can be used for the assessment of interactions between proteins and their ligands. Experiments typically involve immobilizing one molecule (referred to as the ligand) of a binding pair onto the surface of a sensor chip and passing its partner (referred to as the analyte) across the surface of the chip. Changes in the refractive index at the surface where the binding interaction occurs are detected by the hardware and recorded as resonance units (RU).

In order to directly measure the binding affinity between both versions of the D6-N peptide and CCL2, Biacore technology was used. This was achieved by coating the sensor surface of a Biacore chip with neutravidin - a deglycosylated version of streptavidin, and passing biotinylated CCL2 over it to immobilise the CCL2 in the correct orientation for binding. D6-N (s) and D6-N (non-s) were then passed over the CCL2-coated chip at different concentrations, and binding affinity was measured in real time, as response units vs. time on a sensorgram. **Figure 4-10** shows the data resulting from this experiment.

The sensorgram in **Figure 4-10** depicted a large peak of ~900 RU when D6-N (s) was injected at a concentration of 1 mg/ml. A change of 900 RU is indicative of a very strong attraction between D6-N (s) and CCL2, however, there was no evidence of a threshold limit to function, as D6-N (s) injected at a lower concentration of 0.1 mg/ml elicited no response. This was unanticipated, as a peak one tenth of the size of the peak observed when D6-N (s) was injected at 1 mg/ml would be expected. Regeneration of the chip was performed, whereby all analyte is removed from the chip, followed by injection of D6-N (non-s) at 1 mg/ml. The sensorgram showed no detectable response in RU when D6-N (non-s) was injected at 1 mg/ml, suggesting that D6-N (non-s) does not have a high attraction to CCL2.

Although the data from the Biacore experiment looked promising, the peak generated did not resemble peaks which are typically generated from high-affinity protein-protein interactions. The experiment was not repeated because of uncertainties regarding the sensitivity of the Biacore instrument in measuring the binding affinity between small proteins. In addition, indications that both forms of the D6-N peptide exist naturally as dimers and higher order aggregations (detailed in previous sections in this chapter)

discouraged any further use of Biacore to analyse the binding affinity between the D6-N peptide and chemokines, as dimerization and/or aggregation of proteins is likely to cause problems with Biacore analyses.

In summary, the use of Biacore technology to examine the binding affinity between D6-N and CCL2 further suggested that a strong interaction exists between D6-N (s) and CCL2; however these data cannot fully be relied upon due to complexities of the biochemistry of D6-N.

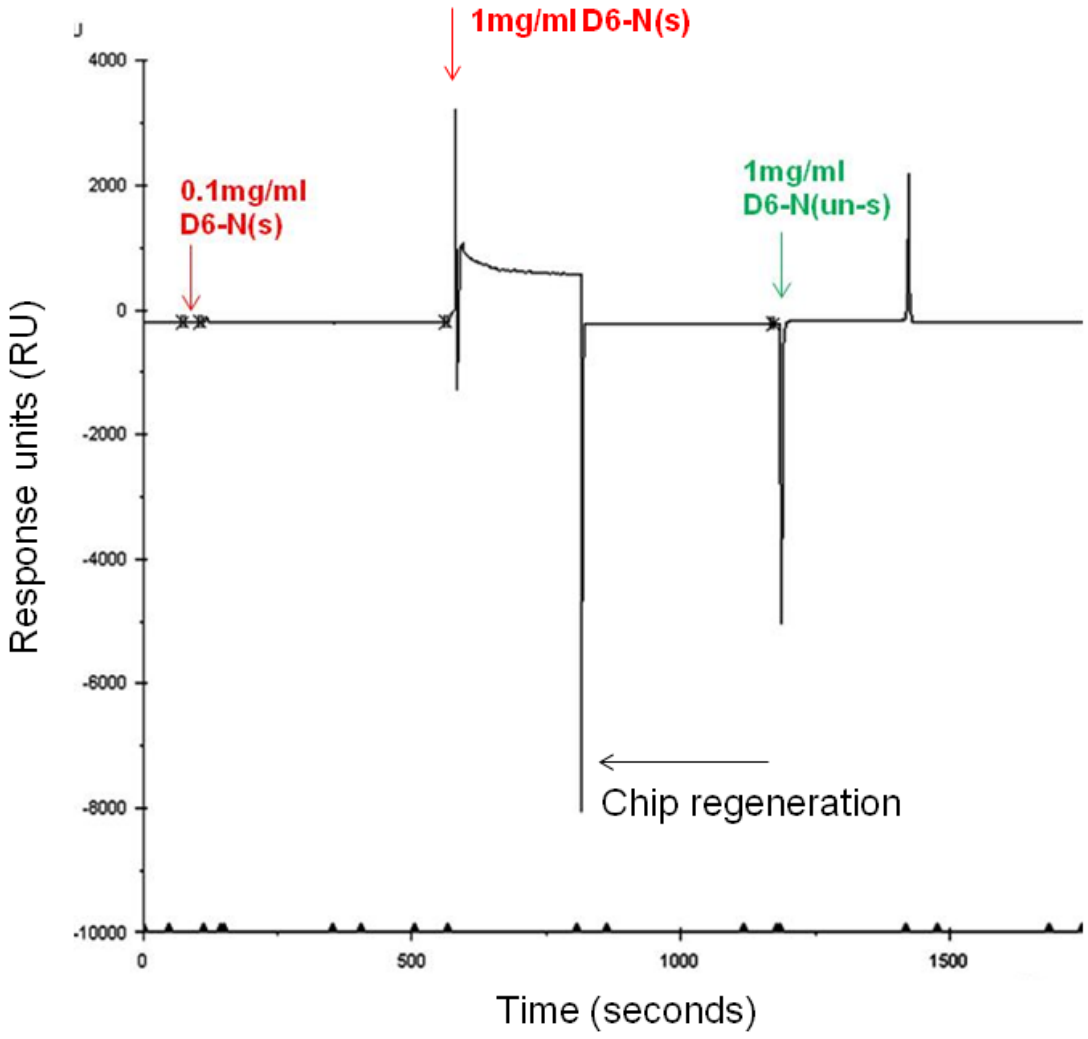


Figure 4-10: Sensorgram displaying results from Biacore experiment.
Times of injection for D6-N (s) at both 0.1 mg/ml and 1 mg/ml are indicated in red, and injection of D6-N (non-s) is indicated in green. Injection of D6-N (s) at 1 mg/ml causes a net increase of approximately 900 RU, whereas injection of D6-N (s) at 0.1 mg/ml or D6-N (non-s) at 1 mg/ml does not induce any change in RU. N=1.

4.5 Addition of D6-N to cells blocks interaction of CCL2 with cognate receptors

With evidence to suggest that D6-N (s) is able to bind to the inflammatory chemokines CCL2 and CCL22, it was important to examine D6-N's functional effects. To investigate this, chemokine uptake assays were performed using different cell lines. This involved addition of AF-CCL2 to cells and measurement of AF-CCL2 uptake by flow cytometry. Full details of the chemokine uptake assays can be found in Chapter 2, **section 2.5.2**. The gating strategy used for this experiment and similar flow cytometry experiments is shown in **Figure 4-11**. The plot shown is representative of a negative control, where AF-CCL2 was not added to cells.

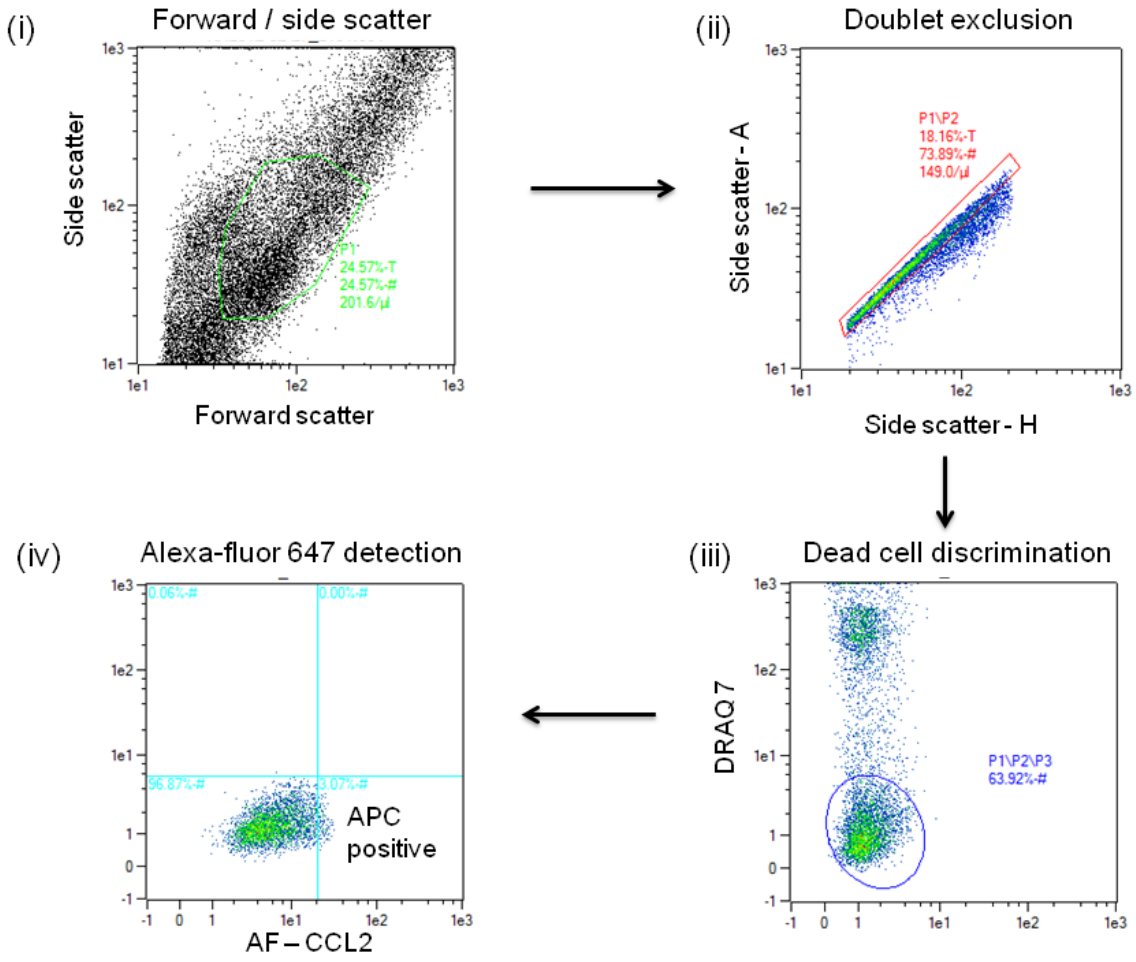


Figure 4-11: Gating strategy for chemokine uptake assays.
(i) Forward and side scatter was used to gate the main population of cells. **(ii)** Cell doublets were excluded, by gating on only single cells. **(iii)** Dead cells were excluded using DRAQ7, a fluorescent chemical which binds to DNA but only stains the nuclei of dead cells **(iv)** Cells were analysed on the APC channel to detect alexa-fluor-647 fluorescence, as a measure of chemokine uptake.

4.5.1 D6-N (s), but not D6-N (non-s) inhibits AF-CCL2 uptake by D6 expressed on HEK D6 transfected cells

To explore the effect of D6-N on chemokine uptake by the full length D6 receptor, both D6-N (s) and D6-N (non-s) were added along with AF-CCL2 to HEK 293 cells transfected with D6 (HEK D6), (see Chapter 2, section 2.4 for details of the transfection process). AF-CCL2 and either D6-N (s) or D6-N (non-s) were added, either directly to cells, or after pre-complexing by incubating peptides and chemokine together at room temperature for 30mins before addition to cells. Cells were incubated at 37°C for 60 minutes before being washed and analysed by flow cytometry. **Figure 4-12** summarises the results. **Figure 4-12 (a)** shows representative flow cytometry profiles from cells only **(i),** with the addition of AF-CCL2 **(ii)** and with the addition of AF-CCL2 and D6-N (s) **(iii)**. The MFIs of cells from each assay were plotted on a dot plot shown in **Figure 4-12 (b)**.

Chemokine uptake assays indicated that when D6-N (s) was added to HEK D6 cells along with AF-CCL2 (**Figure 4-12 (a) (iii)**), the uptake of AF-CCL2 by D6 was significantly decreased as compared to cells that were given AF-CCL2 only. Conversely D6-N (non-s) had no significant effect on uptake of AF-CCL2 by HEK D6 cells (**Figure 4-12 (b)**). This result suggested that sulphation of the D6 peptide is necessary for binding to CCL2 and provides evidence that free D6-N (s) can bind cognate chemokine in solution and inhibit binding to full-length D6. The results also showed that pre-complexing AF-CCL2 with either D6-N (s) or D6-N (non-s) prior to their addition to cells had no significant effect on the inhibition of AF-CCL2 uptake by HEK D6 cells.

In summary, D6-N (s), but not D6-N (non-s), can bind CCL2 in vitro and this binding prevents subsequent chemokine uptake by the full length D6 receptor expressed on the surface of transfected HEK cells.

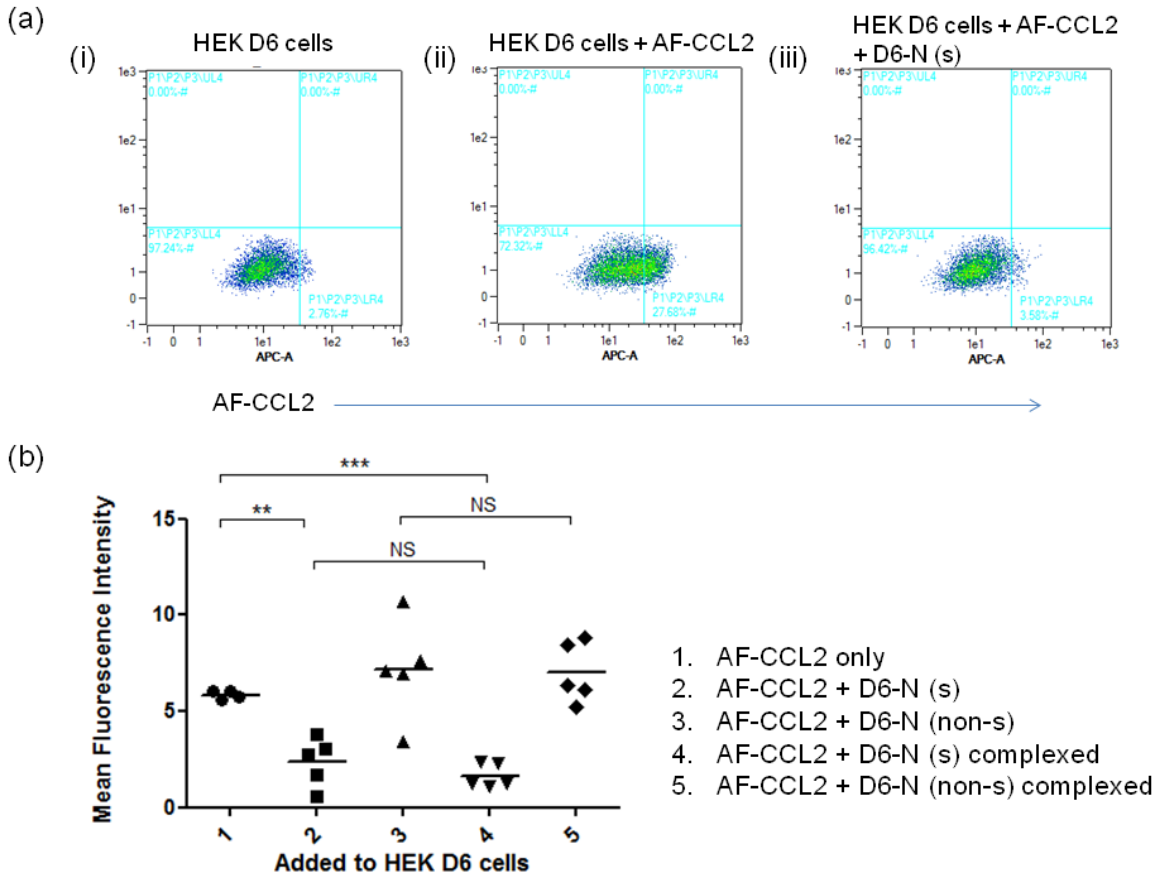


Figure 4-12: D6-N (s) prevents uptake of AF-CCL2 by HEK D6 cells.
(a) Representative flow cytometry plots from chemokine uptake assay displaying AF-CCL2 uptake by HEK D6 cells with **(i)** PBS added, **(ii)** AF-CCL2 added and **(iii)** AF-CCL2 + D6-N (s) added. AF-CCL2 uptake is seen as an increase in MFI. **(b)** Dot plot of MFIs from chemokine binding/uptake assay. Samples in which incubation of D6-N and AF-CCL2 took place before addition to cells are labelled as 'complexed'. N=5 per sample. Data were analysed by unpaired, two tailed student T tests. ***p<0.0001, **p=0.001, NS= no significant difference.

4.5.2 D6-N inhibits AF-CCL2 uptake by CCR2 expressed on THP1 cells

Given that D6-N (s) is capable of binding to inflammatory CC chemokines and inhibiting uptake of AF-CCL2 by D6, we next examined whether this effect could be repeated using cells which naturally express a classical inflammatory chemokine receptor. THP1 cells, which naturally express CCR2, were used in these experiments.

Similar experimental procedures were followed as before with the addition of AF-CCL2 and either D6-N (s) or D6-N (non-s) to THP1 cells. As a separate control, AF-CCL2 was added to the cells along with unlabelled CCL2 at a 20-fold molar excess. This control was included to demonstrate reduction of uptake of AF-CCL2 in the presence of a competitor. All cell samples were incubated for 60 minutes at 37°C, before being washed and analysed by flow cytometry to assess AF-CCL2 uptake. **Figure 4-13** summarises the results. **Figure 4-13 (a)** shows representative flow cytometry profiles from each condition tested. The MFIs of cells from each assay were plotted as shown in **Figure 4-13 (b)**.

These assays indicated that the addition of either D6-N (s) or D6-N (non-s) along with AF-CCL2 to THP1 cells significantly decreased their uptake of AF-CCL2 (**Figure 4-13 (a) (iv)** and **(v)**). Addition of D6-N (s) to cells decreased their MFI to a greater extent than addition of D6-N (non-s) (**Figure 4-13 (b)**); however the result with D6-N (non-s) refines earlier findings suggesting that the addition of D6-N (non-s) had no effect on uptake of chemokine by HEK D6 cells.

In summary, addition of either version of D6-N along with AF-CCL2 reduced the uptake of AF-CCL2 by CCR2 expressing THP1 cells, although D6-N (s) was more effective. This suggests that D6-N could potentially be useful as an inhibitor of inflammatory chemokine receptor interactions.

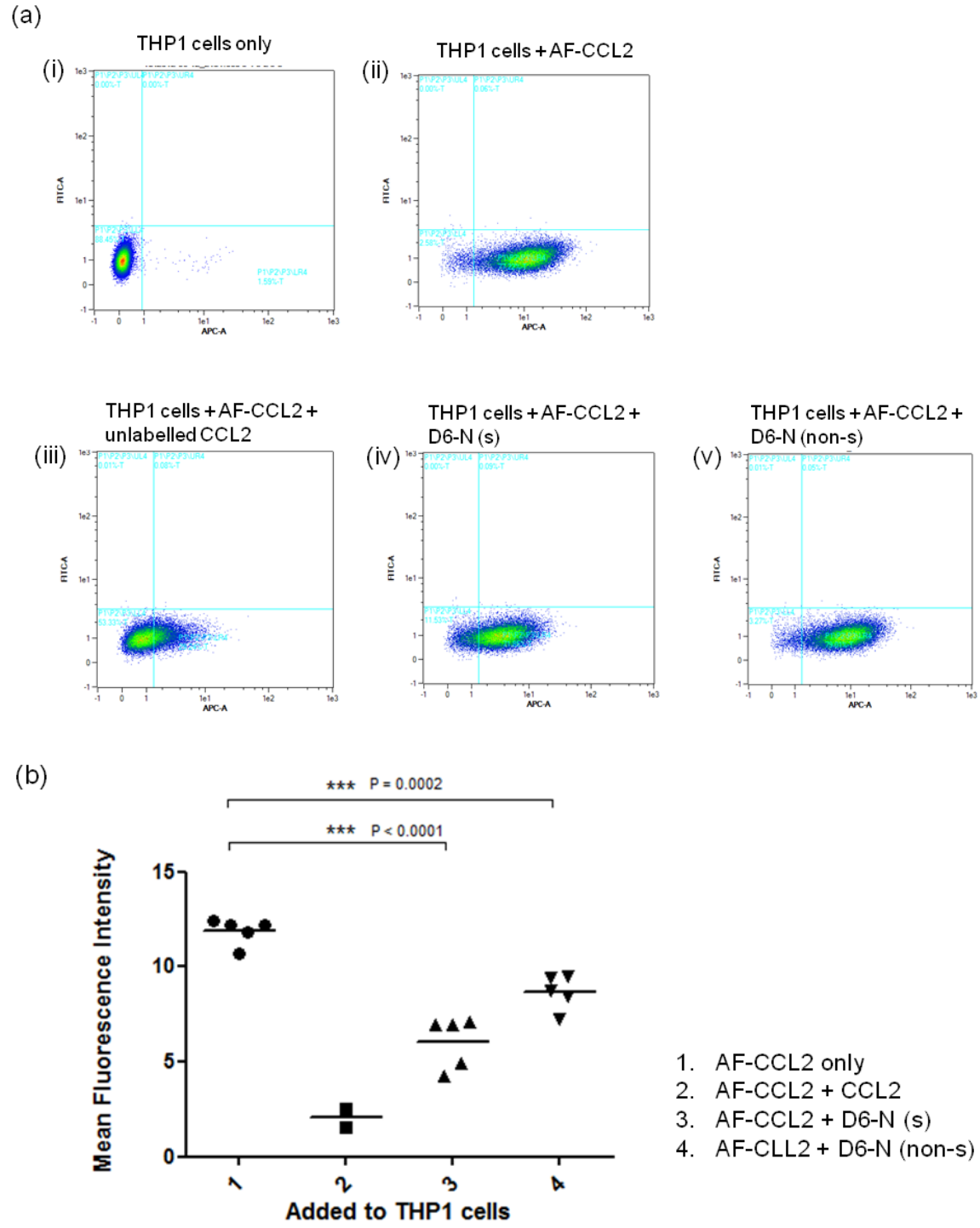


Figure 4-13: D6-N prevents uptake of AF-CCL2 by CCR2 expressed on THP1 cells.
(a) Representative flow cytometry profiles showing AF-CCL2 uptake by THP1 cells with **(i)** PBS added, **(ii)** AF-CCL2 added, **(iii)** AF-CCL2 + unlabelled CCL2 (20 fold molar excess) added, **(iv)** AF-CCL2 + D6-N (s) added, **(v)** AF-CCL2 + D6-N (non-s) added. AF-CCL2 uptake is seen as a 'right-shift' in flow cytometry profiles. **(b)** Dot plot of MFIs from chemokine binding/uptake assay. N=5 per sample, except in case (iii) (lane 2) where N=2. Data were analysed by unpaired, two tailed student T tests.

4.5.3 D6-N inhibits AF-CCL2 uptake of chemokines in a dose-dependent manner

Having established that D6-N (s) is capable of inhibiting the uptake of AF-CCL2 by both HEK D6 and THP1 cells, we next set out to determine the dose response relationship between the amount of D6-N (s) added to cells and the decrease in uptake of AF-CCL2. To this end, assays were performed with increasing concentrations of D6-N (s). D6-N (non-s) was also re-tested for its ability to decrease uptake of AF-CCL2. **Figure 4-14** shows the resulting data.

In this assay, the addition of AF-CCL2 to the cells did not induce the same level of uptake as observed previously. This is most likely due to the disintegration of the fluorophore on CCL2, which can happen as a result of repeated freeze-thaw cycles. In spite of this, the addition of D6-N (s) still resulted in a decrease in AF-CCL2 uptake, as shown in the flow cytometry profiles (**Figure 4-14 (a) (i)** and **(ii)**). This effect was dose dependent, with reduction in uptake gradually increasing as the quantity of D6-N (s) increased (**Figure 4-14 (a) (iii)**) and the most significant difference in binding/uptake occurring when the highest dose of D6-N (s) was added (**Figure 4-14 (b)**). D6-N (non-s) did not have any significant effect on AF-CCL2 uptake in this experiment, but this may be because AF-CCL2 was not as potent in this experiment, rendering the subtle reduction in uptake induced by D6-N (non-s) (observed in Figure 4-13) undetectable in this instance.

In summary, AF-CCL2 uptake by THP1 cells is reduced with the addition of D6-N (s), and this reduction in uptake increases as the amount of D6-N (s) added to the cells increases. In addition, use of D6-N (s) results in a greater reduction in uptake than use of D6-N (non-s), suggesting that D6-N (s) binds to CCL2 with higher affinity than D6-N (non-s).

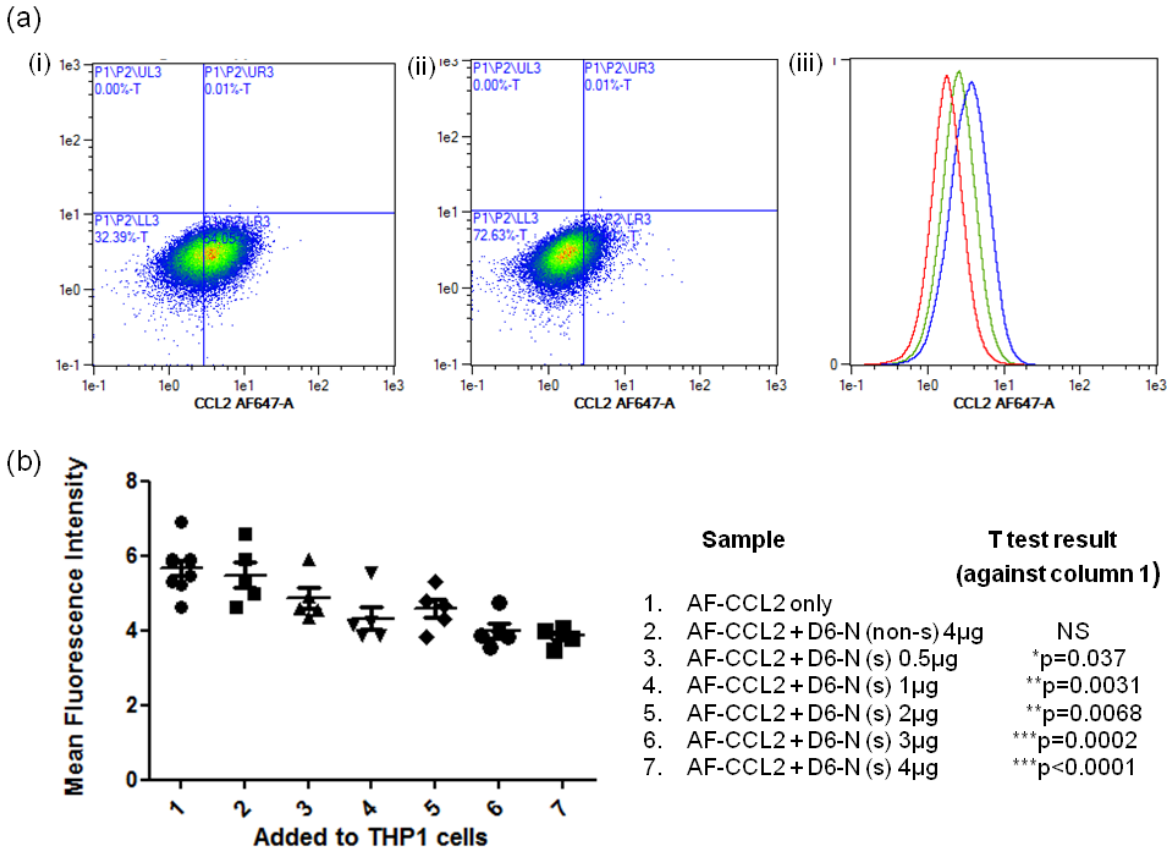


Figure 4-14: D6-N (s) decreases the uptake of AF-CCL2 by THP1 cells in a dose dependent manner, while D6-N (non-s) has no effect.
(a) Representative flow cytometry profiles showing AF-CCL2 uptake by THP1 cells with **(i)** AF-CCL2 + 0.5µg D6-N (s) and **(ii)** AF-CCL2 + 4µg D6-N (s). **(iii)** histogram of data from uptake assays using different quantities of D6-N (s) displaying a gradual decrease in uptake of AF-CCL2, blue line = AF-CCL2 + 0.5µg D6-N (s), green line = AF-CCL2 + 2µg D6-N (s), red line = AF-CCL2 + 4µg D6-N (s). **(b)** Dot plot of MFIs from chemokine binding/uptake assays. Samples are listed along with t test results. N=5 or more per sample. Data were analysed by unpaired, two tailed student T tests.

4.6 The pattern and degree of sulphation of D6-N (s) is crucial to its binding to inflammatory chemokines

With evidence indicating that D6-N (s) is able to significantly reduce chemokine uptake by cells *in vitro*, preparations were made to start experimentation *in vivo.* A new batch of D6-N (s) peptide was ordered as stocks of the existing D6-N (s) peptide had run out. The new batch of D6-N (s) will be referred to as D6-N (s) NEW throughout this thesis.

4.6.1 D6-N (s) and D6-N (s) NEW contain different sulphation patterns

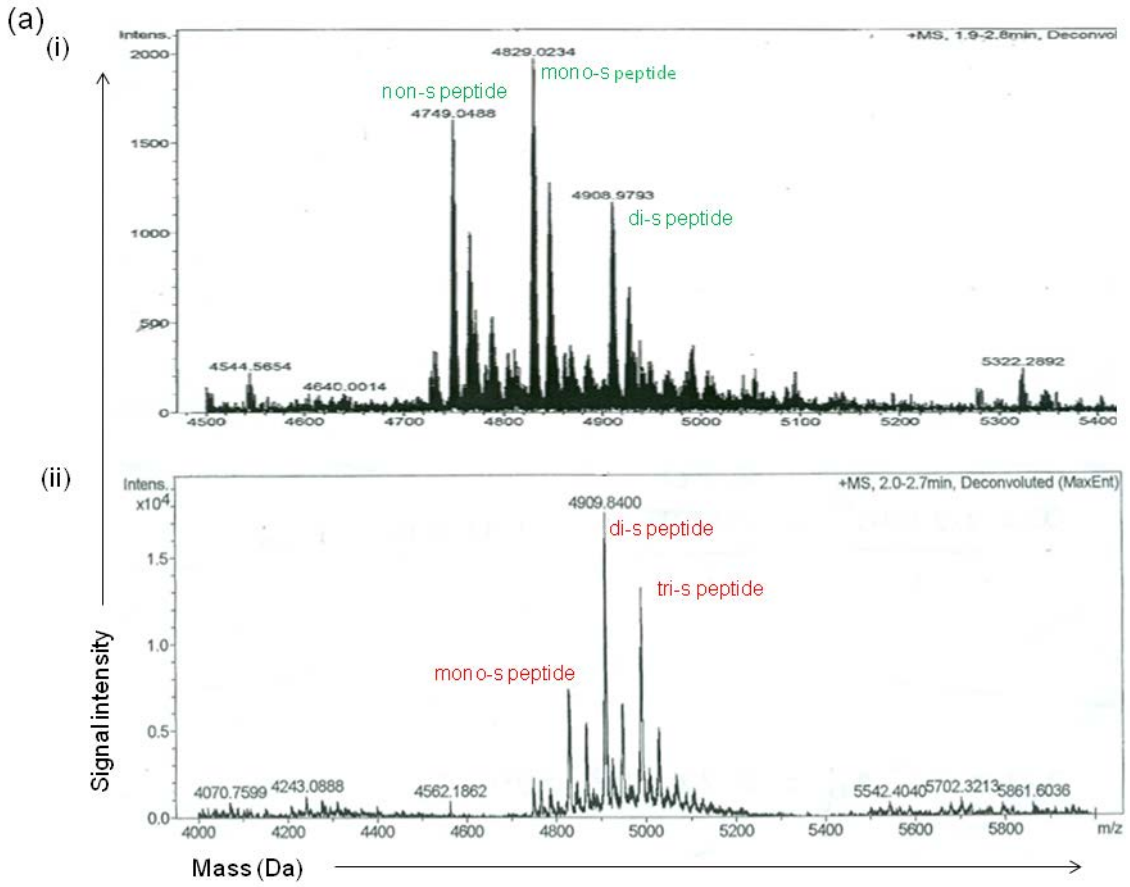
Mass spectrometry analysis of D6-N (s) **(i)** is shown in comparison with D6-N (s) NEW **(ii)** in **Figure 4-15 (a)**. In order to visualise D6-N (s) NEW and compare it to D6-N (s), a Western Blot for D6 was performed following SDS PAGE of both peptides. A cell lysate from HEK D6 cells was used as a positive control. The blot is shown in **Figure 4-15 (b)**.

Both mass spectrometry and Western Blot data indicated that D6-N (s) and D6-N (s) NEW were different, both in composition and abundance of sulphated peptides and in general size and biochemistry. As detailed previously, D6-N (s) was thought to be made up of predominantly mono-sulphated peptides and formed a dimer of ~8-10kDa apparent on SDS PAGE. Conversely mass spectrometry data of D6-N (s) NEW (**Figure 4-15 (a) (ii)**) indicated that it is a mixture of mono-, di- and tri-sulphated peptides, with the signal intensity suggesting that the di-sulphated peptide is most abundant. Western Blot analysis of D6-N (s) NEW (**Figure 4-15 (b)**) showed that, like D6-N (s), it was recognised by the anti-D6 (clone 4A5) antibody, however, the antibody did not recognise bands at ~8-10kDa and ~16kDa, as observed with D6-N (s). Alternatively D6-N (s) NEW seemed to migrate as two bands, one at ~13kDa and one at ~14kDa (indicated by red arrows).

The differences between D6-N (s) and D6-N (s) NEW were not entirely unexpected because of the unpredictability of the chemical sulphation process after peptide synthesis. It is difficult to determine the exact ratios of the differentially sulphated species of peptide

within the two batches, and hence almost impossible to reproduce precisely the make up from one batch to the next.

In summary, D6-N (s) and D6-N (s) NEW represent differentially sulphated peptide preparations. Mass spectrometry results suggested that mono-sulphated peptides were most abundant in D6-N (s), whereas di-sulphated peptides were most abundant in D6-N (s) NEW. The positioning of sulphated peptides in each could not be determined. D6-N (s) NEW is slightly larger in size than D6-N (s) when run on an SDS PAGE gel. Both peptides are recognised by the anti-D6 (clone 4A5) antibody.



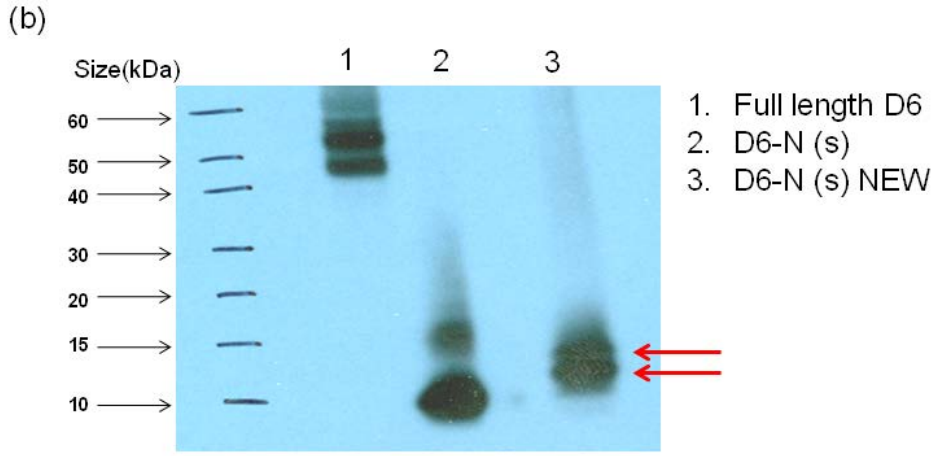


Figure 4-15: Different batches of D6-N (s) contain differently sulphated peptide species. **(a)** Mass spectrometry profiles of **(i)** D6-N (s) and **(ii)** D6-N (s) NEW show that D6-N (s) contains a mixture of non-sulphated, mono-sulphated and di-sulphated peptides (indicated in green), whereas D6-N (s) NEW contains a mixture of mostly di-sulphated and tri-sulphated peptides (indicated in red). **(b)** D6-N (s) and D6-N (s) NEW differ in their size and composition under SDS PAGE conditions, as determined by Western Blotting using the anti-D6 (clone 4A5) antibody.

4.6.2 D6-N (s) NEW does not bind to the inflammatory CC chemokines CCL2 or CCL22

Having established that D6-N (s) and D6-N (s) NEW have different biochemical properties, it was necessary to investigate D6-N (s) NEW further to determine its ability to bind inflammatory chemokines. Similar assays were undertaken as with D6-N (s) to investigate D6-N (s) NEW. This included the binding assay described previously (section 4.4.1) using streptavidin beads and biotinylated chemokines. In addition, chemokine uptake assays were carried out, using similar methods as before (section 4.5.2); to investigate whether D6-N (s) NEW affected the ability of THP1 cells to uptake CCL2. In each assay, D6-N (s) NEW reconstituted in water or PBS was tested. The results of these experiments are shown in **Figure 4-16**.

Western Blotting of samples from the streptavidin bead binding assay (**Figure 4-16 (a) (i)**) showed that D6-N (s) NEW does not bind biotinylated CCL22 or biotinylated CCL19, as indicated by the absence of bands for D6-N (s) NEW in any of the samples. Overnight exposure to x-ray film further confirmed that D6-N (s) NEW was not present in the eluted samples (**Figure 4-16 (a) (ii)**). Western Blots using streptavidin-HRP of the material eluted after the target samples confirmed the presence of either biotinylated CCL22 or biotinylated CCL19 in these samples (**Figure 4-16 (a) (iii)**); therefore there was no problem with the 'bait' used in these binding assays. The addition of D6-N (s) NEW to cells also did not affect the uptake of CCL2 by THP1 cells in the chemokine uptake assay (**Figure 4-16 (b)**). This was the case even when very large quantities of D6-N (s) NEW were added to cells.

In summary, Unlike D6-N (s), D6-N (s) NEW does not bind to CCL2 or CCL22, either in binding assays or chemokine uptake assays. These results compared to those obtained using the original batch of D6-N (s) strongly suggests that the pattern and degree of sulphation of D6-N (s) is crucial to its binding capability to inflammatory chemokines.

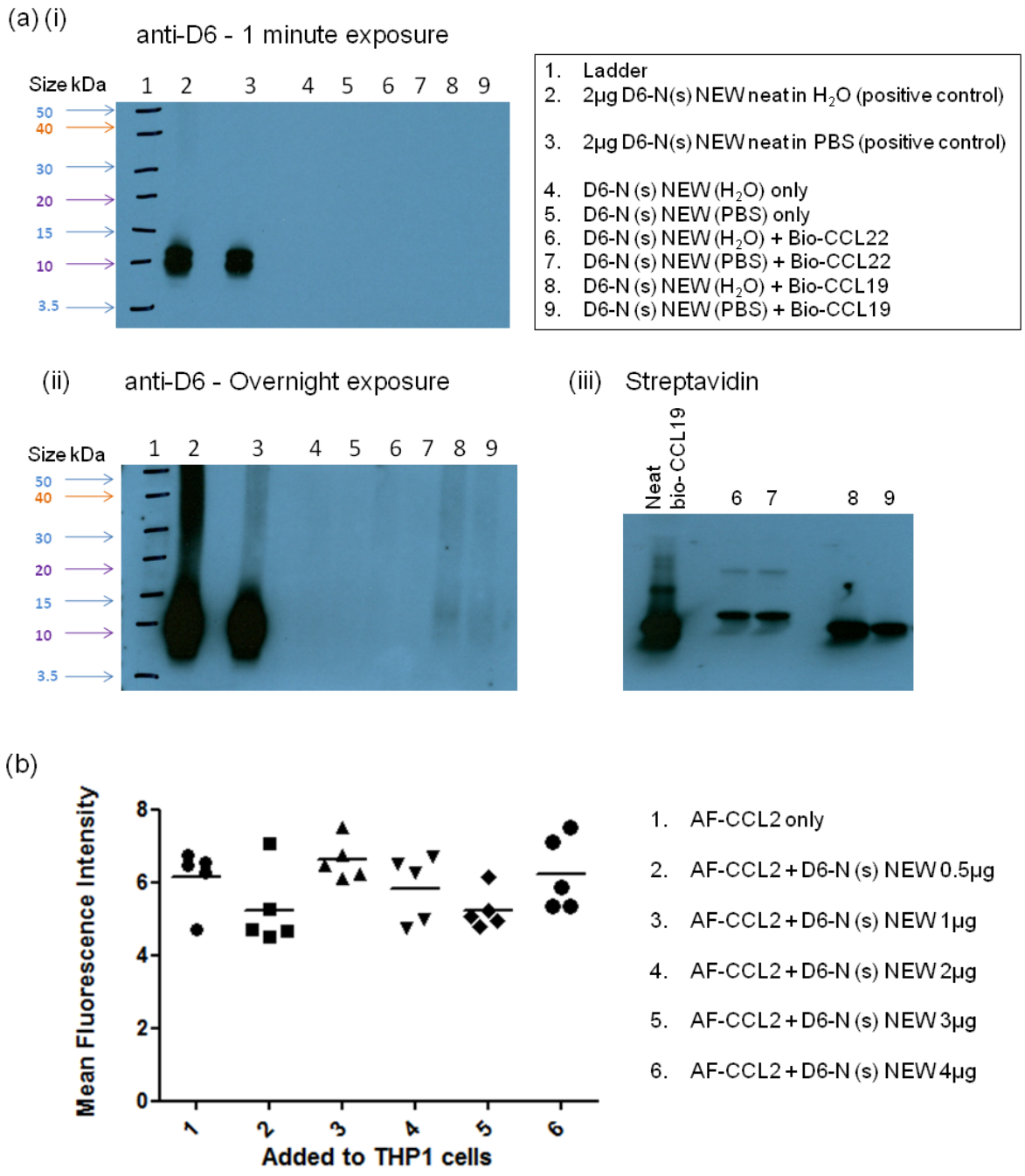


Figure 4-16: D6-N (s) NEW does not bind to inflammatory chemokines.
(a) (i) 1 minute exposure and **(ii)** overnight exposure of Western Blots of samples (listed) using the anti-D6 (clone 4A5) antibody. **(iii)** Western Blotting of 'bait' samples using streptavidin-HRP. **(b)** Dot plot of MFIs from chemokine binding/uptake assays as determined by flow cytometry analysis. N=5 per sample. Data were analysed by one-way ANOVA tests, no significant difference was found between any of the groups, p=0.0839.

4.7 Summary of Chapter 4

This chapter describes experiments designed to investigate (i) the biochemistry of the D6-N peptides (ii) D6-N's ability to bind inflammatory CC chemokines (iii) D6-N's ability to reduce the binding of inflammatory CC ligands to their receptors (iv) whether or not the sulphation status of D6-N affects its chemokine binding capabilities. Such experiments are of value in helping decide if D6-N has the potential to be developed as an anti-inflammatory drug, with the capability to mop up superfluous inflammatory CC chemokines produced during chronic inflammatory diseases. The main findings that are presented in this chapter are the following:

- The sulphated D6-N peptide is a mixture of differentially sulphated peptides which exists predominantly as a dimer at room temperature and forms higher order aggregates when heated. The anti-D6 (clone 4A5) antibody recognises only D6-N (s), suggesting that D6 is naturally sulphated on its *N*-terminus and that the antibody recognises a sulphated epitope. This is in agreement with previous biochemical studies of D6 which demonstrated it was sulphated (Blackburn et al., 2004), but extends this by defining the *N*-terminus as a site of sulphation.

- D6-N (s) binds to the inflammatory CC chemokines CCL2 and CCL22, which are natural high-affinity D6 ligands. Preliminary data generated from Biacore experiments support this finding by showing D6-N (s) binds to CCL2.

- D6-N (s) can prevent CCL2 binding to either D6 expressed on HEK transfectants or CCR2 expressed on THP1 cells. This suggests that the addition of D6-N (s) could potentially inhibit inflammatory chemokine/chemokine receptors interactions. It also suggests that sulphation of tyrosine residues on the D6-N peptide increases its binding affinity for CCL2.

- The pattern and degree of sulphation of D6-N (s) is crucial to its ability to bind inflammatory CC chemokines, however unfortunately it is difficult to determine the different species of peptide present in either batch of D6-N (s).

It is reasonable to assume that the optimum pattern of sulphation for D6-N (s) in order to bind chemokines would be that which exists on the full length D6 receptor. Further investigation of the consequences of sulphation for the full-length D6 receptor and a possible natural version of D6-N will be discussed in the following chapters.

Chapter 5 - Results

N-terminal processing of D6 and possible mechanisms of cleavage

5.1 Introduction

In recent years it has become apparent that certain chemokine receptors can be regulated by proteolytic cleavage of their extracellular domains. As discussed in section 1.2.2.3, CXCR1, CXCR2 and CXCR4 have all been shown to be regulated in this way by the action of different proteolytic enzymes (Laarman et al., 2012, Hartl et al., 2007, Valenzuela-Fernandez et al., 2002). Interestingly in the case of CXCR1, the resulting cleaved peptide has been shown to have biological activity, and its downstream effects include inducing the release of CXCL8; a CXCR1 ligand (Hartl et al., 2007). Such studies highlight an important role for proteases in the regulation of chemokine receptor function. **Figure 5-1** summarises some examples of chemokine receptor cleavage.

Previous studies on the characterization of the atypical chemokine receptor D6 revealed that the *N*-terminus of D6 is subject to processing, giving rise to a truncated receptor of approximately 38kDa (Blackburn et al., 2004). This suggests that the resultant cleaved *N*-terminal peptide should be ~11kDa, and may have post translational modifications including glycosylation and sulphation of tyrosine residues. Taking into account the previous findings for other chemokine receptors, investigations were initiated to examine potential mechanisms involved in *N*-terminal cleavage of D6 and the nature of the *N*-terminal cleavage product.

Key questions were:

- Can previous studies demonstrating the existence of a truncated D6 protein be repeated?
- Can the mechanism of cleavage be determined and the effect enhanced?
- Does cleavage of D6 modify its ability to bind inflammatory chemokines?
- Can the *N*-terminal cleavage product be identified and isolated, and if so can it be characterised?

In order to confirm previous studies which indicated the existence of a truncated D6 protein, it was decided that heterologous transfectants expressing the HA-D6 plasmid would be employed (see chapter 3, section 3.2.1 for details on this plasmid). Transfected cells are able to express extremely high levels of D6; therefore it was thought that such cells would provide an effective and valuable resource to study the biochemical properties of the D6 protein.

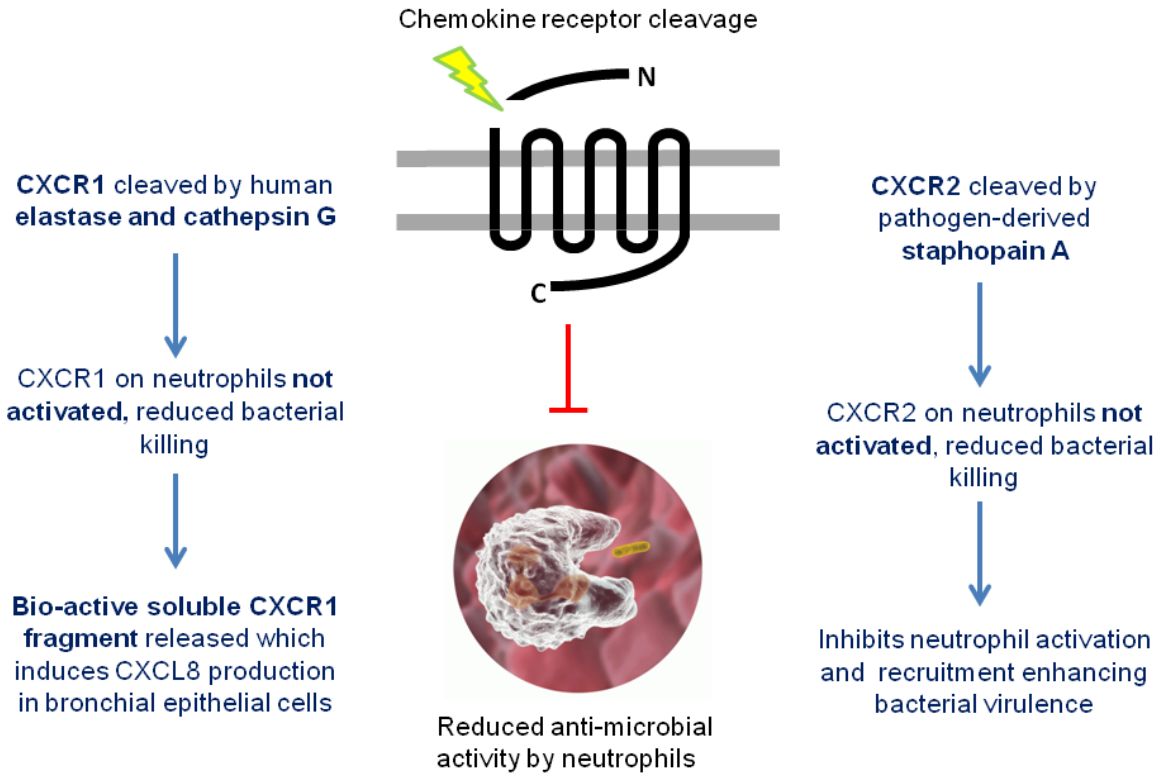


Figure 5-1: Examples of chemokine receptor cleavage and its biological consequences. Diagram highlighting previous reports of proteolytic processing of CXCR1 and CXCR2, resulting in inactivation of the receptor and important downstream effects, including reduced anti-microbial activity by neutrophils. Created using information from (Laarman et al., 2012) and (Hartl et al., 2007).

5.2 Seeking evidence for a ‘shed’ D6 *N*-terminal peptide

5.2.1 HEK 293 cells

The Human embryonic kidney (HEK) 293 cell line was originally derived from human embryonic kidney cells grown in tissue culture and has been experimentally transformed with adenovirus 5 DNA. HEK 293 cells are easy to grow and transfect and are commonly used as a vehicle to analyse receptor function. For these reasons, HEK 293 cells were used to analyse the possible shedding of a D6 *N*-terminal peptide.

5.2.2 Western Blot antibodies

Western Blotting was used as the principle means to detect an *N*-terminally derived D6 peptide. It was therefore essential that the antibodies used recognised epitopes associated specifically with the *N*-terminus or the *C*-terminus of D6. Consequently the anti-D6 (clone 4A5) antibody and an anti-HA antibody were used for detection of the *N*-terminus, and anti-D6 (Sigma) antibody was used for detection of the *C*-terminus.

5.2.3 Transfection of HEK 293 cells with HA-D6

HEK 293 cells were stably transfected with pcDNA3.1 HA-D6 using effectene (as described in Chapter 2, section 2.4). Cells were selected using G418 and assessed for D6 expression by quantifying D6 using flow cytometry. Surface expression of D6 was confirmed using the anti-D6 (clone 4A5) antibody with a FITC-conjugated secondary antibody. Flow cytometry profiles and histograms are shown in **Figure 5-2** which compares **(a)** un-transfected HEK 293 and **(b)** HEK D6**.**

Flow cytometry analysis indicated that transfection was a success with over 75% of cells expressing high levels of D6 (**Figure 5-2 (b) (i) and (ii)**).

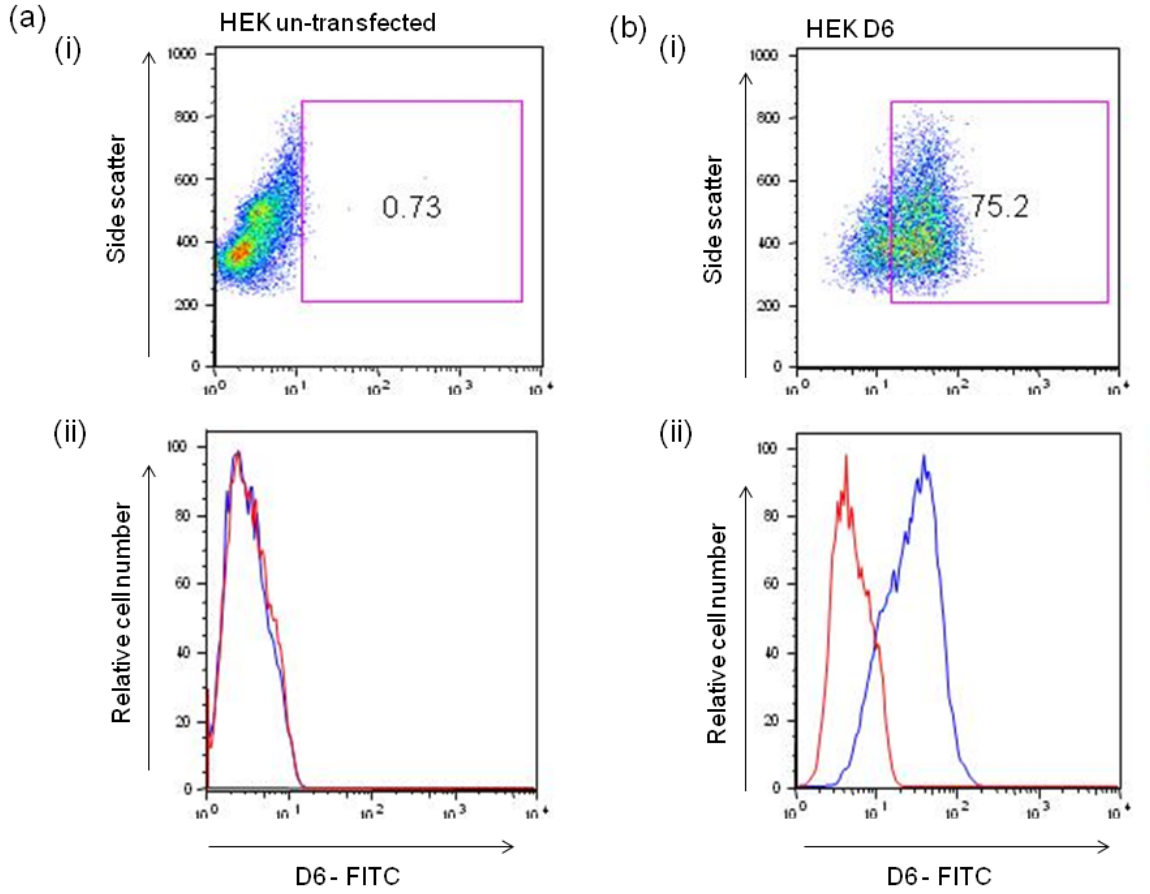


Figure 5-2: D6 expression of the HEK 293 cell line before and after transfection.
D6 expression was determined on **(a)** HEK 293 and **(b)** HEK D6 cells by flow cytometry using the mouse anti-human D6 antibody (clone 4A5) and an anti-mouse FITC secondary antibody. Flow cytometry profiles are shown in **(i)** and histograms in **(ii)**. Histograms show the isotype controls in red and D6 expression in blue. Cells positive for D6 expression are FITC positive and this is shown as a right shift on the flow cytometry profiles.

5.2.4 Detection of an *N*-terminally 'shed' D6 peptide

In order to verify previous results which indicated that D6 is subject to *N*-terminal processing, detection methods were employed as outlined in **Figure 5-3**. Immunoprecipitation (IP) assays were performed by mixing the conditioned media with magnetic anti-HA microbeads and 'on-column' binding. The microbeads are held magnetically in the column, and any material binding to the microbeads is also retained. Material held in the column can be eluted using hot SDS buffer.

Western Blot analysis of samples of un-immunoprecipitated conditioned media taken directly from cultures of both un-transfected HEK and HEK D6 cells is shown in **Figure 5-4 (a)**. Western blotting using either the **(i)** anti-HA or the **(ii)** anti-D6 (clone 4A5) antibody did not detect any shed D6-specific peptides. Many of the bands detected seem to be non-specific and are most likely attributed to proteins in the growth media (indicated as 'non-specific' in **Figure 5-4 (a) (i)**). Red arrows highlight full length D6 detected in HEK D6 cell lysates using both antibodies (**Figure 5-4 (a) (i)** and **(ii)**). Next samples were subjected to IP before SDS PAGE and Western Blot analysis with the anti-D6 (clone 4A5) antibody or silver stain analysis (**Figure 5-4 (b)**). The conditioned media samples labelled HEK D6 1 and HEK D6 2 are from cell cultures that were 80% confluent and 100% confluent respectively. Bands were present in immunoprecipitated HEK D6 conditioned media, but absent in HEK media in both the Western Blot (**Figure 5-4 (b) (i)**) and the silver stain (**Figure 5-4 (b) (ii)**), and are indicated using a red box. These bands are ~15kDa, which is near to the expected size of a cleaved D6 *N*-terminal peptide. Further analysis of this band is needed in order to confirm that it is an *N*-terminal cleavage product of D6.

In summary, cultured HEK D6 cells release a ~15kDa peptide into the growth media, which can be enriched by IP using anti-HA beads and detected on Western Blots using an anti-D6 antibody which is specific for the D6 N-terminus. This peptide is not released by un-transfected cells, and therefore potentially represents a shed N-terminal peptide.

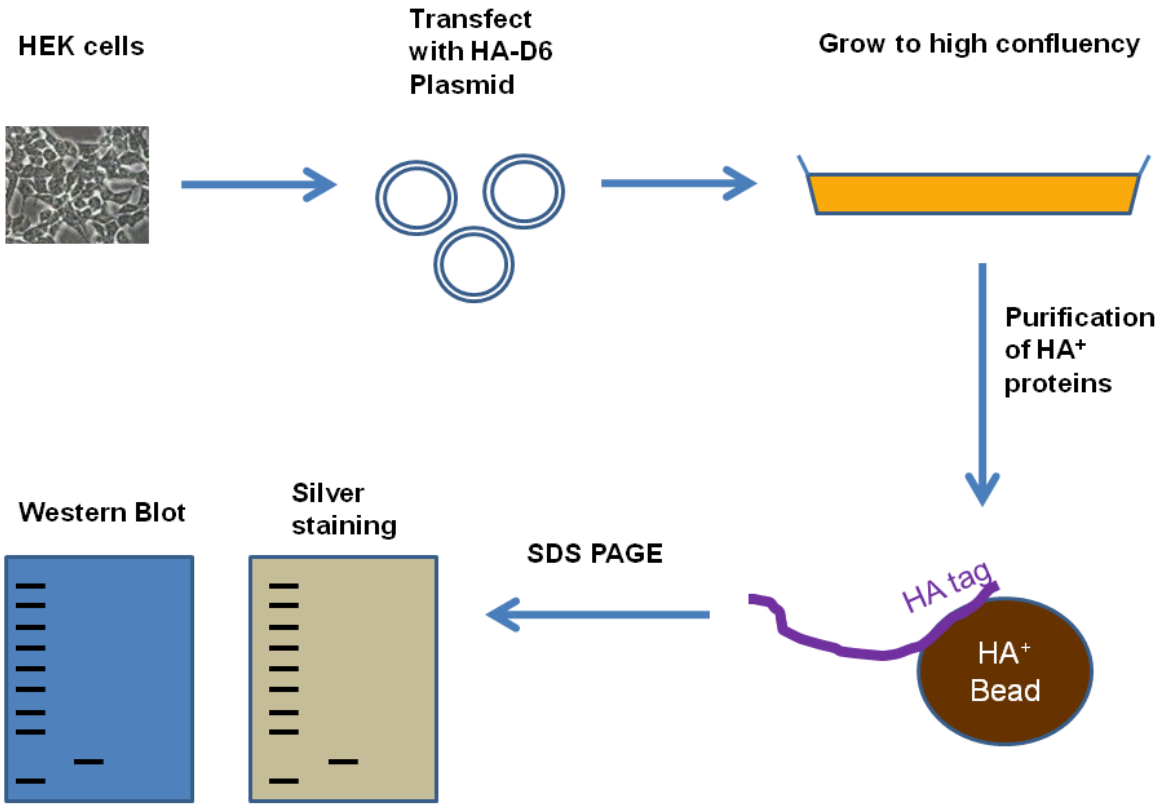


Figure 5-3: Detection method for 'shed' D6 *N*-terminal peptide.
HEK D6 transfectant cells were cultured until highly confluent. The conditioned media from cell cultures was collected and either tested directly by Western Blotting or purified using an IP assay, whereby anti-HA beads were used to capture any material present in the media which bears an HA-tag. Material bound to the anti-HA beads was eluted and resultant samples were subjected to SDS PAGE and analysed by Western blotting and/or silver staining.

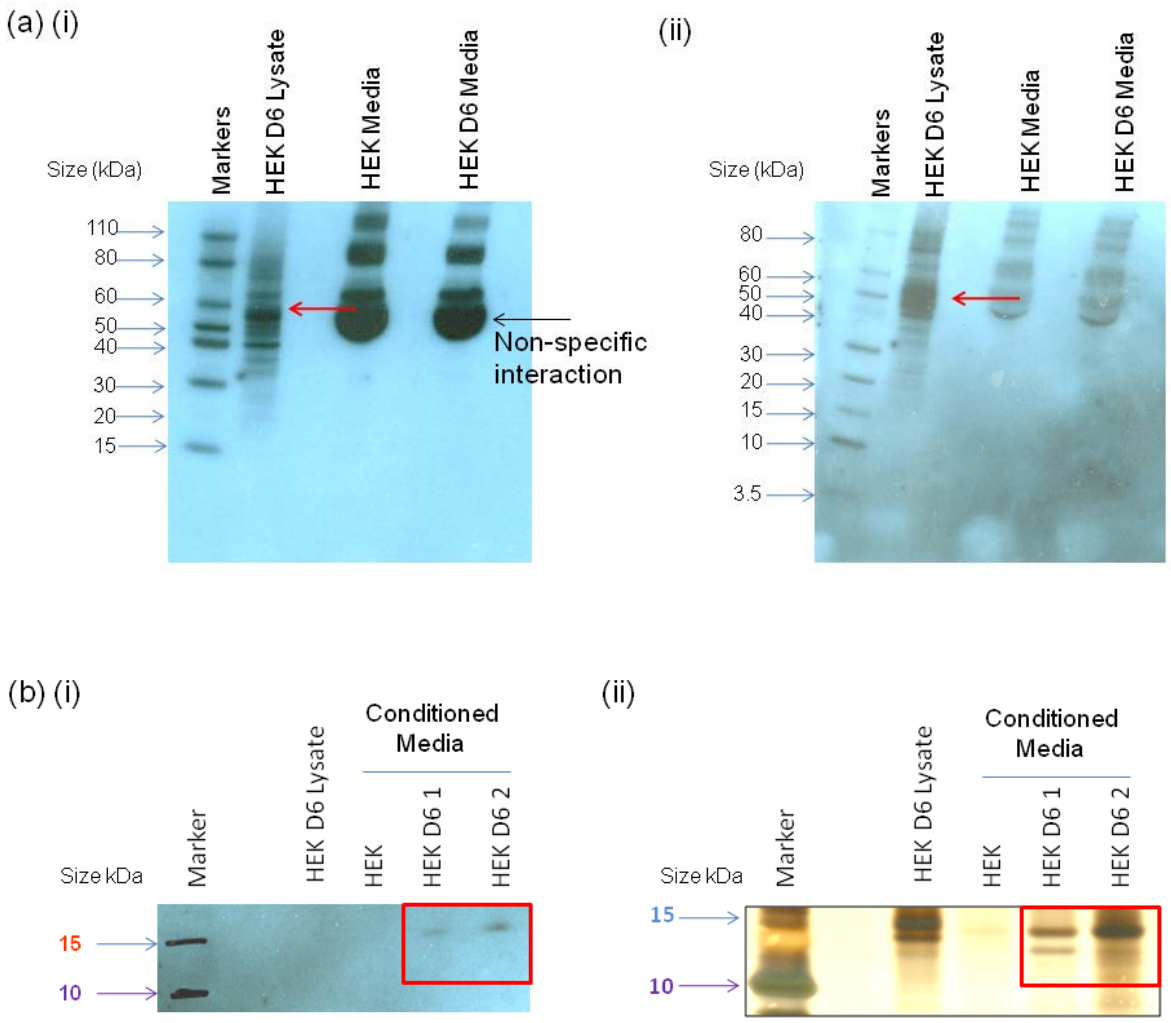


Figure 5-4: Detection of a shed *N*-terminal peptide derived from D6.
After collection of conditioned media from HEK and HEK D6 cell cultures, and also HEK D6 cell lysates, samples were either **(a)** analysed directly by SDS PAGE and Western Blots using **(i)** an anti-HA antibody or **(ii)** the anti-D6 (clone 4A5) antibody. The red arrows indicate full length D6 detected in HEK D6 cell lysates. **(b)** Conditioned media was enriched by IP using anti-HA beads and resulting samples were analysed by SDS PAGE and **(i)** Western Blotting using the anti-D6 (clone 4A5) antibody or **(ii)** silver staining. HEK D6 1 and HEK D6 2 are from cell cultures that were 80% confluent and 100% confluent respectively. Red boxes highlight a ~15kDa band present in conditioned media from HEK D6 cell cultures after enrichment with anti-HA beads. The Western Blot shown in (b) (i) is representative of 3 separate experiments.

5.2.5 Detection of truncated D6 protein

With evidence to suggest that an *N*-terminally derived D6 peptide is present in conditioned media from cultures of HEK D6 cells, we next examined the D6 protein expressed by these cells for evidence of truncation. As shown in Figure 5-4 (a) (ii), Western Blot analysis of HEK D6 cell lysates using the anti-D6 (clone 4A5) antibody detected a full length D6 protein of the expected size of ~49kDa. However this antibody only recognises the *N*-terminus therefore it is not appropriate for detection of a truncated D6 protein, from which the *N*-terminus has been cleaved. Therefore a commercially available anti-D6 antibody (Sigma), which recognises a sequence of amino acids on the *C*-terminus of D6, was used for Western blot analysis (**Figure 5-5**).

Analysis of D6 species detected using antibodies specific to either the *N*-terminus (**Figure 5-5 (a)**) or the *C*-terminus (**Figure 5-5 (b)**) revealed that both antibodies detect full length D6 protein (49kDa) (indicated by one asterisk), however the *C*-terminal antibody also detects a ~30kDa truncated D6 protein which is not detected by the *N*-terminal antibody (indicated by 2 asterisks). This suggests that the *N*-terminus has been cleaved from this truncated protein and supports previous studies by Blackburn et al., demonstrating that D6 is subject to *N*-terminal processing. The truncated D6 protein detected in **Figure 5-5 (b)** is smaller than the 38kDa protein detected in the previous study, however the recombinant D6 protein used in previous studies had a 10-histidine tag at its *C*-terminus. Therefore the discrepancy in size may be partly due to sequence differences in the proteins expressed and also differences in the type of gels used to analyse the samples.

In summary, analysis of HEK cells transfected with D6 revealed a truncated version of D6 (~30kDa), which can be detected using an antibody which recognises the C-terminus. This truncated D6 protein is not detected with an antibody that recognises the N-terminus, suggesting that the N-terminus has been cleaved. This result, along with the detection of a D6-derived ~15kDa peptide in conditioned media from HEK D6 cells, confirm previous studies indicating that D6 is susceptible to N-terminal processing.

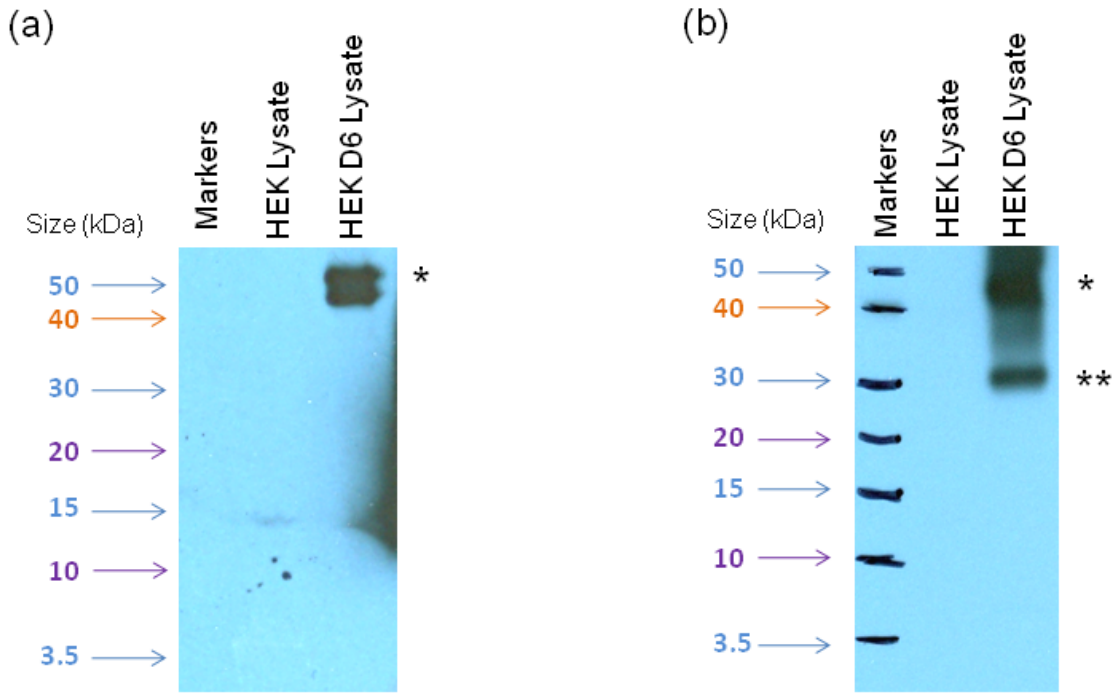


Figure 5-5: Detection of a truncated D6 protein.
Cell lysates from HEK or HEK D6 cells were analysed by SDS PAGE and run on Western blots using **(a)** the D6 (clone 4A5) antibody which recognises the *N*-terminus or **(b)** a commercially available (sigma) D6 antibody which recognises the *C*-terminus.

5.3 Seeking mechanisms of cleavage of D6

Data gathered thus far indicate that *N*-terminal processing of D6 occurs constitutively during cell growth. We next carried out experiments to determine if cleavage of D6 can be enhanced by the addition of proteases.

5.3.1 Treatment of HEK D6 cells with proteases

As mentioned in the introduction to this chapter, the chemokine receptors CXCR1 and CXCR2 are susceptible to cleavage by the action of different proteases. The serine proteases human neutrophil elastase and cathepsin G, which are commonly released by neutrophils, are both able to cleave the extracellular region of CXCR1 (Hartl et al., 2007). In addition, the cysteine protease staphopain A, a virulence factor secreted by the bacterial pathogen *Staphylococcus aureus*, is able to cleave the *N*-terminus of CXCR2 (Laarman et al., 2012) . In light of these findings, neutrophil elastase, cathepsin G and staphopain A were tested for their ability to cleave D6.

In the initial experiment, various concentrations of each protease were added to HEK D6 cells and incubated for different time periods (see chapter 2, section 2.5.4 for specific details). The conditioned media were collected and stored and the cells were lysed using cell lysis buffer. Samples were analysed by SDS PAGE and Western blotting was performed using an anti-HA antibody to detect the *N*-terminus and the anti-D6 (Sigma) antibody to detect the *C*-terminus of D6.

Treatment of HEK D6 cells with neutrophil elastase or cathepsin G at any of the concentrations given did not alter the relative balance of full length and truncated D6 protein, as shown by Western blotting of cell lysates for detection of the *N*-terminus (**Figure 5-6 (a)**) and the *C*-terminus (**Figure 5-6 (b)**). The *C*-terminal anti-D6 antibody (Sigma) once again detected both the full length (~49kDa) and the truncated form of the D6 protein (indicated by one and two asterisks respectively). Western blotting of cell supernatants with the anti-HA antibody did not detect any D6-specific proteins, but as before, albumin from growth media supplemented with serum was detected with this antibody (indicated on **Figure 5-6 (a) (i)** and **(ii)**). Notably however, after treatment with staphopain A, the density of the full length D6 protein was noticeably decreased (highlighted by red boxes in **Figure 5-6 (a) (ii)** and **(b) (ii)**), while the truncated protein

remained detectable (**Figure 5-6 (b) (ii)**). This result suggested that treatment of HEK D6 cells with staphopain A induces a change in the D6 protein, presumably by cleavage of the *N*-terminus, making the *N*-terminally cleaved D6 protein non-detectable with the anti-HA antibody but still detectable by the anti-D6 (Sigma) antibody.

In summary, D6 is unaffected when treated with neutrophil elastase and cathepsin G, however initial analysis suggested that treatment with staphopain A noticeably reduces the amount of full length D6 protein that can be detected on Western Blots.

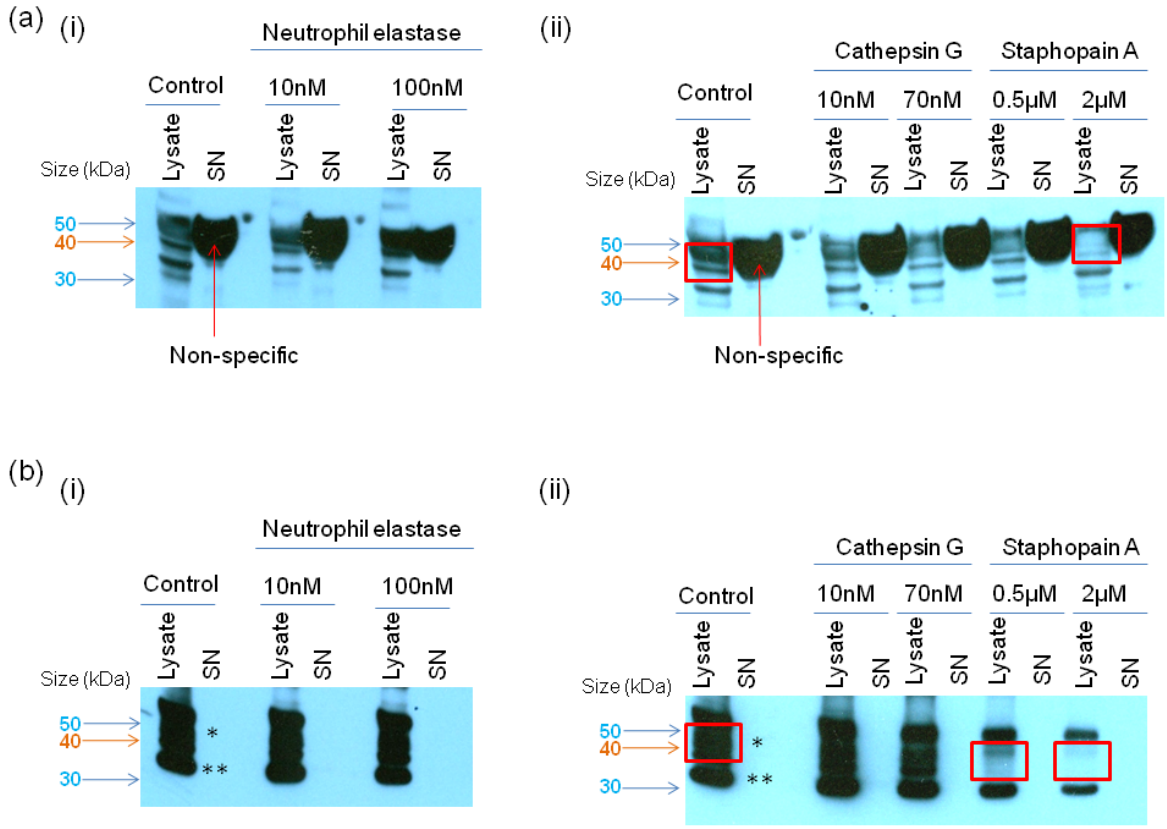


Figure 5-6: Treatment of HEK D6 cells with different proteases.
Cell lysates and conditioned media (labelled SN for supernatant) from HEK D6 cells treated with PBS (labelled control) or different concentrations (indicated) of **(i)** neutrophil elastase, **(ii)** cathepsin G or staphopain A. Samples were analysed by SDS PAGE and Western blots were performed to detect D6 using the **(a)** anti-HA antibody and **(b)** the anti-D6 (Sigma) *C*-terminal antibody. One asterisk = full length D6, two asterisks = 30kDa D6 band. Red boxes highlight full length D6.

5.3.2 Analysing the activity of staphopain A

Staphopain A used in the study mentioned previously (Laarman et al., 2012) was purified from *S. aureus* cultures, however the staphopain A used in this study was obtained from Sigma. In order to further assess the activity of staphopain A from Sigma, experiments were set up to test its ability to cleave CXCR2, a known target. To do this, staphopain A was added to CXCR2-expressing THP-1 cells and cell lysates were subsequently analysed by Western blotting using an *N*-terminal specific anti-CXCR2 antibody (**Figure 5-7 (a) (i)**). The blot was stripped and re-probed with an anti-beta actin antibody (**Figure 5-7 (a) (ii)**). The Western blot in **Figure 5-7 (a) (i)** shows a reduction in CXCR2 after staphopain A treatment, which suggested cleavage of CXCR2 by staphopain A. Image J software was used to normalise this reduction against the loading control (**Figure 5-7 (b)**). Image J analysis indicated that there was a 50% reduction in the density of the CXCR2 band after treatment with staphopain A. This confirms staphopain A's ability to act on its substrates.

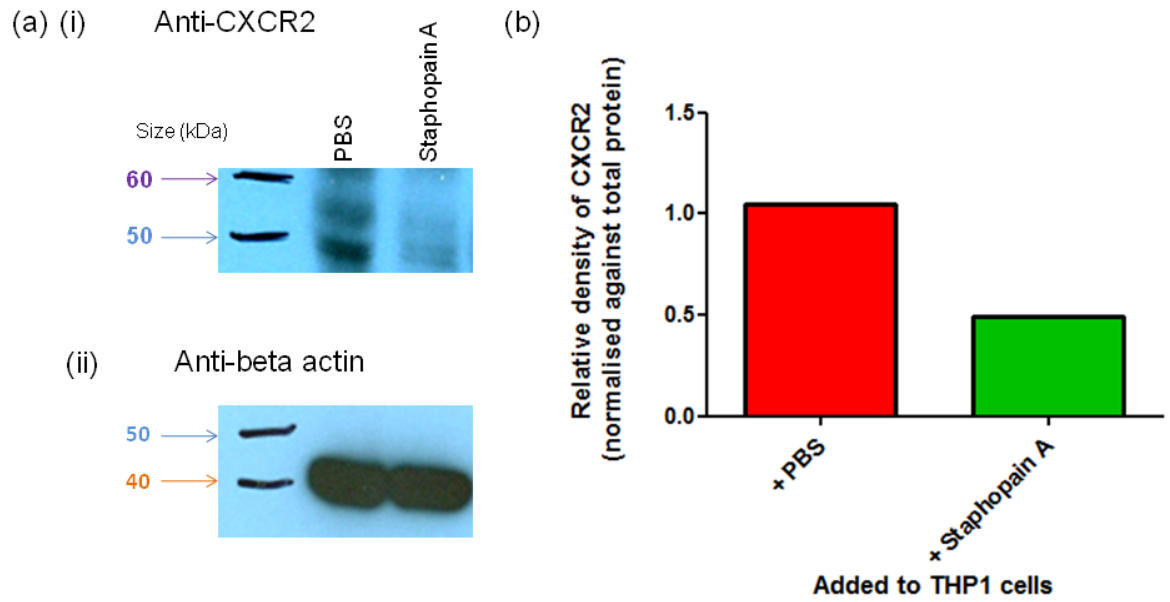


Figure 5-7: Staphopain A treatment of CXCR2-expressing THP-1 cells.
(a) Western blots of cell lysates from THP-1 cells treated with PBS or staphopain A. Samples were subjected to SDS PAGE and Western blots were performed to detect **(i)** CXCR2 using an antibody against the CXCR2 *N*-terminus. Blots were then stripped and re-probed to detect **(ii)** beta-actin using an anti-beta actin antibody. **(b)** Bands were analysed using image J software and normalised against loading controls. Results are presented in a histogram. N=1.

5.3.3 The effect of staphopain A on D6

The result from earlier experiments suggests that staphopain A can cleave D6 at its *N*-terminus. To analyse this further, methods were considered that may enhance staphopain A's proteolytic effectiveness. It is widely known that serum proteins can inhibit the action of enzymes by competing with the substrate (Shulman, 1952), therefore it was thought that staphopain A activity may be affected by the presence of serum proteins, and that their removal may therefore increase the activity of staphopain A. Since the previous experiment involved the addition of staphopain A to cell cultures incubating in complete growth media containing 10% FCS, the effect of incubating cells in PBS for the duration of the experiment (15 minutes, 1 hour and 2 hours) was investigated. Staphopain A was used at a concentration of 2μM, since in the previous experiment this concentration produced the most pronounced truncation of D6. After treatment with staphopain A, cell lysates were analysed by Western blotting using both the *C*-terminal anti-D6 (Sigma) antibody and the *N*-terminal anti-D6 (clone 4A5) antibody. The supernatants were also collected and analysed by Western blotting with both D6 antibodies. The results are shown in **Figure 5-8**.

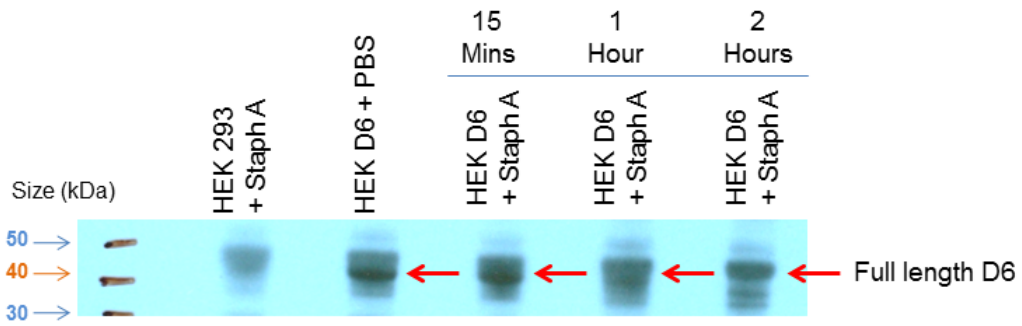
The Western blot in **Figure 5-8 (a) (i)**, using the anti-D6 (clone 4A5) antibody, shows that the density of the full length D6 protein (highlighted by red arrows) decreases with increasing time of staphopain A treatment. Western blotting with the *C*-terminal anti-D6 (Sigma) antibody revealed the presence of two truncated D6 proteins (~35kDa and ~30kDa) (labelled with asterisks in **Figure 5-8 (a) (ii)**), both of which increased in density with increasing time of staphopain A incubation. The change in full length (i) and truncated (ii) D6 species after staphopain A treatment was further supported by densitometry analysis, shown in **Figure 5-8 (b)**. These results indicated that the *N*-terminus of D6 is cleaved by staphopain A treatment, and that this cleavage may result in two products of different sizes.

Western Blots of the supernatants from this experiment are shown in **Figure 5-8 (c)**. The *N*-terminal D6 antibody detects a ~20kDa band in lanes where HEK D6 cells were treated with staphopain A, but not in lanes where HEK D6 cells were treated with PBS or where un-transfected HEK cells were treated with staphopain A (**Figure 5-8 (c) (i)**). This blot had to be exposed to X-ray film overnight in order to detect any bands; therefore the quality of the image is not optimal and contains a lot of background. The *C*-terminal antibody does

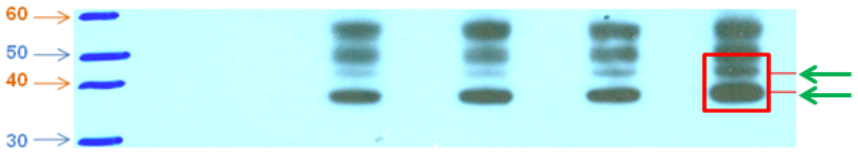
not detect any bands, even after overnight exposure to X-ray film ((**Figure 5-8 (c) (ii)**). These results suggest that this band is an *N*-terminal cleavage product of D6.

In summary, the proteolytic activity of staphopain A was confirmed by its ability to cleave CXCR2. D6 is subject to N-terminal enzymatic processing by staphopain A. A ~20kDa peptide is present in supernatants after HEK D6 cells are treated with staphopain A. This peptide is recognised by the anti-D6 (clone 4A5) N-terminal antibody, but not the anti-D6 (Sigma) C-terminal antibody, suggesting it may be a cleaved N-terminal D6 peptide.

(a) (i) *N*-terminal D6 antibody

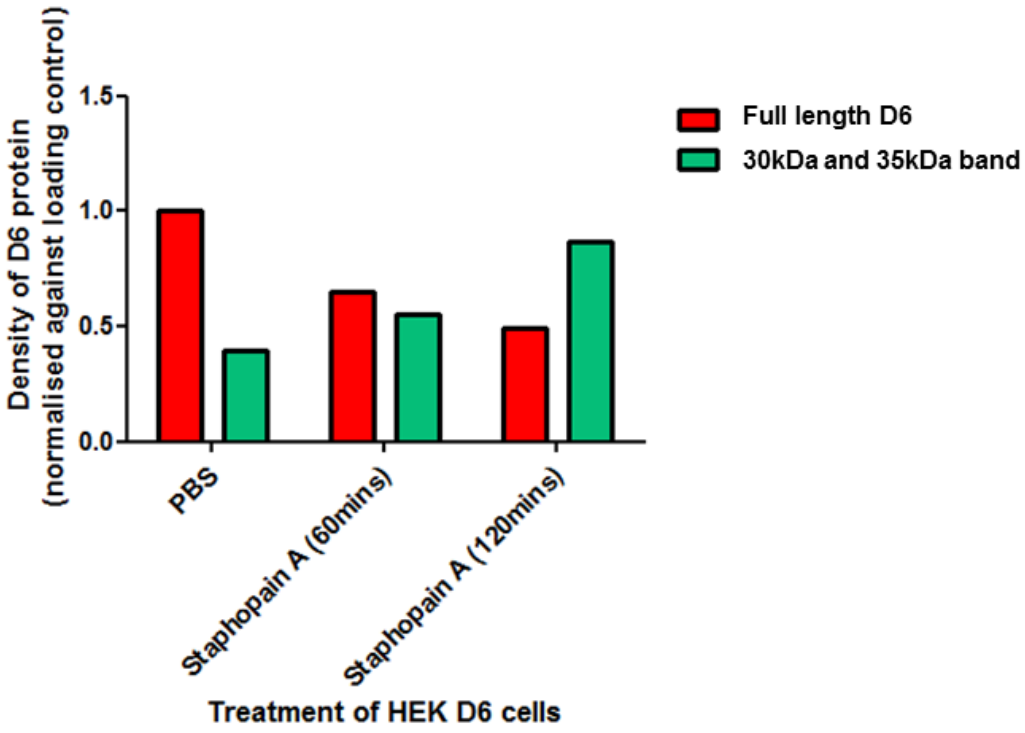


(ii) *C*-terminal D6 antibody



(iii) Beta-tubulin antibody

(b)



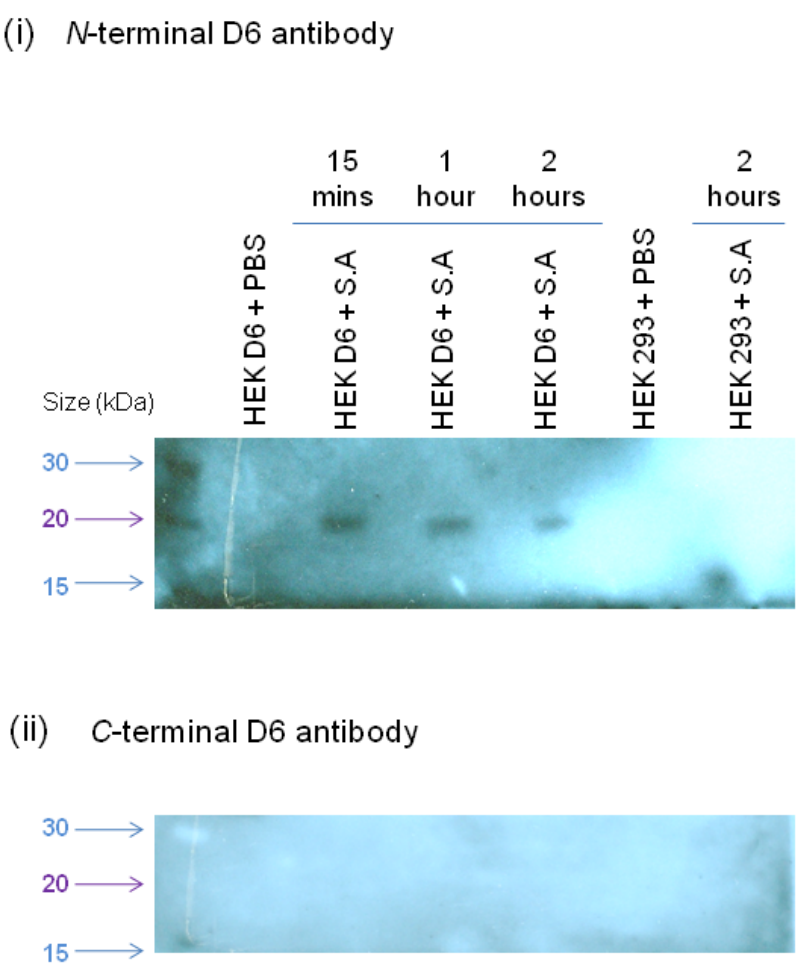


Figure 5-8: Staphopain A treatment of HEK D6 cells in PBS.
Western blots of **(a)** cell lysates from HEK (negative control) or HEK D6 cells treated with PBS or staphopain A for increasing time periods. Cells were incubated in PBS during the time period of treatment with staphopain A. D6 was detected using **(i)** the anti-D6 (clone 4A5) *N*-terminal antibody or **(ii)** the anti-D6 (Sigma) *C*-terminal antibody. Blots were also stripped and re-probed using **(iii)** anti-beta-actin as a loading control. Blots are representative of 3 separate experiments. Full length D6 is indicated by red arrows. Truncated D6 proteins are indicated by a red box and green arrows. **(b)** Bands from blot (a) were analysed using image J software and normalised against loading controls, results are presented in a histogram. S.A. = staphopain A. **(c)** Supernatants from samples in experiment (a) were analysed by Western blotting with **(i)** the anti-D6 (clone 4A5) *N*-terminal antibody or **(ii)** the anti-D6 (Sigma) *C*-terminal antibody.

5.3.4 Attempts to purify the D6 N-terminal cleaved peptide

Experimental data from Figure 5-8 suggested that treatment of HEK D6 cells with staphopain A results in the cleavage of a peptide of ~20kDa which can be detected by Western blotting using the anti-D6 (clone 4A5) *N*-terminal antibody. If this peptide is indeed an *N*-terminal cleavage product of D6, it should carry an HA-tag. We therefore attempted to purify and characterise the peptide from cell supernatants using the HA-bead IP method described previously (see section 5.2.4 and Figure 5-3). Supernatant samples before and after IP were then analysed by SDS PAGE and Western blots to detect D6. Alternatively silver stains were performed.

Both the Western blot and the silver stain shown in **Figure 5-9 (a)** and **(b)** respectively detect a ~20kDa band in supernatants from staphopain A treated HEK and HEK D6 cells before IP, however this band is not present in pre-IP supernatant samples where PBS was added to HEK or HEK D6 cells. This result contradicts the Western blot analysis in Figure 5-8 (c), which did not detect a band in the supernatant from HEK 293 cells treated with staphopain A. In all samples that were immunoprecipitated with use of anti-HA magnetic microbeads, a strong ~25kDa band can be detected in both the Western blot in **Figure 5-9 (a)** and the silver stain in **(b)**. This band is most likely the light chain of the anti-HA antibody which is present in large quantities on the anti-HA magnetic microbeads. No other bands are visible in the region of ~20kDa in these samples after IP, indicating that IP of supernatants was unable to purify any D6-specific peptides.

These results taken together imply that the bands detected in Figure 5-8 (c) were not *N*-terminal cleavage products of D6, but more likely to be the result of a non-specific interaction between the anti-D6 (clone 4A5) antibody and staphopain A. Correspondingly staphopain A has a predicted molecular weight of 19.9kDa. Further support for the idea that the band in question is staphopain A comes from comparison of the density of this band on the Western blot and the silver stain. The silver stain shows an intense band, however the band on the Western blot is very faint, suggesting a non-specific interaction with the anti-D6 (clone 4A5) antibody. If the ~20kDa band was an *N*-terminal cleavage product of D6, it would be expected that, given the high density of this protein on the silver stain, it would be highly reactive to the anti-D6 (clone 4A5) antibody and would also be easily purified by IP with anti-HA microbeads.

In summary, attempts to purify an N-terminal D6 peptide using IP were unsuccessful. The band previously thought to be an N-terminal cleavage product of D6, is more likely to be staphopain A, as this band is also present in supernatants taken from un-transfected HEK cells treated with staphopain A.

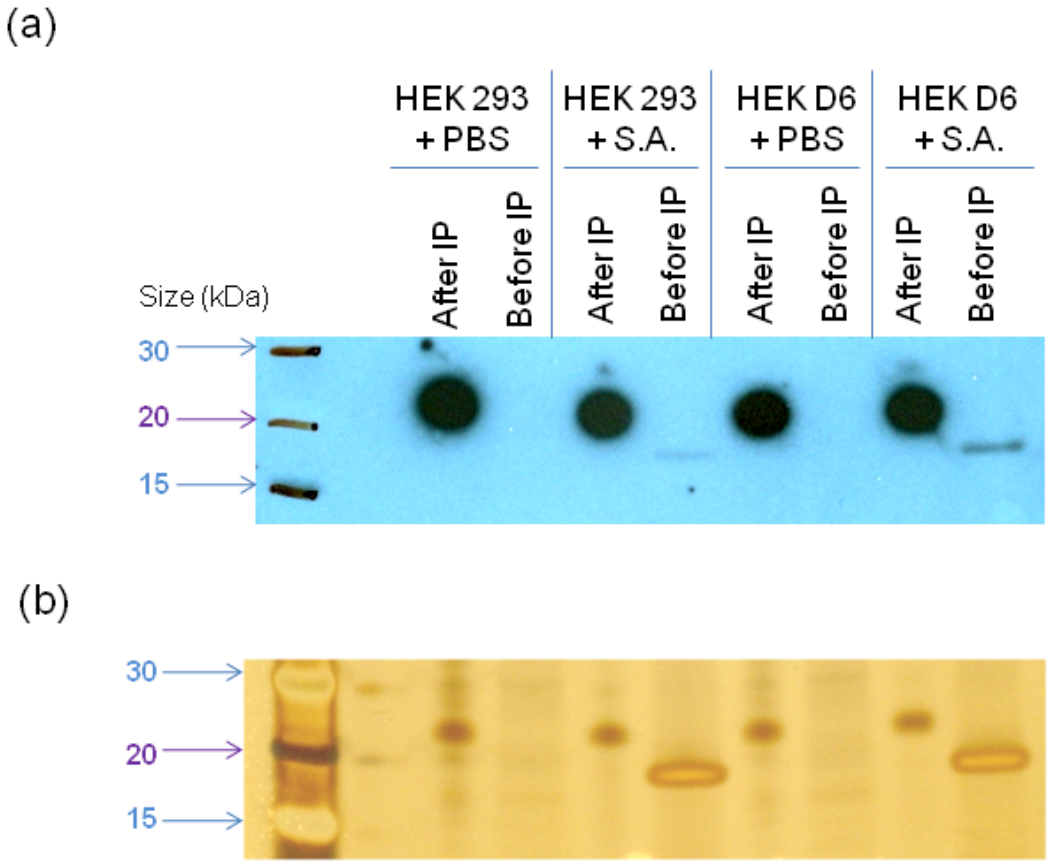


Figure 5-9: Supernatants of cells before and after IP.
IP assays using anti-HA magnetic microbeads were performed on supernatants from HEK 293 (negative control) or HEK D6 cells treated with either PBS or staphopain A. Samples from before and after IP were analysed by SDS PAGE and **(a)** Western blots were performed using the anti-D6 (clone 4A5) antibody or **(b)** gels were silver stained.

5.3.5 D6 activity decreases when treated with staphopain A

Having established that treatment of HEK D6 cells with staphopain A results in truncation of D6, we next examined whether this proteolytic event had effects on the cells' ability to bind and uptake chemokines. To test this, HEK D6 cells were treated with staphopain A or PBS, before being washed and used in chemokine uptake assays with AF-CCL22, a high-affinity D6 ligand with a fluorescent tag. After incubation with AF-CCL22, cells were washed and analysed by flow cytometry to measure their MFI.

Treating HEK D6 cells with staphopain A before performing the chemokine uptake assay significantly reduced the ability of D6 to uptake AF-CCL22 compared to treatment with PBS. This is apparent by comparing flow cytometry profiles (**Figure 5-10 (a) (i)**) of PBS treated and **(ii)** staphopain A treated cells, and also by analysis of MFI values on a histogram (**Figure 5-10 (b)**). Notably this was an incomplete inactivation of D6 activity, suggesting that functional D6 molecules were left on cell surfaces after treatment with staphopain A.

Upon consideration, the assay was further developed to allow adherent D6-expressing cells to remain attached to the surface of a tissue culture plate throughout the assay. This was thought to be closer to an *in-vivo* situation because D6 is mostly expressed on stromal cells such as LECs, therefore an assay whereby cells expressing D6 are not floating in suspension but remain adhered to a surface was thought to be more physiologically relevant. Consequently a fluorescence-based assay was developed as an alternative method in order to measure D6-mediated binding and uptake of AF-CCL22 after exposure to staphopain A (see Chapter 2, section 2.5.6 for details of the assay). CHO K1 hD6 cells were used in place of HEK D6 as these cells are more adherent to the plates required for this assay. Cells were treated with staphopain A and washed before adding AF-CCL22 and incubating for 1 hour at 37°C to allow uptake and internalisation of ligand. Cells were washed and then analysed using a plate reader detecting fluorescence at the appropriate wavelength (650-690nm).

Figure 5-10 (c) shows the results of the fluorescence assay. Addition of staphopain A to CHO K1 hD6 cells resulted in a more marked inhibition of ligand uptake and internalisation than observed with the HEK D6 cells. This inhibition was dose dependent;

with 2μM staphopain A causing the greatest reduction in ligand binding by D6-expressing CHO K1 cells (approximately 50%).

In summary, treatment of two separate D6-transfected cell lines with staphopain A significantly reduces the ability of D6 to bind and take up ligand. These data therefore suggest that D6 is a natural substrate for staphopain A, which results in cleavage of the D6 N-terminus.

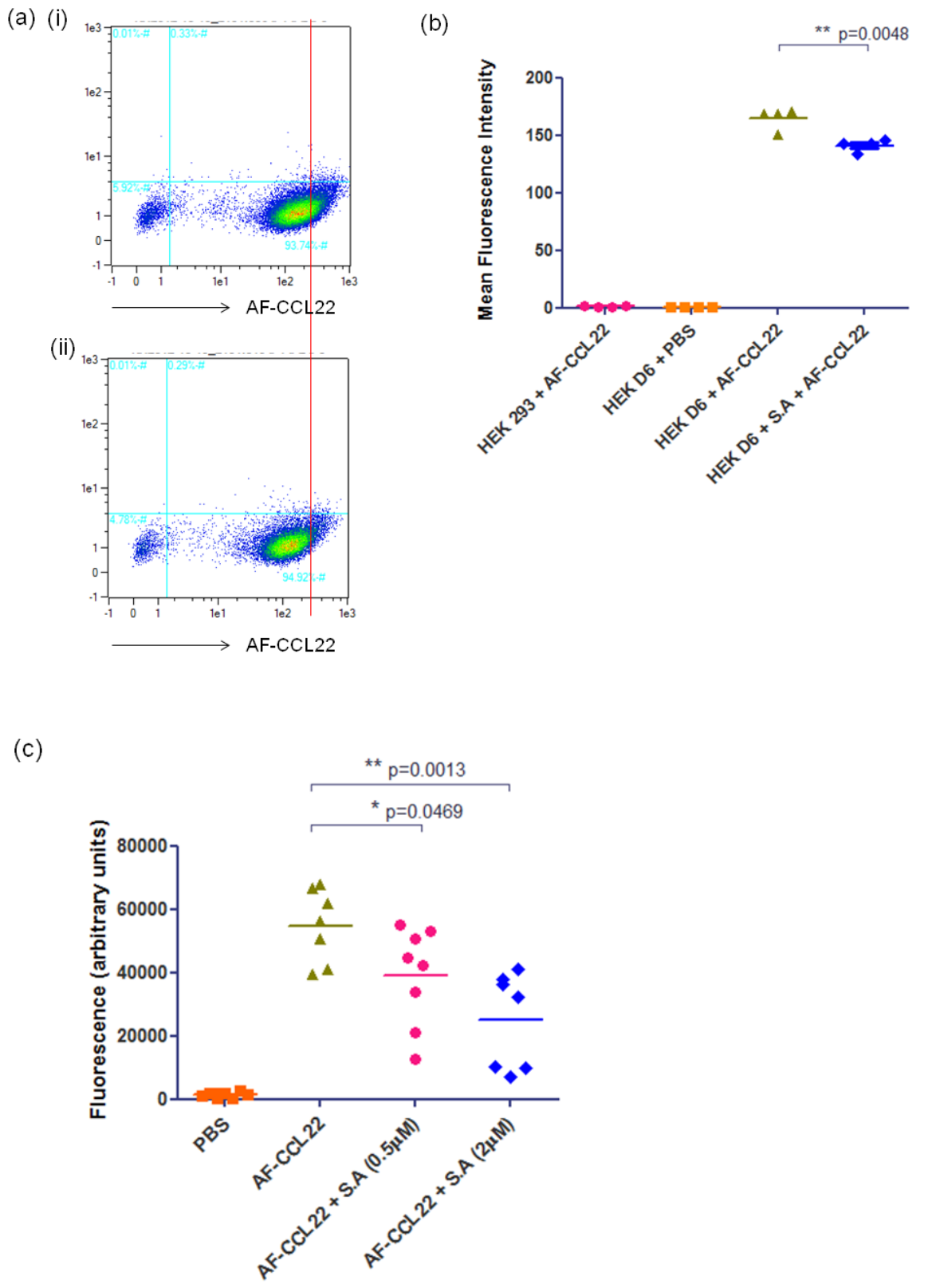


Figure 5-10: Staphopain A treatment of HEK D6 cells reduces D6 activity.
(a) Representative flow cytometry plots displaying AF-CCL22 uptake by HEK D6 cells treated with **(i)** PBS and **(ii)** 2μM staphopain A. AF-CCL22 uptake is seen as a 'right-shift' in flow cytometry profiles. **(b)** Dot plot of MFI measurements from chemokine uptake assays using HEK D6 cells treated with and without 2μM staphopain A prior to addition of AF-CCL22, as determined by flow cytometry. N=4 per group. Data were analysed by unpaired, two tailed student T tests. **(c)** Dot plot of fluorescence measurements from fluorescence assays using CHO K1 hD6 cells treated with and without different concentrations (indicated) of staphopain A prior to the addition of AF-CCL22. N=7 or more per group. Data were analysed by unpaired, two tailed student T tests.

5.4 Chapter 5 Summary

This chapter describes experiments designed to (i) examine the existence of a truncated D6 protein, produced after the *N*-terminal peptide has been shed, (ii) investigate the mechanism of cleavage of D6 and its biological consequences, and (iii) detect and study the *N*-terminal cleavage product of D6. These experiments were important to find out if, like other chemokine receptors, D6 can be modified by post-translational proteolytic cleavage, which may have significant consequences for its biological function *in vivo*. The main findings that are presented in this chapter are the following:

- HEK D6 cells release a ~15kDa peptide into growth media which can be detected by the *N*-terminal-specific anti-D6 (clone 4A5) antibody.
- A truncated D6 protein can be detected in cell lysates from HEK D6 cells using the *C*-terminal D6 antibody (sigma).
- *N*-terminal processing of D6 on HEK D6 cells is enhanced by treatment with staphopain A.
- Staphopain A treatment of HEK D6 or CHO K1 hD6 cells significantly reduced the ability of D6 to uptake ligand.

It was hoped that purification and analysis of the *N*-terminal cleavage product of D6 would provide new information regarding its biochemical properties, including its sulphation pattern and ability to bind chemokines. Since this was not possible, different experimental approaches were employed to analyse the biochemical features of the D6 *N*-terminus. Such investigations are described in the final results chapter (Chapter 6).

Chapter 6 - Results

Functional analysis of post-translational sulphation on the D6 *N*-terminus

6.1 Introduction

As described in section 1.2.2.1 of the Introduction, a conserved structural feature of chemokine receptors is the incidence of clusters of tyrosine residues at the *N*-terminus. Tyrosine residues, particularly those following a neutral or acidic amino acid, can be post-translationally sulphated by tyrosyl-protein sulphotransferases 1 and 2 (TPST -1 and TPST-2) in the Golgi apparatus. Evidence from studies on many different chemokine receptors suggests that such tyrosine sulphation is important, and often essential, for ligand binding and function (Cormier et al., 2000, Fong et al., 2002, Gutierrez et al., 2004). For example sulphation of CCR5 is essential for ligand binding and importantly for the entry of HIV into the cell (Cormier et al., 2000, Farzan et al., 1999). Mutation of tyrosine residues in the *N*-terminal regions of many chemokine receptors including CCR5 (Seibert et al., 2002), CCR8 (Gutierrez et al., 2004), CXCR4 (Veldkamp et al., 2006) and DARC (Choe et al., 2005) has been shown to reduce the level of metabolic sulphate labelling of these receptors, which subsequently reduces the binding affinity they have for their ligands.

D6 has a cluster of four tyrosine residues at its *N*-terminus, and it has been shown previously that D6 is sulphated (Blackburn et al., 2004). Three out of the four tyrosine residues present in this motif, as well as the neighbouring acidic amino acids, are highly conserved throughout various mammalian species, as shown by alignment of the primary amino acid sequences of the *N*-terminus of D6 from different species (**Figure 6-1**).

The aim the work described in this chapter was to examine the functional significance of tyrosine sulphation for D6. Key questions were:

- Are tyrosine residues on the *N*-terminus of D6 required for binding of ligands?
- Does sulphation of tyrosine residues on the *N*-terminus enhance the binding affinity of ligands for the *N*-terminus of D6?
- Does D6 have a particular sulphation pattern that is essential for ligand binding?
- Is D6 sulphated by TPST-1 or TPST-2?

The results presented in this chapter shed new light on this topic and provide evidence to show that the *N*-terminal region of D6 is a key determinant of ligand binding.

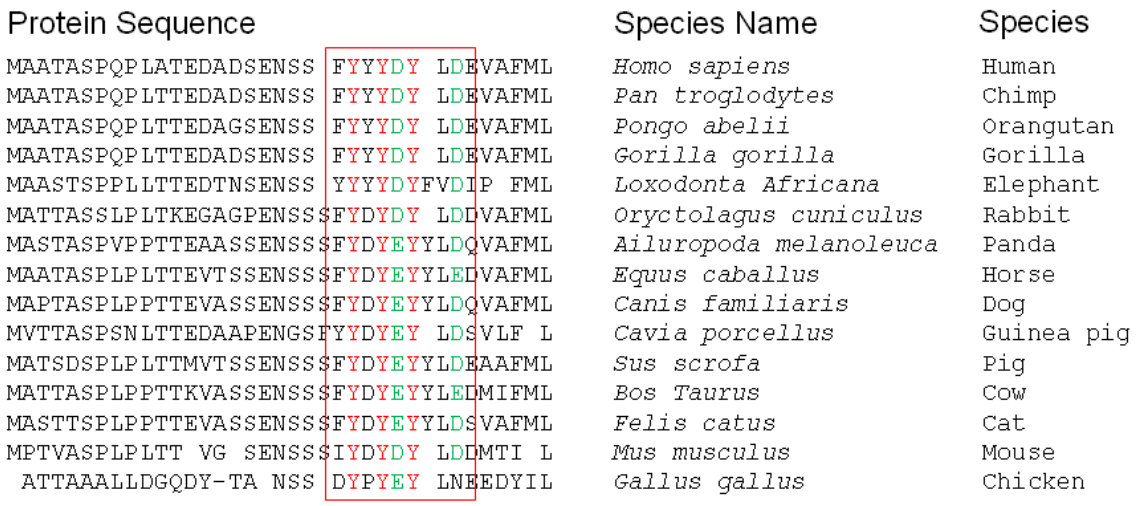


Figure 6-1: Evolutionary conservation of the tyrosine motif on the D6 *N*-terminus.
The amino acid sequences of D6 from different species are aligned showing the conservation of tyrosine residues (Y) which are highlighted in red. The acidic amino acids aspartic acid (D) and glutamic acid (E) are also conserved in this motif and are highlighted in green.

6.2 Inhibition of tyrosine sulphation by sodium chlorate treatment

Post-translational sulphation can be inhibited by decreasing the availability of the universal sulphate donor PAPS, thereby inhibiting tyrosine sulphation in the Golgi apparatus. This is achieved by growing cells in the presence of sodium chlorate, which competitively inhibits the formation of PAPS (Safaiyan et al., 1999). This method has been employed previously to examine the effect of decreasing sulphation on CCR2 function (Tan et al., 2013a). Therefore HEK D6 cells (see Chapter 2, section 2.4.1, and Chapter 5, section 5.2.3 for details on HEK transfection with D6) were grown in complete growth media supplemented with sodium chlorate over increasing time periods.

6.2.1 Inhibition of protein sulphation reduces D6 activity

6.2.1.1 Temporal analysis of sodium chlorate treatment

Detection of D6 activity after sodium chlorate treatment involved the addition of AF-CCL22 to cells and their subsequent incubation at 37°C for 1 hour to allow D6-mediated uptake and internalisation of chemokine. Afterwards cells were washed twice to remove excess chemokine and subsequently analysed by flow cytometry. DRAQ7 was used to

exclude dead cells from the analysis. D6 expression on HEK D6 cells was also measured throughout the experiments by flow cytometry in order to ensure that D6 expression levels were not affected by sodium chlorate treatment. **Figure 6-2** shows the results of these experiments.

The flow cytometry profiles in **Figure 6-2 (a)** show that the fluorescence of cells was at its highest for untreated cells **(i)**. The fluorescence of cells decreased with increasing time periods grown in sodium chlorate, with the greatest reduction after growth in sodium chlorate for 6 days **(iii)**. MFI measurements from the chemokine uptake assay (**Figure 6-2 (b)**) show that chemokine uptake by HEK D6 cells grown in the presence of sodium chlorate was significantly reduced compared to the control, and the size of this reduction was time dependent, with the largest reduction in chemokine uptake being seen after 6 days of sodium chlorate treatment. This result suggests that the sulphation of tyrosine residues on D6 is important for its binding affinity and ability to uptake CCL22. Despite the significant reduction in chemokine uptake, the effect of sodium chlorate treatment was modest, with approximately a 30% reduction in D6 activity after 6 days of sulphation inhibition. This suggested that the concentration of sodium chlorate given (30mM) may not be adequate to completely inhibit tyrosine sulphation.

In summary, D6 activity is reduced when HEK D6 cells are grown in media supplemented by sodium chlorate, indicating that sulphation is important for D6 function.

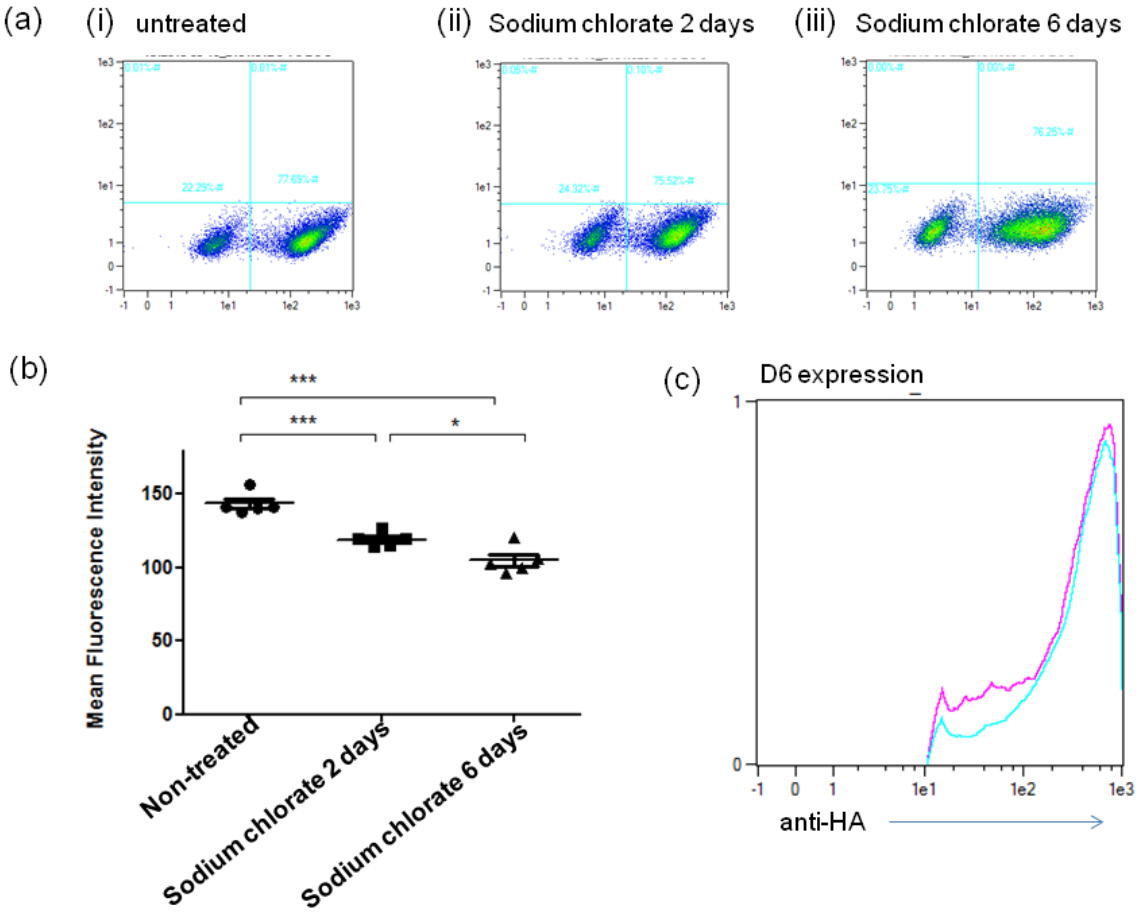


Figure 6-2: Inhibition of protein sulphation over time reduces D6 activity.
(a) Flow cytometry profiles of HEK D6 cells after the addition of AF-CCL22 **(i)** in complete growth media or **(ii)** complete growth media supplemented with sodium chlorate for 2 days and **(iii)** 6 days. **(b)** Dot plot of MFI values from chemokine uptake assay, as determined by flow cytometry. N=5 per group. Data were analysed by unpaired, two tailed student T tests. **(c)** Flow cytometry histogram showing D6 expression of HEK D6 cells grown in normal growth media (cyan line) or media supplemented with sodium chlorate for 6 days (magenta line). D6 expression was measured using an anti-HA biotin antibody and streptavidin-PE secondary.

6.2.1.2 Increasing concentration of sodium chlorate

To further examine the ability of sodium chlorate to reduce ligand binding and internalisation by D6, HEK D6 cells were grown for 2 days in complete growth media supplemented with increasing concentrations of sodium chlorate. Firstly, to confirm the inhibition of sulphation by high concentrations of sodium chlorate, cell lysates from untreated HEK D6 cells and cells grown in 150mM sodium chlorate were analysed by Western blotting with an antibody that is able to detect sulphated tyrosines (Hoffhines et al., 2006) (**Figure 6-3 (a)**). Subsequent densitometry analysis using image J software (**Figure 6-3 (b)**) confirmed that sulphation of total cell protein was inhibited by growth in media supplemented with 150mM sodium chlorate.

Next, cells grown in different concentrations of sodium chlorate were used in chemokine uptake assays, as described previously (section 6.2.1.1) to measure D6-mediated binding and internalisation of AF-CCL22. As shown in **Figure 6-3 (c) (i)**, whilst concentrations of sodium chlorate as high as 100mM partially reduced ligand uptake by D6, concentrations of 150mM induced a considerably more marked, and significant, reduction compared to untreated cells. This was also shown by comparison of the flow cytometry profiles generated by untreated and 150mM sodium chlorate treated cells (**Figure 6-3 (c) (ii)**), which shows a considerable 'left shift' in cells treated with 150mM sodium chlorate. This result suggested an involvement of sulphation in ligand binding and internalisation by D6.

In summary, post-translational tyrosine sulphation in HEK D6 cells is inhibited by growth in high concentrations of sodium chlorate. This, in turn, reduces the chemokine uptake capability of D6.

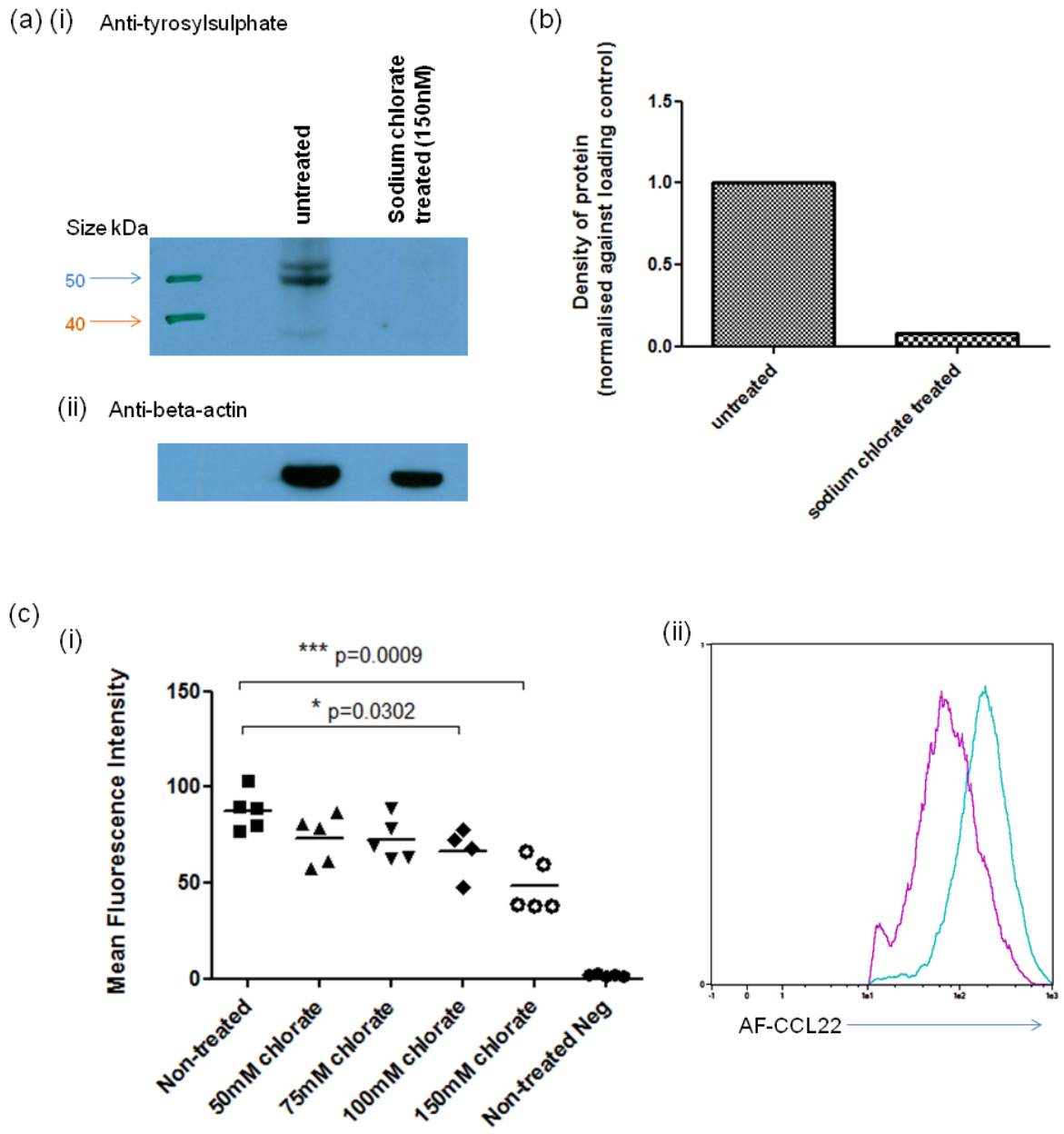


Figure 6-3: D6 activity decreases as sodium chlorate concentration increases.
(a) Western blot of HEK D6 lysates after growth in normal or sodium chlorate supplemented media for 2 days using **(i)** anti-tyrosylsulphate and **(ii)** anti-beta actin. **(b)** Histogram showing absolute density of protein. Bands were analysed using image J software and normalised against loading controls. **(c) (i)** Dot plot of MFI values from chemokine uptake assay, as determined by flow cytometry. N=5 per group. Data were analysed by unpaired, two tailed student T tests. **(ii)** Flow cytometry profile comparing uptake of AF-CCL22 by non-treated cells (cyan line) and cells grown in 150mM sodium chlorate (magenta line).

6.3 Site directed mutagenesis of D6

With evidence to suggest that sulphation of tyrosine residues on the D6 *N*-terminus is important for ligand binding, conventional mutagenesis was employed as an alternative experimental approach in order to examine this further.

Site-directed mutagenesis (described fully in chapter 2, section 2.3.2) was used to mutate the four tyrosine residues present on the *N*-terminus of D6. Phenylalanine was chosen to be substituted in place of tyrosine because both amino acids are similar in structure, with both containing a phenyl group; however the absence of a hydroxyl group in phenylalanine means it cannot be post-translationally sulphated.

Site-directed mutagenesis was performed using the original HA-D6 plasmid (described in chapter 2, section 2.3.1) as a template. In order to make the tyrosine to phenylalanine substitutions, the codon requires only one base pair change. Primers were designed which incorporated these base pair substitutions and the mutated plasmid was generated using PCR and cloning techniques. **Figure 6-4** summarizes the site-directed mutagenesis process. Subsequently the plasmid sequence containing mutated D6 was verified and transfected into HEK 293 cells in order to generate cells expressing a mutated version of D6 (henceforth referred to as mutant 1). **Figure 6-5** compares wildtype D6 with mutant 1.

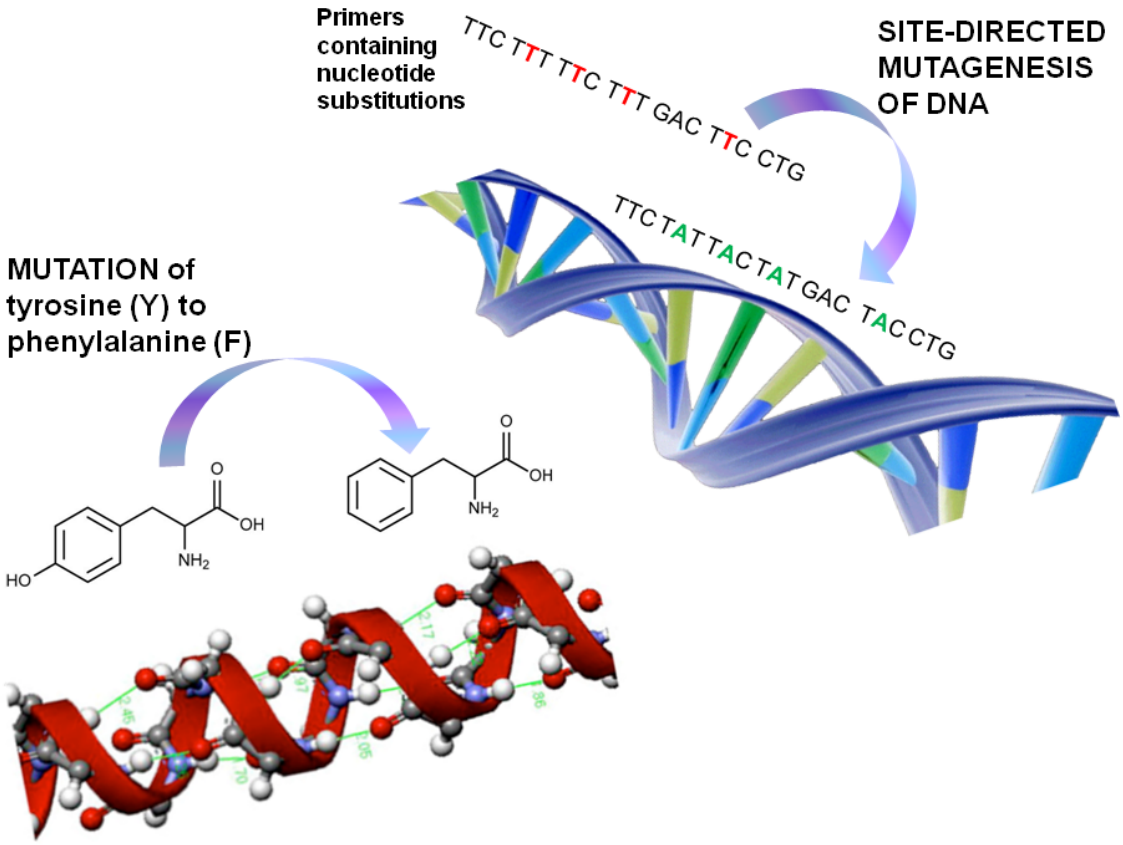


Figure 6-4: Site-directed mutagenesis
Site directed mutagenesis is achieved by using primers which incorporate nucleotide substitutions into the coding sequence of the DNA. By use of PCR techniques and cloning, these substitutions result in the production of a new plasmid with amino acid changes, in this case tyrosine to phenylalanine.

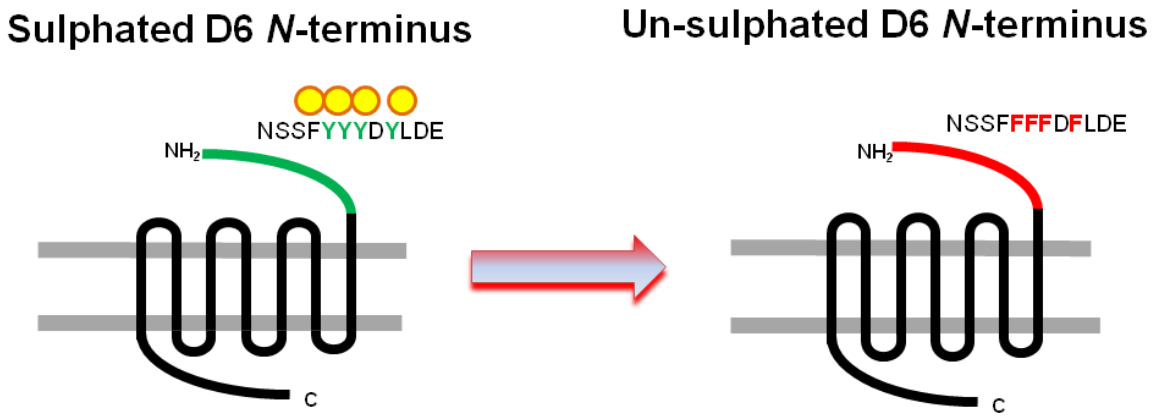


Figure 6-5: Comparing wildtype D6 and mutant 1 D6.
The human D6 *N*-terminus contains a tyrosine motif (YYYDY) which is thought to be post-translationally sulphated, although whether or not every tyrosine residue is sulphated is unknown. After site-directed mutagenesis, all the tyrosines have been substituted to phenylalanine, making this site biochemically unavailable for sulphation. Yellow circles are representative of possible sulphate groups (SO_4).

6.3.1 Stable transfection of HEK 293 cells with mutant 1

HEK 293 cells were stably transfected with the pcDNA3.1 plasmid encoding mutant 1 using effectene (as described in Chapter 2, section 2.4). Transfected mutant 1 cells were enriched for D6 positivity using the same method outlined in Figure 3-3, chapter 3. Two pools of mutant 1 cells resulting from enrichment were analysed alongside HEK D6 wt cells to assess their levels of D6 expression. Extracellular D6 levels were detected by flow cytometry using both an anti-HA biotin antibody followed by a secondary streptavidin-PE antibody, and the anti-D6 (clone 4A5) antibody followed by a secondary antibody conjugated to FITC. Flow cytometry plots for HEK D6 wt and the two different pools of mutant 1 transfectants are shown in **Figure 6-6**.

Figure 6-6 shows flow cytometry profiles of HEK D6 wt and the pools of mutant 1 cells using **(a)** anti-D6 and **(b)** anti-HA antibodies and shows that D6 expression was high in all three populations of cells. Mutant 1, pool 1 displayed very similar levels of D6 expression to HEK D6 wt, as shown by the MFI values from analysis using two different detection antibodies for D6.

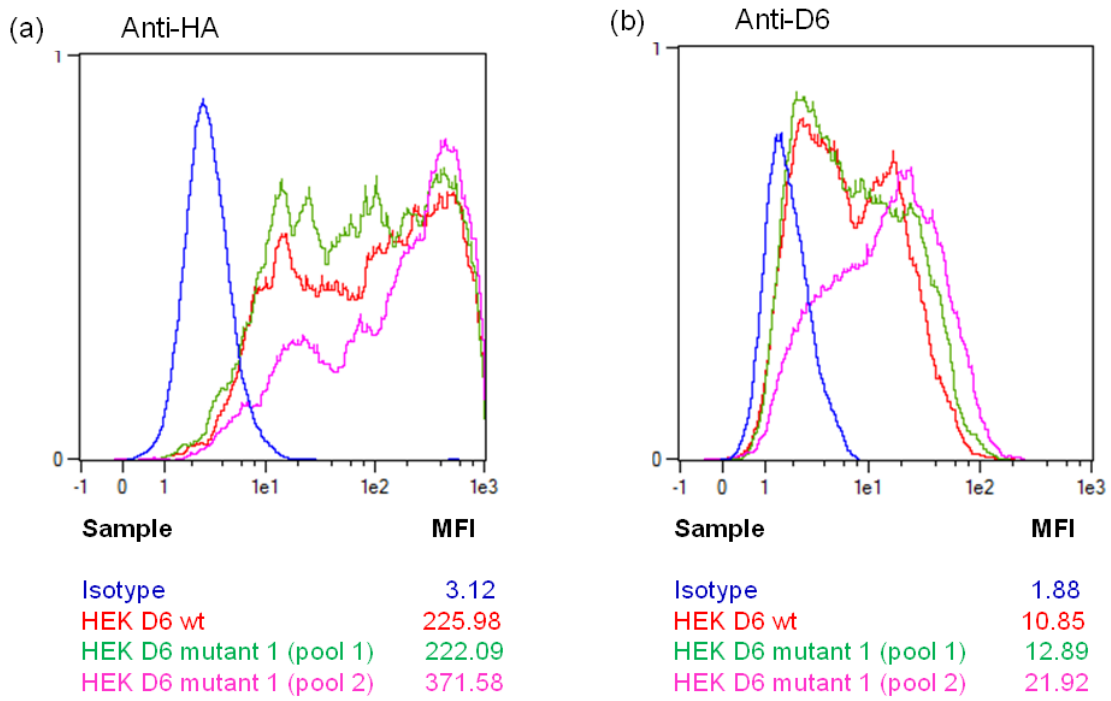


Figure 6-6: HEK D6 wt and mutant 1 (pool 1) express D6 at very similar levels.
Cells were assayed using **(a)** a mouse anti-HA (biotin) antibody or a mouse (biotin) isotype control and a streptavidin conjugated to R-phycoerythin (PE) secondary, or **(b)** the D6 (clone 4A5) antibody or a mouse isotype control and an anti-mouse FITC secondary antibody. The mean fluorescence intensities (MFIs) of D6-expressing cells are indicated underneath each flow cytometry plot.

6.3.2 Sulphation of the D6 *N*-terminus is reduced in mutant 1

Mutation of all tyrosine residues to phenylalanine on the *N*-terminus of D6 should, in principle, block post-translational sulphation, however in order to confirm this, cell lysates were prepared from HEK D6 wt, mutant 1 and un-transfected HEK 293 cells and subjected to SDS PAGE before Western blotting with the anti-tyrosylsulphate antibody. The resulting Western blot is shown in **Figure 6-7 (a)**. Sulphated tyrosines are present on many proteins expressed by HEK 293 cells and transfectants, as shown by the multitude of bands present on the Western blot in **Figure 6-7 (a)**. No conclusions could be drawn regarding the sulphation of D6 from this Western blot, as the blot highlighted every protein that possessed sulphated tyrosines, and D6 could not be distinguished from other proteins.

In order to improve comparisons of sulphation of D6 in the different cell lines, HA-tagged D6 was immunoprecipitated from HEK D6 wt and mutant 1 cell lysates using anti-HA magnetic microbeads (methods detailed in chapter 2, section 2.8.12). Samples were then analysed by SDS PAGE and Western blotting using both an anti-HA antibody to detect D6 and the anti-tyrosylsulphate antibody to detect sulphated tyrosines. Such Western blots are shown in **Figure 6-7 (b)**. Western blotting with an anti-HA antibody (**Figure 6-7 (b) (i)**) shows that D6 was successfully immunoprecipitated from HEK D6 wt and mutant 1 cell lysates. Probing immunoprecipitated D6 with the anti-tyrosylsulphate antibody (**Figure 6-7 (b) (ii)**) confirmed that wildtype D6 is sulphated, however sulphation of mutant 1 was considerably reduced and this was evident using two different pools of mutant 1, as shown on the anti-tyrosylsulphate Western blot.

In summary, mutation of all four tyrosine residues to phenylalanine on the N-terminus of D6 inhibits post-translational sulphation of this protein.

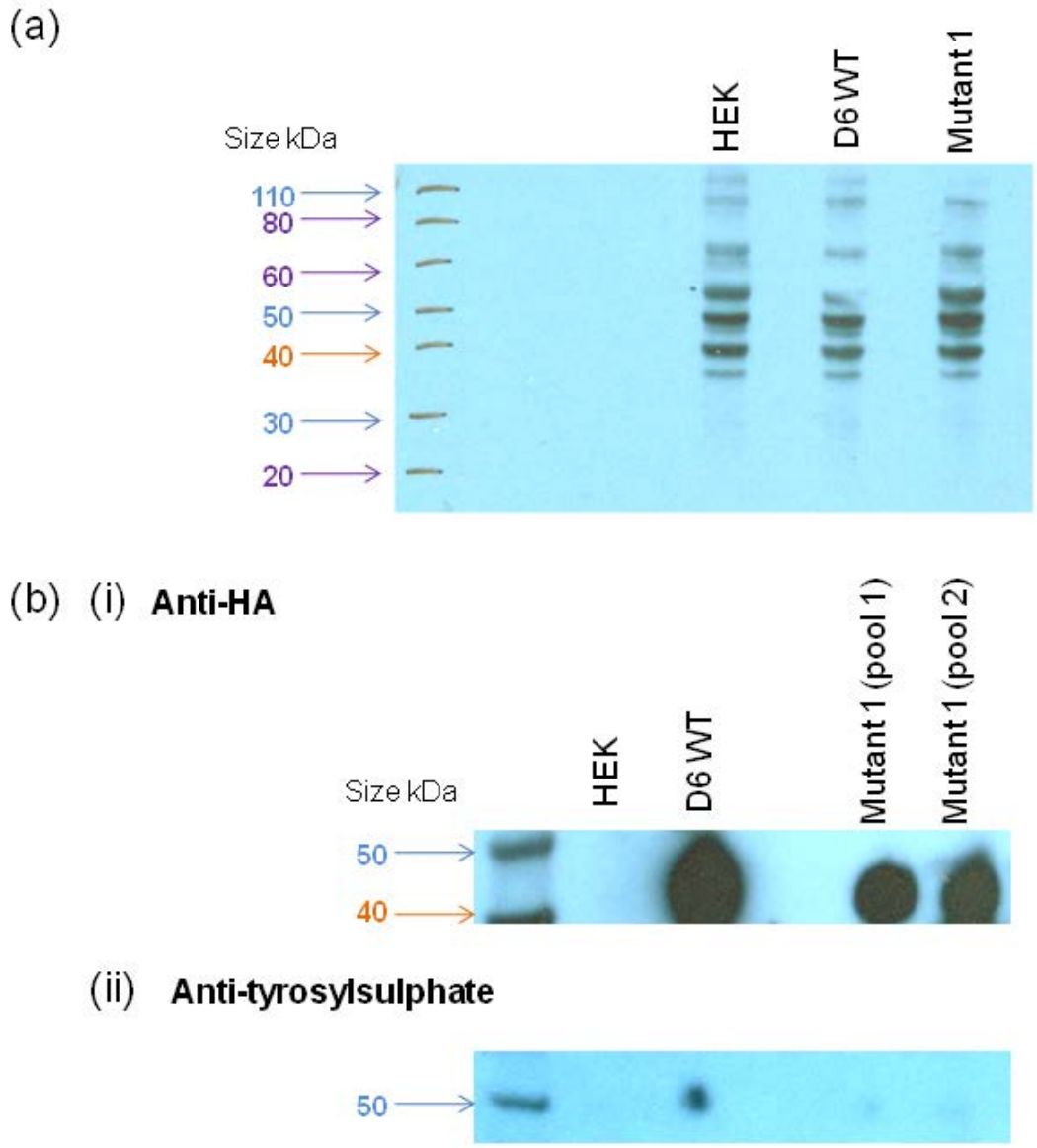


Figure 6-7: Sulphation of Mutant 1 is reduced compared to wildtype D6.
(a) Western blot of cell lysates from HEK 293, HEK D6 wt and Mutant 1 cells using the anti-tyrosyl sulphate antibody. **(b)** Western blot of immunoprecipitated D6 from different cell lysates (labelled) using **(i)** an anti-HA antibody which detects HA-tagged D6 and **(ii)** the anti-tyrosyl sulphate antibody.

6.4 Analysis of TPST 1 and TPST 2 expression

Tyrosine sulphation is dependent on the action of specific enzymes called tyrosylprotein-sulphotransferases (TPSTs). Two different TPSTs (TPST-1 and TPST-2) are known to be expressed, and functioning, in mammalian cells.

6.4.1 TPST-1 and TPST-2 expression in different tissue / cell types

To gain insights into which of these may be involved in the post-translational sulphation of D6, their expression was examined in tissues either associated with, or not associated with, D6 expression using QPCR (details on relevant methods can be found in chapter 2, sections 2.6 and 2.7). Briefly, RNA from different tissues or cell types was extracted and converted to cDNA and used in QPCR assays to determine the expression of TPST-1 and TPST-2 in these cell types. TATA-binding protein (TBP) was used as the house-keeping gene in this assay and expression of TPST-1 and TPST-2 is reported as numbers of transcripts per 10,000 copies of TBP. The cDNA from tissues or cell types used that are associated with D6 expression included keratinocytes (Singh et al., 2012), human dermal lymphatic endothelial cells (HD LECs) (Nibbs et al., 2001, McKimmie et al., 2013), placenta and trophoblasts (Madigan et al., 2010). Monocyte and osteoclast cDNA was used to represent cell types that are not associated with D6 expression. The resulting data are shown in **Figure 6-8**.

Figure 6-8 shows that although both TPST-1 and TPST-2 genes are expressed in every cell type, very little expression of TPST-1 was detected in any of the cell types examined. TPST-2, however, was expressed most strongly in HD LECs and placenta, which are both sites of high D6 expression. This suggests that TPST-2 may be more likely to contribute to tyrosine sulphation of D6 than TPST-1.

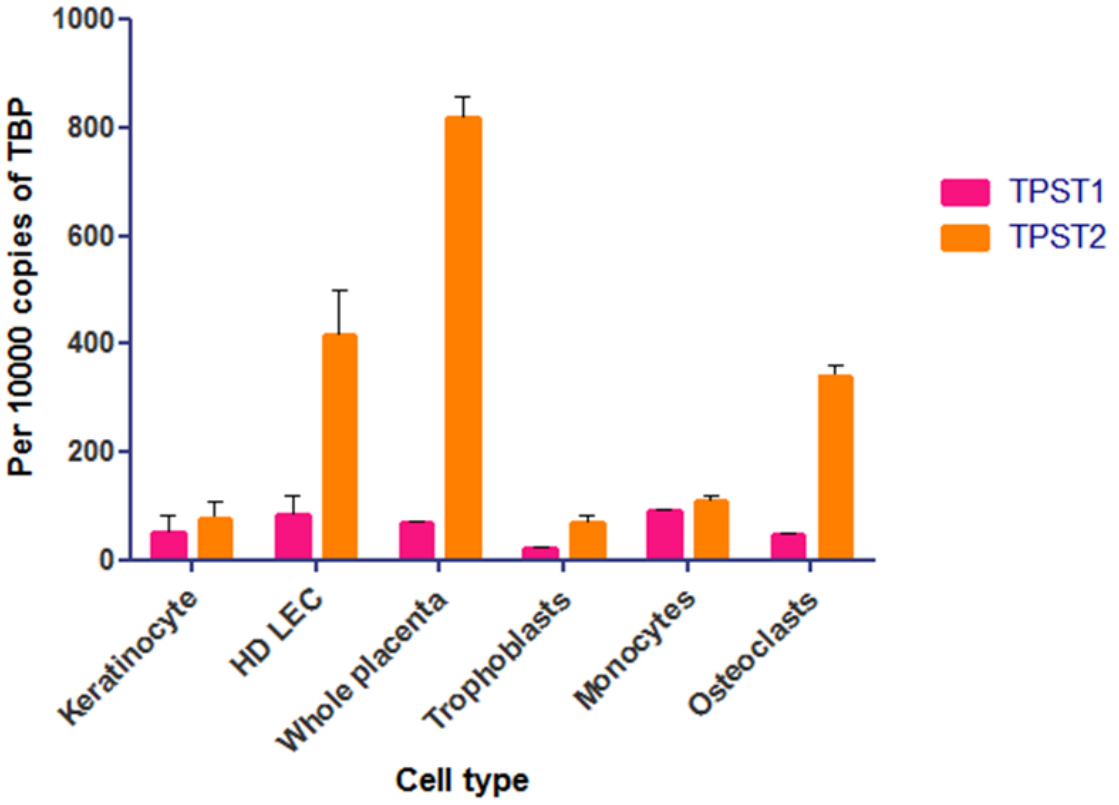


Figure 6-8: TPST-1 and TPST-2 expression in different tissues / cells.
QPCR analysis of the expression of TPST-1 and TPST-2 in the indicated cell types. Expression is reported as numbers of transcripts per 10,000 copies of Tata-binding protein (TBP). N=3 per sample, standard error of the mean (SEM) is plotted.

6.4.2 TPST-1 and TPST-2 expression in HEK 293 cell lines

As this study has used HEK D6 cells throughout, we next examined TPST-1 and TPST-2 in this cell line. The expression of TPSTs by transfected and un-transfected HEK 293 cells was examined at both the mRNA and protein level. THP1 cells are a type of monocyte that do not express D6 and were used as a control in these assays. RNA was extracted from HEK 293, HEK D6 wt and THP1 cells, converted to cDNA and used in QPCR assays to detect transcripts of TPST-1, TPST-2 and D6. Subsequently, cell lysates were produced from HEK 293, HEK D6 wt and THP1 cells and protein expression analysed by Western blotting using antibodies for TPST-1 and TPST-2. The data generated from such experiments are shown in **Figure 6-9**.

QPCR analysis revealed that TPST-1 and TPST-2 expression was detected in both transfected and un-transfected HEK 293 cells and in THP1 cells as shown in **Figure 6-9 (a)**. Notably D6 is strongly expressed in HEK D6 wt. The Western blots shown in **Figure 6-9 (b)** confirm that TPST-1 and TPST-2 proteins are expressed by HEK 293 cells and that transfection with D6 does not seem to alter the level of expression. Interestingly the Western blot suggests that both TPSTs are more highly expressed by THP1 cells than HEK 293 cells, although the QPCR data does not reflect this.

In summary, TPST-1 and TPST-2 are differentially expressed in all the tissues and cell types tested. Cell types and tissues that naturally express D6 at high levels also express high levels of TPST-2, suggesting that this may be the preferred enzyme for D6 sulphation. Both TPSTs are expressed by HEK 293 cells and transfection of these cells with D6 does not alter their expression of TPSTs. This indicates that HEK 293 cells grown in cell culture are likely to be capable of post-translational sulphation of proteins.

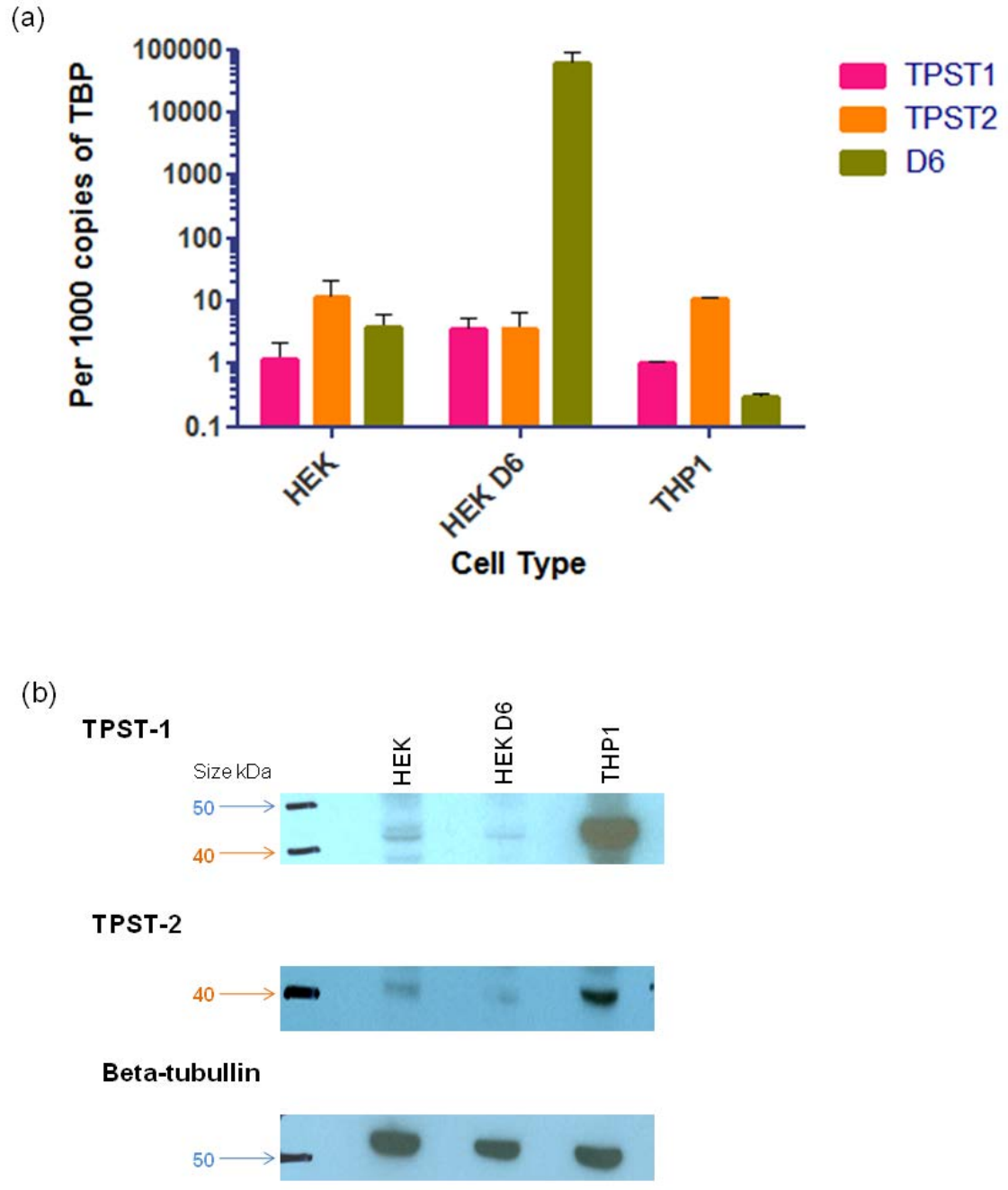


Figure 6-9: TPST-1 and TPST-2 are expressed by HEK 293 cells.
(a) QPCR analysis of the expression of TPST-1, TPST-2 and D6 in HEK 293, HEK D6 wt and THP1 cells. Expression is reported as numbers of transcripts per 1000 copies of Tata-binding protein (TBP). N=3 per sample, standard error of the mean (SEM) is plotted. **(b)** Western blot of cell lysates from HEK 293, HEK D6 wt and THP1 cells using antibodies for TPST-1, TPST-2 and beta tubulin. Beta tubulin was used as a housekeeping gene to show the equal protein loading to each well. Western blots are representative of 3 separate experiments.

6.4.3 TPST-1 and TPST-2 are involved in D6 sulphation

Having shown that TPST-1 and TPST-2 in HEK and HEK D6 cells have equivalent expression at the mRNA and protein levels, the importance of these enzymes for D6 sulphation and function in transfected HEK cells was examined. To do this, HEK 293 cells were pre-treated with TPST-1 siRNA, TPST-2 siRNA, or a mixture of both for 24 hours before transient transfection with D6 using the HA-D6 plasmid described previously. After another 24 hours cells were assayed for their ability to bind and internalise AF-CCL22 in chemokine uptake assays (**Figure 6-10 (a)**). D6 expression of each of the cell groups was also assessed by Western blotting and densitometry analysis (**Figure 6-10 (b)**).

Chemokine uptake assays, shown in **Figure 6-10 (a)**, revealed that treatment with TPST-1 siRNA or TPST-2 siRNA alone had no significant effect on ligand binding and uptake by D6, however simultaneous treatment with siRNA to both enzymes significantly reduced ligand binding. Western blotting of cell lysates from each of the treatment groups (**Figure 6-10 (b) (i)**) and subsequent densitometry analysis against a loading control **(ii)** showed that D6 expression was similar in all transfected cell groups, suggesting that the effect of siRNA treatment was not associated with any change in D6 protein expression. Together these results suggest that both TPST-1 and TPST-2 are independently able to contribute to sulphation of D6. The efficiency of siRNA to knockdown TPST 1 and TPST 2 was not assessed directly in this assay; therefore the result could also be due to off target effects of siRNA transfection.

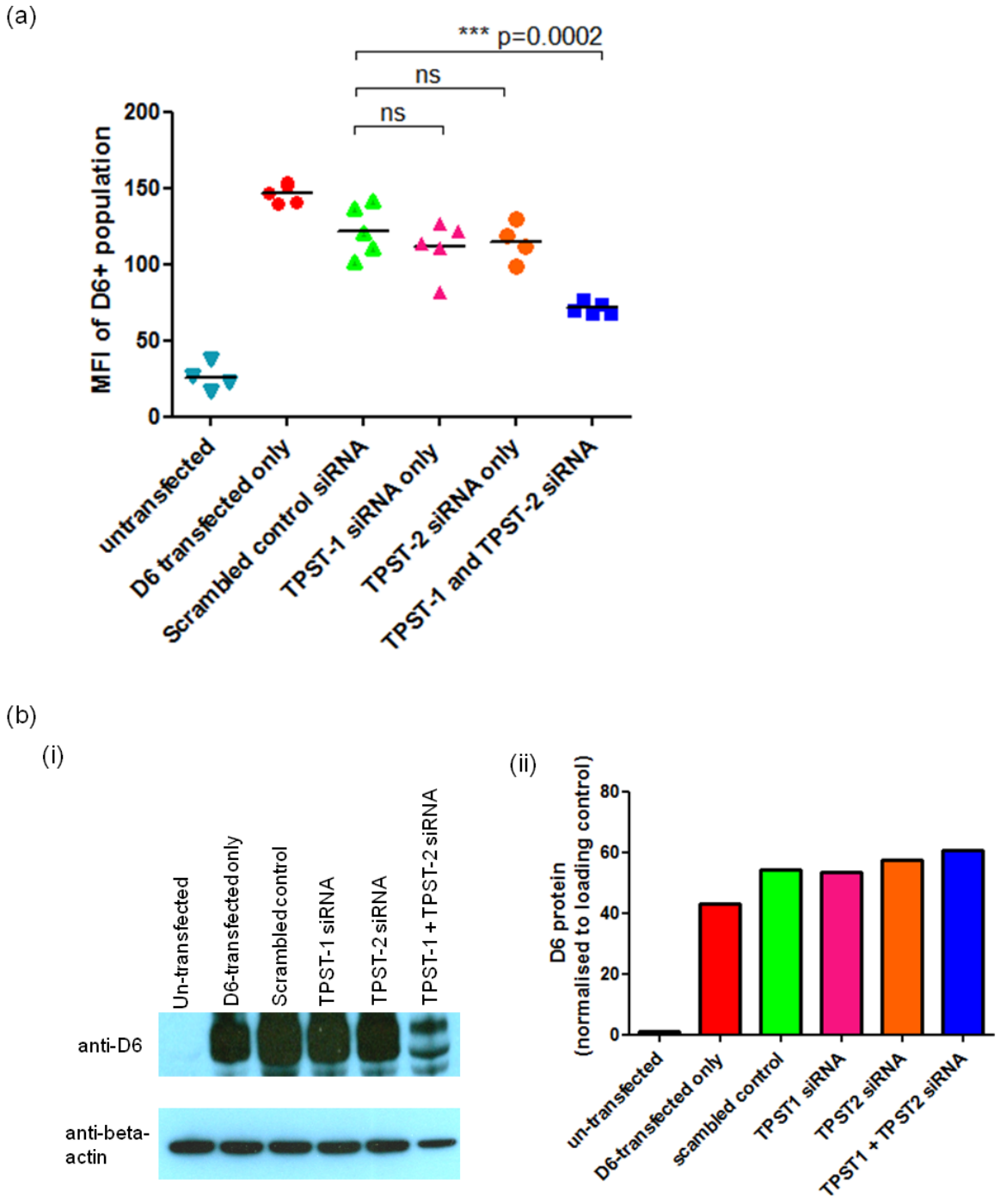


Figure 6-10: D6 activity is reduced by simultaneous down-regulation of TPST-1 and TPST-2. **(a)** Scatter plot indicating the results of multiple chemokine uptake experiments with AF-CCL22. Cells were analysed by flow cytometry and the MFI values for each experiment are plotted. Statistical analysis was performed using two-tailed unpaired t tests. N=4 or more per sample. ns = not significantly different. **(b)** Cell lysates from each treatment group were analysed by **(i)** Western blotting using an anti-D6 (Sigma) antibody and an anti-beta actin antibody. **(ii)** Densitometry analysis of bands from (i) using image J software. siRNA knockdown efficiency was not directly assessed in these experiments.

6.5 Ligand binding by D6 is greatly enhanced by receptor sulphation

Having obtained populations of HEK D6 wt and mutant 1 which express similar levels of D6, and having established that mutant 1 is not post-translationally sulphated, experimental procedures were implemented in order to establish whether the ability of D6 to uptake chemokine was affected by a lack of sulphation.

6.5.1 Mutant 1 has reduced ability to uptake ligand

To directly compare the ligand binding capabilities of HEK D6 wt and mutant 1, chemokine uptake assays were performed. AF-CCL2 was added to cells which were then incubated at 37°C for 1 hour to allow uptake and internalisation of fluorescently-labelled chemokine. Subsequently cells were washed twice to remove unbound chemokine, and DRAQ7 was added to cells before flow cytometry analysis in order to detect and exclude dead cells. **Figure 6-11** shows analysis and results of the chemokine uptake experiments.

Typical flow cytometry plots for HEK D6 wt and mutant 1 are shown in **Figure 6-11 (a)** after the addition of 0.6μg/ml AF-CCL2. The plots demonstrated that whilst HEK D6 wt can uptake fluorescently-labelled chemokine avidly, uptake of AF-CCL2 is greatly reduced in mutant 1. The histogram in **Figure 6-11 (b)** shows uptake of AF-CCL2 at a concentration of 0.6μg/ml by HEK 293, HEK D6 wt and mutant 1 cells, and allows easy comparison of the chemokine uptake capabilities of all three cell lines. This histogram clearly shows the increased capability of HEK D6 wt cells to uptake chemokine compared to cells expressing mutant 1. **Figure 6-11 (c)** shows the results from multiple repeat chemokine uptake experiments using increasing concentrations of AF-CCL2. MFI values of HEK D6 wt increase in a dose-dependent fashion after addition of AF-CCL2, however it was clear that mutant 1 displayed a significantly reduced ability to uptake ligand at each of the chemokine concentrations tested. There was no difference between mutant 1 and HEK 293 when 0.2 or 0.4μg/ml AF-CCL2 was added to cells. Even at the highest chemokine concentration (0.6μg/ml), mutant 1 displayed limited chemokine uptake capabilities, and this was greatly impaired compared to HEK D6 wt.

In summary, chemokine uptake capabilities are greatly reduced in a tyrosine-phenylalanine mutated version of D6 compared to wildtype. This suggests that post-translational sulphation of the D6 N-terminus is an important requirement for function of D6.

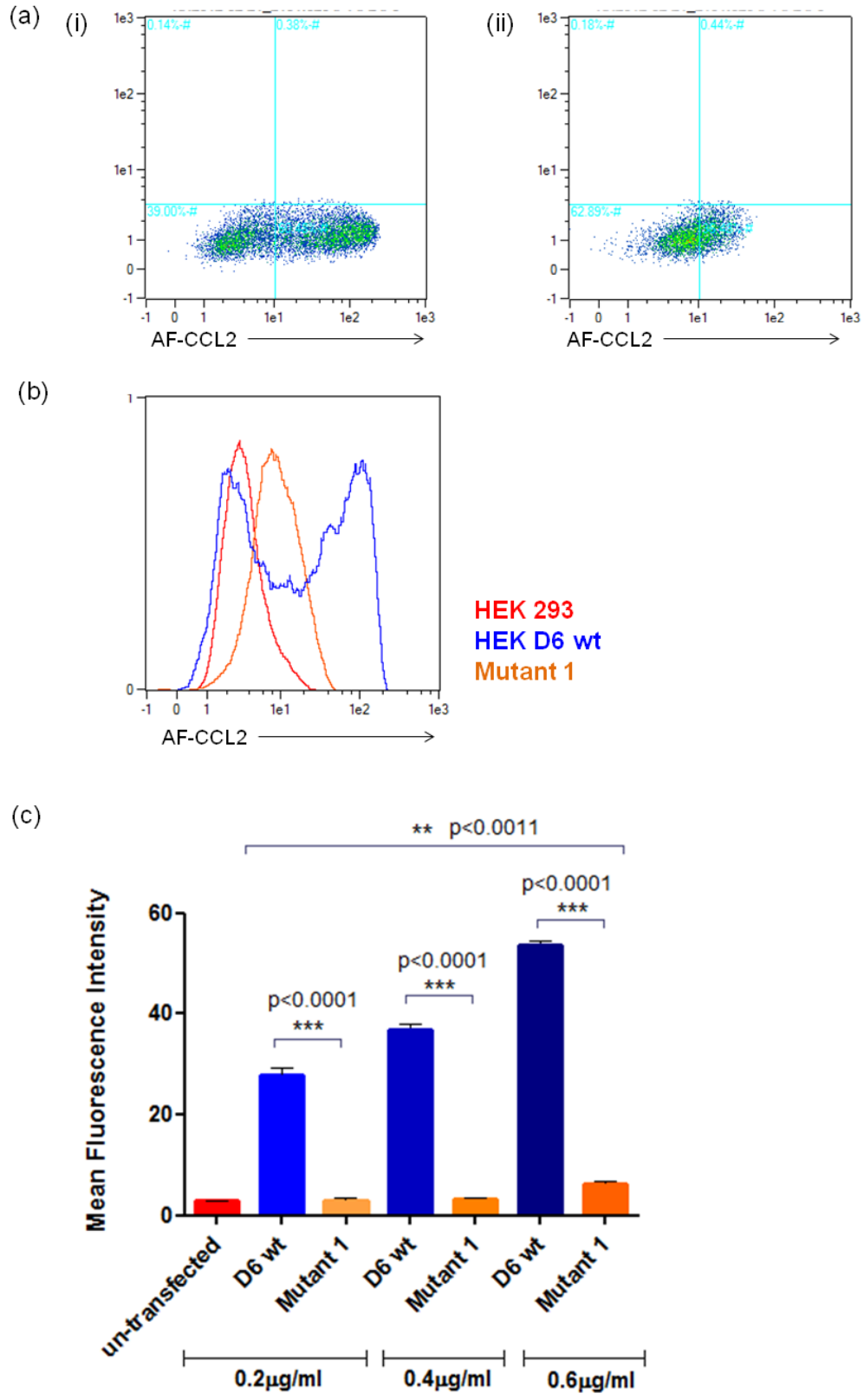


Figure 6-11: Mutant 1 has greatly reduced chemokine uptake capability.
(a) Flow cytometry profiles of **(i)** HEK D6 wt and **(ii)** mutant 1 after the addition 0.6µg/ml AF-CCL2 **(b)** Flow cytometry histogram allowing easy comparison of AF-CCL2 binding and uptake between HEK 293, HEK D6 wt and mutant 1. **(c)** Bar chart indicating the results of multiple chemokine uptake experiments using 0.2, 0.4 and 0.6µg/ml of AF-CCL2. Cells were analysed by flow cytometry and the MFI values for each experiment are plotted showing mean with standard deviation indicated. Statistical analysis was performed using two-tailed unpaired t tests. Means were found to be significantly different where indicated. N=5 per sample.

6.5.2 Mutant 1 has a reduced ability to degrade ligand

D6 is a highly effective scavenger of inflammatory CC chemokines and as well as binding to and internalising its ligands it also targets them for lysosomal degradation. Having confirmed that mutant 1 has a reduced ability to uptake ligand, we next tested the ability of HEK D6 wt and mutant 1 to degrade ligand. To investigate this, the ability of wildtype D6 and mutant 1 to remove CCL2 from its environment was examined. A chemokine degradation assay was performed whereby biotinylated CCL2 (bio-CCL2) was added to HEK D6 wt and two different pools of mutant 1 cells and incubated over a time course. Media were then collected and the amount of bio-CCL2 left in the media determined using Western blotting with streptavidin-HRP and measuring the relative density of each bio-CCL2 band using image J software.

Western blotting, shown in **Figure 6-12 (a)**, indicated that bio-CCL2 was degraded over time by HEK D6 wt cells and both pools of mutant 1 cells; however after 24 and 30 hours of incubation, there was a considerable difference in the extent of degradation between HEK D6 wt and the mutant 1 pools. Western blots showed that HEK D6 wt had degraded much more bio-CCL2 than cells expressing mutant 1 after incubation for 24 and 30 hours, indicating that wildtype D6 was able to degrade chemokine at a faster rate than non-sulphated D6. **Figure 6-12 (b)** shows the relative densities of bio-CCL2 bands in each of the media samples, further emphasizing the decreased rate of degradation by mutant 1 expressing cells compared with cells expressing wildtype D6.

In summary, the rate of D6-mediated degradation is reduced in a tyrosine-phenylalanine mutated version of D6 compared to wildtype. This is most likely due to the reduced ligand uptake capabilities of this mutant.

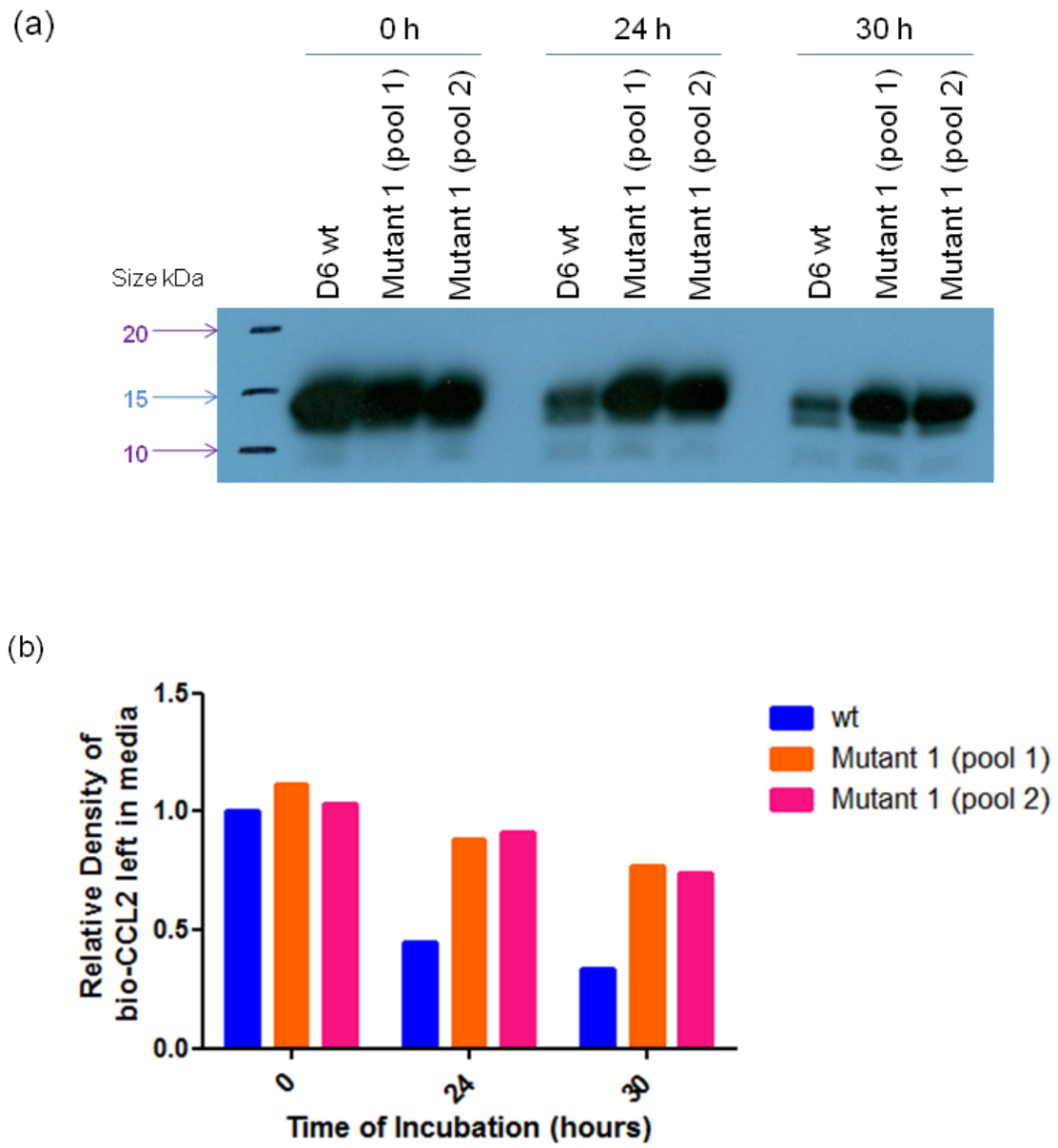


Figure 6-12: The speed of D6-mediated degradation of CCL2 is reduced in mutant 1. **(a)** Western blot using streptavidin-HRP of samples from a chemokine degradation assay. Bio-CCL2 was added to HEK D6 wt or different pools of mutant 1 cells and samples collected at different time points (indicated). **(b)** The relative density of bands from the Western blot shown in (a) were analysed using image J software and results are presented in a histogram. N=1.

6.5.3 Chemokine uptake is not blocked in mutant 1

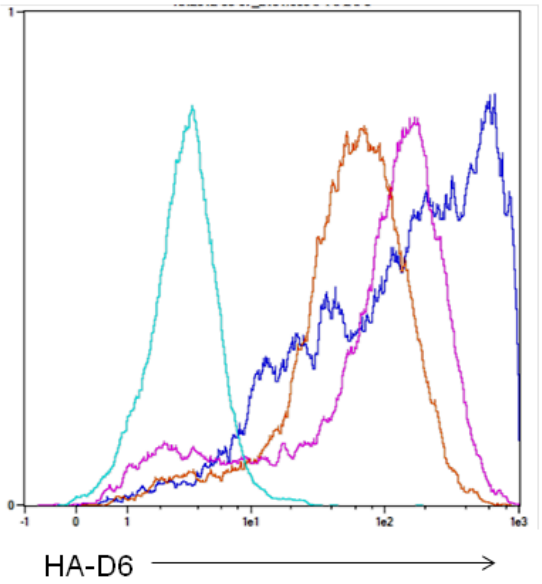
Previous experiments established that inhibiting post-translational sulphation of D6 drastically decreased its ability to uptake CCL2, even when relatively high concentrations of chemokine were tested. In further experiments, levels of D6 expression were re-analysed by flow cytometry with an anti-HA antibody to ensure that each cell line was still expressing similar levels of D6 protein (**Figure 6-13**). Next, more chemokine uptake experiments were performed with HEK D6 wt and the two different pools of mutant 1 cells, however this time the cells were exposed to extremely high concentrations of AF-CCL2 (1.2µg/ml) in order to test if the mutant receptor was fully incapable of chemokine uptake even at super-physiological chemokine levels. MFI values were recorded and the resultant data, as well as representative flow cytometry plots, are displayed in **Figure 6-14**. The data plotted in **Figure 6-14 (c)** have been normalised by taking into account the relative D6 expression levels in the cell lines.

Interestingly, the flow cytometry plot shown in **Figure 6-13** revealed that levels of D6 protein on the surface of cells expressing the D6 mutant (pools 1 and 2 of mutant 1) had both decreased almost 3 fold compared to levels that were previously recorded (Figure 6-6), however the levels of D6 expression in HEK D6 wt had slightly increased in this period of time. This observation suggests that as well as being important for ligand binding, tyrosine residues on the D6 *N*-terminus may also be an important pre-requisite for optimal trafficking of the protein to the cell surface. However it could also be a consequence of expression levels of transfected cells drifting over time.

Typical flow cytometry plots for HEK D6 wt **(i)** and the two different pools of mutant 1 **(ii)** and **(iii)** are shown in **Figure 6-14 (a)** after the addition of 1.2µg/ml AF-CCL2. The histogram shown in **Figure 6-14 (b)** allows easy comparison of the chemokine uptake capabilities of all three cell lines at this high chemokine concentration. **Figure 6-14 (c)** shows the resulting MFI values of multiple repeat chemokine uptake experiments. The results again demonstrate that both pools of mutant 1 had significantly reduced uptake capabilities compared to wildtype D6, however the difference between uptake values for HEK D6 wt and mutant 1 decreased compared to the values recorded in the previous uptake experiment from a ~10-fold difference at 0.6µg/ml AF-CCL2 (see Figure 6-11 (c)) to a ~2.3-fold difference at 1.2µg/ml AF-CCL2 (**Figure 6-14 (c)**). This result suggests that mutant 1 is not incapable of binding and taking up CCL2, however it is less equipped to do

so compared to wildtype D6, presumably as a result of a weaker binding affinity between un-sulphated D6 and CCL2.

In summary, mutant 1's chemokine uptake capability was consistently reduced compared to wildtype D6; however it was not completely incapable of taking up chemokine when exposed to very high concentrations. The amount of mutant 1 protein present on the cell surface decreased over time in cell culture, suggesting that tyrosines on the N-terminus of D6 may also contribute to optimal trafficking of the receptor to the cell surface.



Sample	MFI
Isotype	7.25
HEK D6 wt	290.74
Mutant 1, pool 1	79.10
Mutant 1, pool 2	130.47

Figure 6-13: Surface expression levels of mutant 1 decrease over time in cell culture. Cells (indicated) were assayed using a mouse anti-HA (biotin) antibody or a mouse (biotin) isotype control and a streptavidin conjugated to R-phycoerythin (PE) secondary. The mean fluorescence intensities (MFI's) of each cell type are indicated beside the flow cytometry plot.

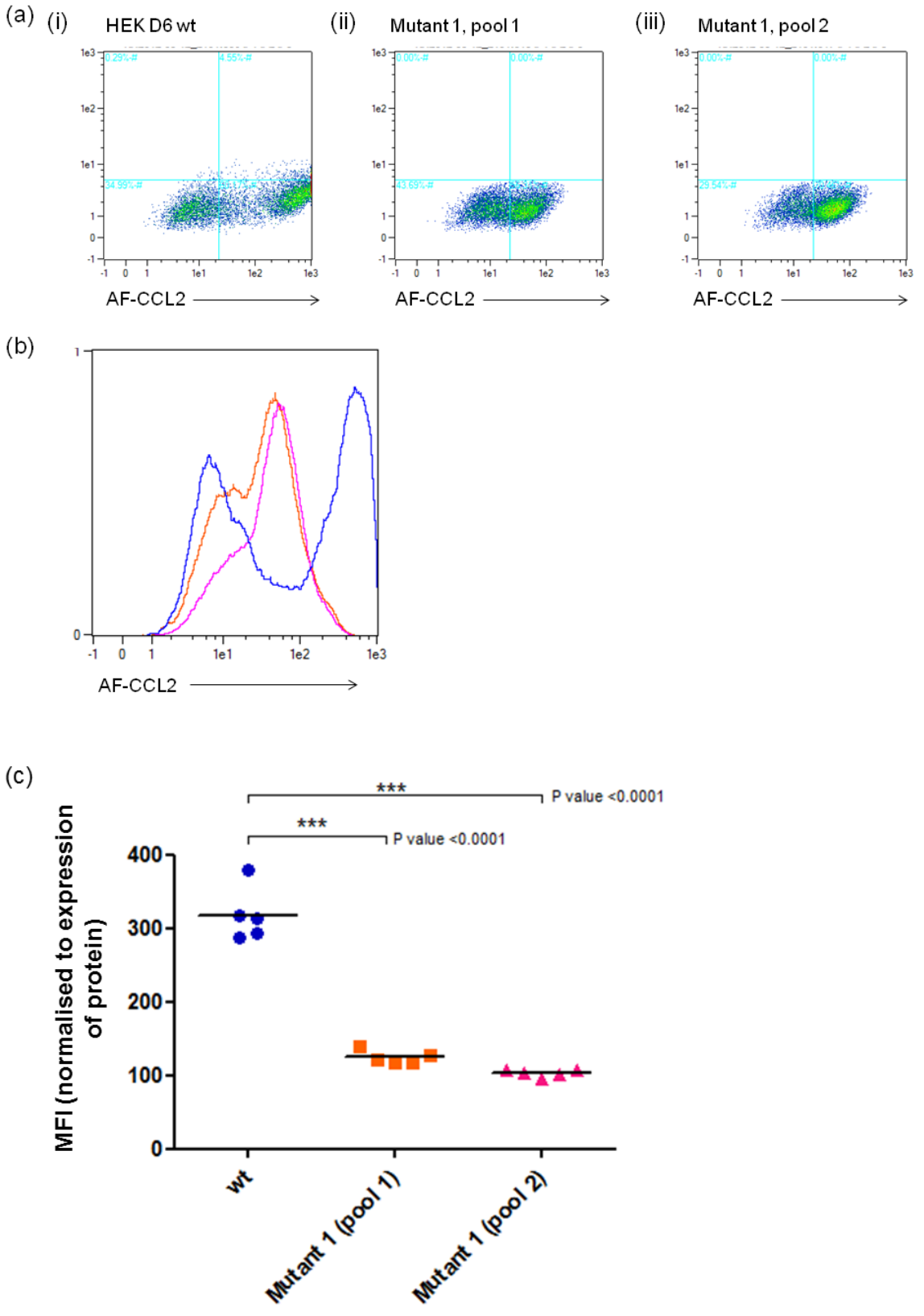


Figure 6-14: Mutant 1 is functional at high CCL2 concentrations.
(a) Flow cytometry profiles of **(i)** HEK D6 wt **(ii)** mutant 1, pool 1 and **(iii)** mutant 1, pool 2 after the addition 1.2µg/ml AF-CCL2. **(b)** Flow cytometry histogram allowing comparison of AF-CCL2 binding and uptake between HEK D6 wt (blue), mutant 1, pool 1 (orange) and mutant 1, pool 2 (pink). **(c)** Dot plot indicating the results of multiple chemokine uptake experiments using 1.2µg/ml of AF-CCL2. Cells were analysed by flow cytometry and the MFI values for each experiment were normalised against the D6 and Mutant 1 expression measurements shown in Figure 6-13. Statistical analysis was performed using two-tailed unpaired t tests. N=5 per sample.

6.6 Investigating the effect of single tyrosine mutations

As detailed in the introduction to this chapter, D6 has a conserved tyrosine motif (YYYDY) at its *N*-terminus which is thought to be sulphated, although it is not known if all four tyrosine residues are sulphated or if there is a specific pattern of sulphated residues. Results presented in Chapter 4 regarding the sulphated D6 peptide and the previous results in this chapter indicate that sulphation of the D6 *N*-terminus enhances ligand binding; however the optimal pattern of sulphation for ligand affinity is unknown.

6.6.1 Generation of different sulphation mutants

In order to try to establish if a single tyrosine residue is essential for ligand binding, more specific mutagenesis studies were performed, in which individual tyrosines within the conserved motif were mutated to phenylalanines. Previous analysis of the evolutionary conservation of the tyrosine motif confirmed that the first, third and fourth tyrosines are highly conserved within higher order primates (see Figure 6-1), therefore mutants were generated in which either the first, third or fourth tyrosine in the motif were substituted with phenylalanine. **Table 6-1** summarises these new mutants, which are referred to as mutant 2, mutant 3 and mutant 4 throughout this thesis.

Mutant Name	Description	Predicted *N*-terminal sulphation status	Protein sequence of tyrosine region
D6 WT	All tyrosines present	S-S-S-x-S	YYYDY
Mutant 1	All tyrosines mutated	U-U-U-x-U	FFFDF
Mutant 2	First tyrosine mutated	U-S-S-x-S	FYYDY
Mutant 3	Third tyrosine mutated	S-S-U-x-S	YYFDY
Mutant 4	Fourth tyrosine mutated	S-S-S-x-U	YYYDF

Table 6-1: Description of versions of D6 mutants with variations of the conserved tyrosine motif. U = un=sulphated, S = sulphated.

Plasmids encoding the different D6 mutants were generated by site directed mutagenesis of the original wildtype-D6 encoding plasmid, similar to processes described in section 6.3. Subsequently the plasmids were transfected into HEK 293 cells using methods detailed in chapter 2, section 2.4. After transfection, D6 expression was assessed by flow cytometry using an anti-HA biotin antibody followed by a secondary streptavidin-PE antibody. Levels of D6 expression were variable for each mutant; overall, however D6 expression was low, with ~20% of cells expressing D6 mutants on their surface. In order to increase the number of D6 (mutant) expressing cells to correspond with HEK D6 wt levels, several rounds of $D6^+$ cell enrichment were performed with the use of the anti-HA biotin antibody and anti-biotin microbeads (Figure 3-3 in chapter 3 illustrates this process). The cells collected after the enrichment process were cultured and left to grow to confluency before performing the next enrichment. **Figure 6-15** shows representative flow cytometry profiles of mutant 2 after the initial transfection and subsequent to each enrichment step, which resulted in almost 80% of cells expressing D6 following the final enrichment. Mutants 2, 3 and 4 were enriched in this manner, with similar purities of $D6^+$ cells being obtained for each.

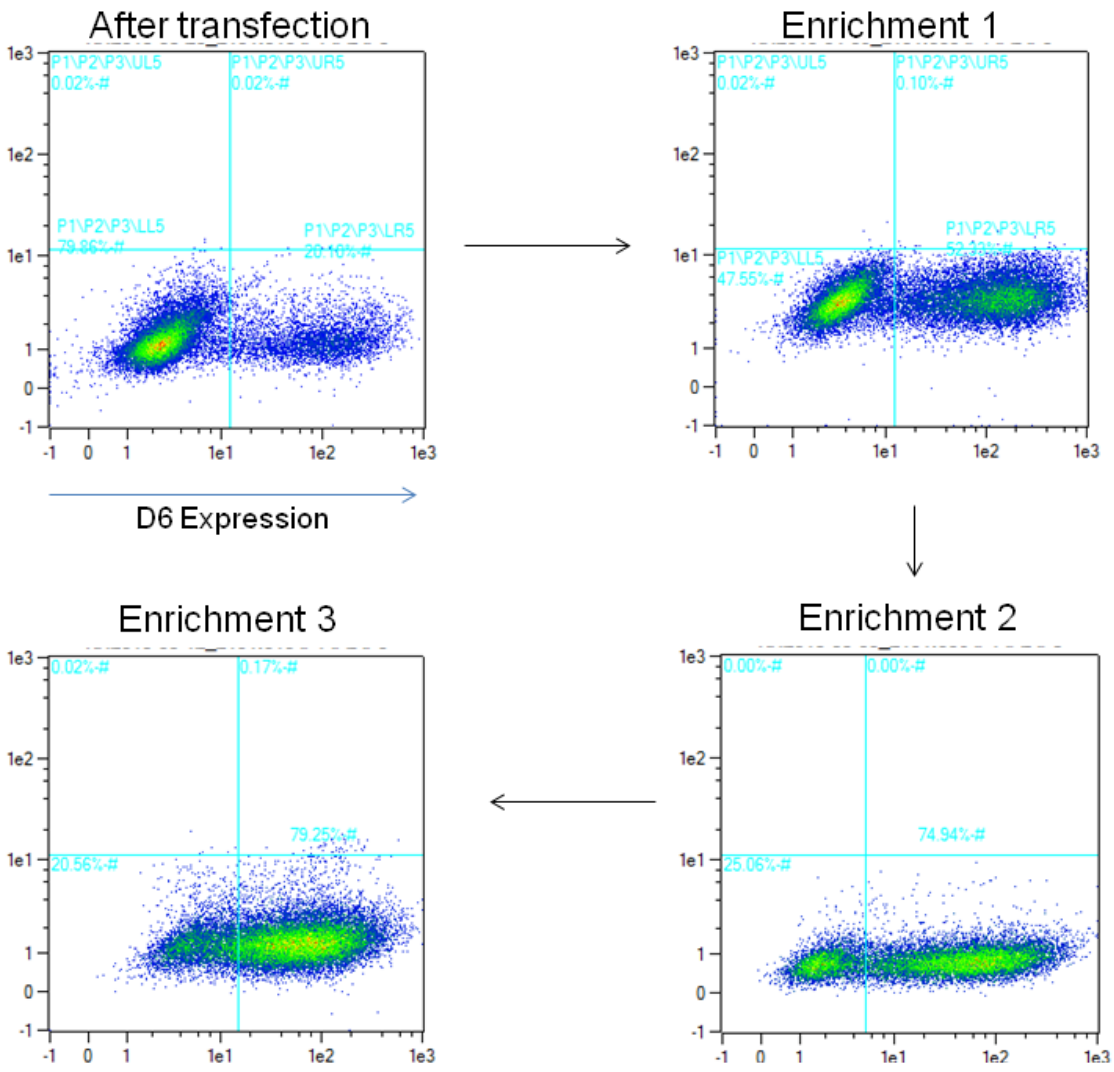


Figure 6-15: Enrichment for D6 (mutant) expressing cells after transfection.
Cells were assessed for D6 expression using a mouse anti-HA (biotin) antibody or a mouse (biotin) isotype control and a streptavidin conjugated to R-phycoerythin (PE) secondary. Flow cytometry plots after transfection and subsequent to each enrichment are shown. The percentage of D6 positive cells increases from ~20% after transfection to ~80% after enrichment 3. The profiles shown are from mutant 2 but were very similar for each mutant.

6.6.2 No single tyrosine residue on the D6 *N*-terminus is essential for ligand binding

To directly compare the ligand binding capabilities of HEK D6 wt and each of the single tyrosine mutants (mutants 2, 3 and 4), chemokine uptake assays were performed on each cell line as detailed previously (section 6.5.1). In order to ensure comparisons could be made between each cell line, D6 expression of HEK D6 wt and mutants 2, 3 and 4 was assessed shortly before the chemokine uptake assay by flow cytometry. The resultant flow cytometry plots are shown in **Figure 6-16 (a)**. Ligand uptake capability was then assessed with the use of high concentrations of AF-CCL2 (1μg/ml). **Figure 6-16 (b)** shows the results of several repeat chemokine uptake experiments.

The flow cytometry plots in **Figure 6-16 (a)** confirm that D6 expression levels were similar for each of the D6 expressing cell lines cell lines (**(ii)**, **(iii)**, **(iv)** and **(v)**), at the time of experimentation. The data presented in **Figure 6-16 (b)** show that CCL2 uptake by mutant 2, 3 and 4 was not significantly different from that of HEK D6 wt. The scatter plots do draw attention to a trend suggesting that there was a slightly decreased MFI for each mutant compared with wildtype D6, although these differences were not significant. This result demonstrated that mutation of only one of the three conserved tyrosines to phenylalanine did not significantly impair ligand binding compared to wildtype D6.

In summary, mutation of individual tyrosine residues in positions 1, 3 or 4 of the conserved tyrosine motif on the N-terminus of D6 does not impair the ability of D6 to uptake CCL2.

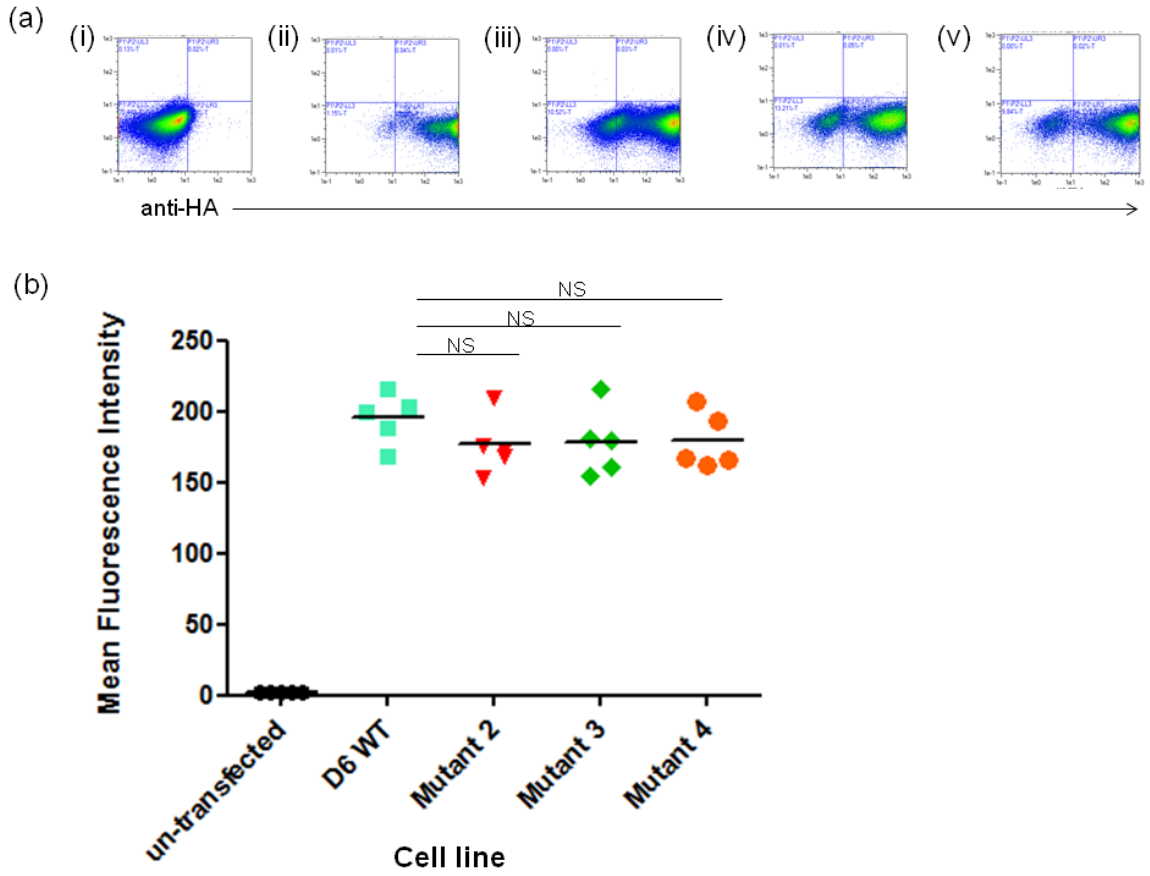


Figure 6-16: No single tyrosine residue on the D6 *N*-terminal is essential for ligand binding. **(a)** Flow cytometry profiles of **(i)** un-transfected HEK 293 cells, **(ii)** HEK D6 wt, **(iii)** Mutant 2, **(iv)** Mutant 3, **(v)** Mutant 4. Cells from each cell line were assayed for D6 expression using a mouse anti-HA (biotin) antibody or a mouse (biotin) isotype control and a streptavidin conjugated to R-phycoerythin (PE) secondary. **(b)** Dot plot indicating the results of multiple chemokine uptake experiments using 1µg/ml of AF-CCL2. Cells were analysed by flow cytometry and the MFI values for each experiment are plotted. Statistical analysis was performed using two-tailed unpaired t tests. N=5 per sample. NS = not significantly different.

6.7 Generation of a catalogue of D6 mutants

Having already established that Mutant 1's capability to uptake ligand is greatly reduced compared to wildtype D6, and with data to suggest that mutation of only one tyrosine residue does not alter the ability of D6 to uptake CCL2; it was decided that the reciprocal experiment should be performed, in order to establish if the presence of a single tyrosine residue at any of the four positions is sufficient to rescue ligand binding capability. In order to achieve this, cell lines were generated which expressed mutant versions of D6 with only one of the four tyrosine residues retained on the D6 *N*-terminus. These were named mutants 6-9, with mutant 6 retaining only the first tyrosine residue and mutant 7 retaining only the second tyrosine residue etc. Similar to the generation of previous mutants of D6,

site-directed mutagenesis of a plasmid encoding D6 was performed before sequence verification and transfection into HEK 293 cells. As all but one tyrosine residue was mutated to phenylalanine in each of these new mutants, the plasmid encoding Mutant 1 was used as the template for the site-directed mutagenesis reaction as this plasmid required only one base-pair change in order to make mutants 6-9. A mutant was also generated in which the non-conserved tyrosine in position 2 was mutated to phenylalanine but tyrosines in positions 1, 3 and 4 were retained. Originally this mutant was not thought to be important because of the absence of this tyrosine in non-primate mammals, however upon consideration the mutant was generated in order to ascertain if the gain of a tyrosine in position 2 of the highly conserved motif had any functional significance for higher order primates. The plasmid encoding wildtype D6 was used as the template for this mutant; named Mutant 5. All the mutants generated in this study are summarised in **Table 6-2**.

Mutant Name	Description	Predicted N-terminal sulphation status	Protein sequence of tyrosine region
Mutant 1	All tyrosines mutated	U-U-U-x-U	FFFDF
Mutant 2	First tyrosine mutated	U-S-S-x-S	FYYDY
Mutant 3	Third tyrosine mutated	S-S-U-x-S	YYFDY
Mutant 4	Fourth tyrosine mutated	S-S-S-x-U	YYYDF
Mutant 5	Second tyrosine mutated	S-U-S-x-S	YFYDY
Mutant 6	Only first tyrosine present	S-U-U-x-U	YFFDF
Mutant 7	Only second tyrosine present	U-S-U-x-U	FYFDF
Mutant 8	Only third tyrosine present	U-U-S-x-U	FFYDF
Mutant 9	Only fourth tyrosine present	U-U-U-x-S	FFFDY

Table 6-2: Description of all the D6 mutants generated with variations of the conserved tyrosine motif.

6.8 Investigating the activity of different D6 mutants

6.8.1 Analysis of mutant cell lines for D6 expression

In order to obtain populations of cells expressing similar levels of each D6 mutant, Mutants 5-9 were enriched by the same method detailed previously (section 6.6.1) with the aim of isolating D6 expressing cells. Following the enrichment process, all mutant cell lines and HEK D6 wt were analysed by flow cytometry using an anti-HA biotin antibody followed by a secondary streptavidin-PE antibody. The data are presented in the histograms in **Figure 6-17**.

Both histograms in **Figure 6-17** are set out in such a way to group mutants with similar tyrosine patterns together, therefore HEK cells expressing wildtype D6 and Mutants 2, 3, 4 and 5, which each have only one tyrosine substituted to phenylalanine, are side by side. Likewise Mutant 1, which has all its tyrosines substituted to phenylalanine, and Mutants 6, 7, 8 and 9, which each have all but one tyrosine substituted to phenylalanine, are depicted side by side. **Figure 6-17 (a)** demonstrates that all the cell lines have a high percentage (>75%) of D6-expressing cells. **Figure 6-17 (b)** shows that despite all cell lines displaying high levels of transfection, the levels of cell surface D6, as measured by mean fluorescence intensity, are reduced in the cell lines expressing mutants of D6 with only one or no tyrosine residues on the *N*-terminus.

In summary, all cell lines showed high levels of transfection with D6; however there was a marked difference in cell surface D6 expression between cell lines and this seemed to correlate with the number of tyrosines present on the D6 N-terminus.

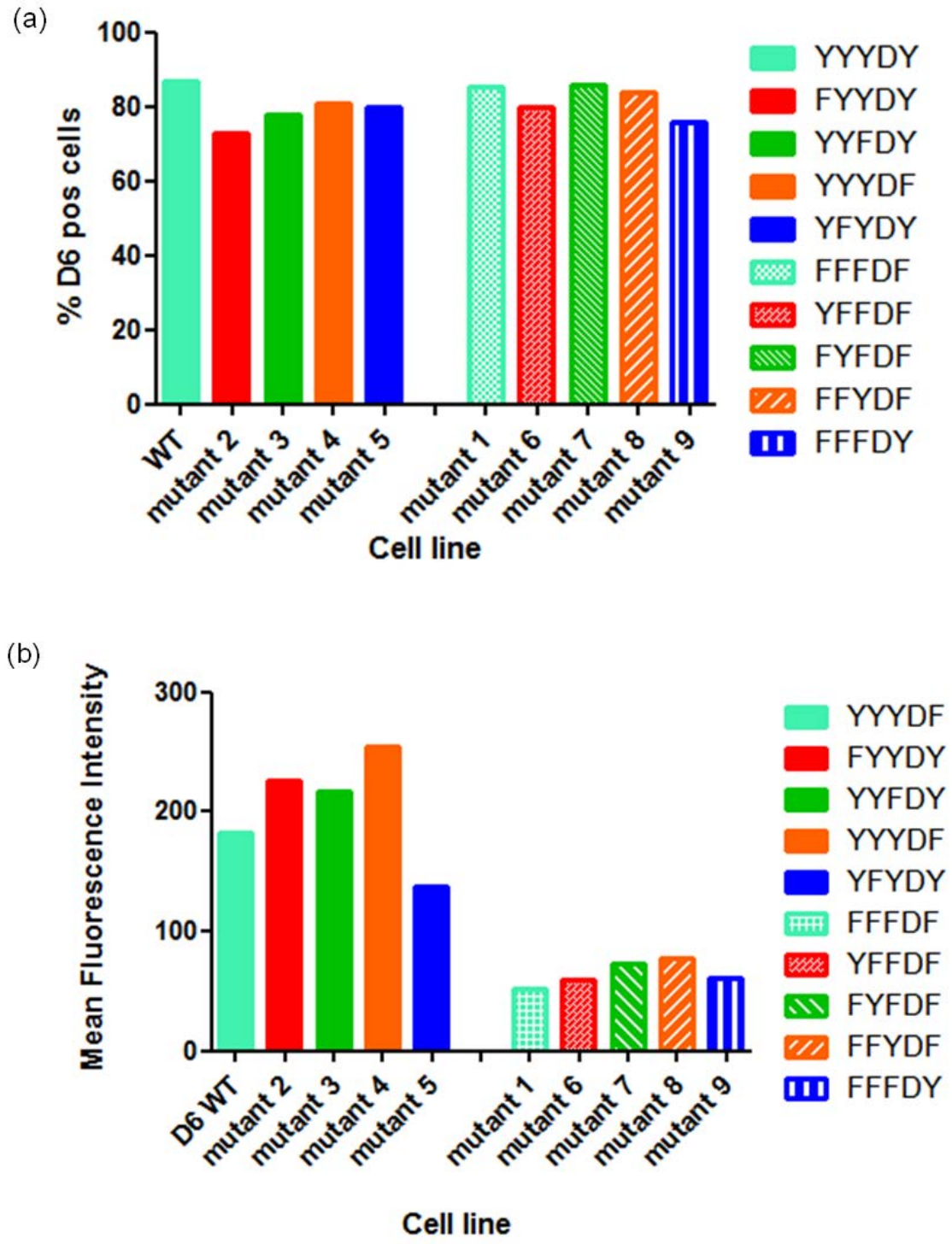


Figure 6-17: Percentage and level of D6 expression shown by mutant cell lines.
Each cell line was assayed for D6 expression using a mouse anti-HA (biotin) antibody or a mouse (biotin) isotype control and a streptavidin conjugated to R-phycoerythin (PE) secondary. **(a)** Histogram showing the percentage of D6-expressing cells of each cell line. **(b)** Histogram showing the level of extracellular D6 expression shown by each cell line measured by their mean fluorescence intensities. 1 x 10^7 cells analysed per mutant.

6.8.2 Mutant 5 has enhanced ability to uptake CCL22 compared to WT D6

Previous experiments using chemokine uptake assays to compare Mutants 2, 3 and 4 to WT D6 revealed that the ligand uptake and scavenging phenotype of WT D6 is unaffected by individually altering three out of the four tyrosine residues on the *N*-terminus. In further experiments, the newly generated Mutant 5 was tested alongside WT D6 and Mutants 2, 3 and 4 for its ability to uptake fluorescently labelled chemokine. In these experiments AF-CCL22 was used in place of AF-CCL2 in order to compare different D6 ligands for their ability to bind and be taken up by the different D6 mutants. **Figure 6-18** shows the results of the chemokine uptake assay as well as flow cytometry profiles displaying the D6 expression levels of each mutant compared to WT D6.

The flow cytometry profiles depicted in **Figure 6-18 (a)** show that the cell lines expressing WT D6 or Mutant 2, 3, 4 and 5 had very similar levels of cell surface D6 expression at the time of experimentation and could therefore be compared. The data presented in **Figure 6-18 (b)** show that AF-CCL22 uptake by Mutant 2 was slightly reduced compared to WT D6 but this difference was not significant. On the other hand, uptake of AF-CCL22 by Mutant 3 and Mutant 4 was significantly reduced compared to WT D6, and this result is in contrast to the data generated while using AF-CCL2, where the reduction in uptake capability by Mutant 3 and Mutant 4 was not significant (see Figure 6-16). Most surprisingly however, AF-CCL22 uptake by Mutant 5 was enhanced compared to WT D6, and this result was highly significant. Interestingly, the tyrosine mutated to phenylalanine in Mutant 5 is not a conserved tyrosine in non-primate mammals and may therefore represent an evolutionary adaptation in human and primate D6 that alters the ability of D6 to bind CCL22. The contrasting results for Mutants 3 and 4 with CCL2 and CCL22 demonstrated that there may be subtle differences in ligand binding affinity and uptake capability depending on the ligand and depending on peptide sequence of the mutant in question.

In summary, Mutant 5 has an enhanced ligand binding and uptake capability compared to WT D6. The differences in ligand binding and uptake capability between each mutant may be altered depending on the ligand in question.

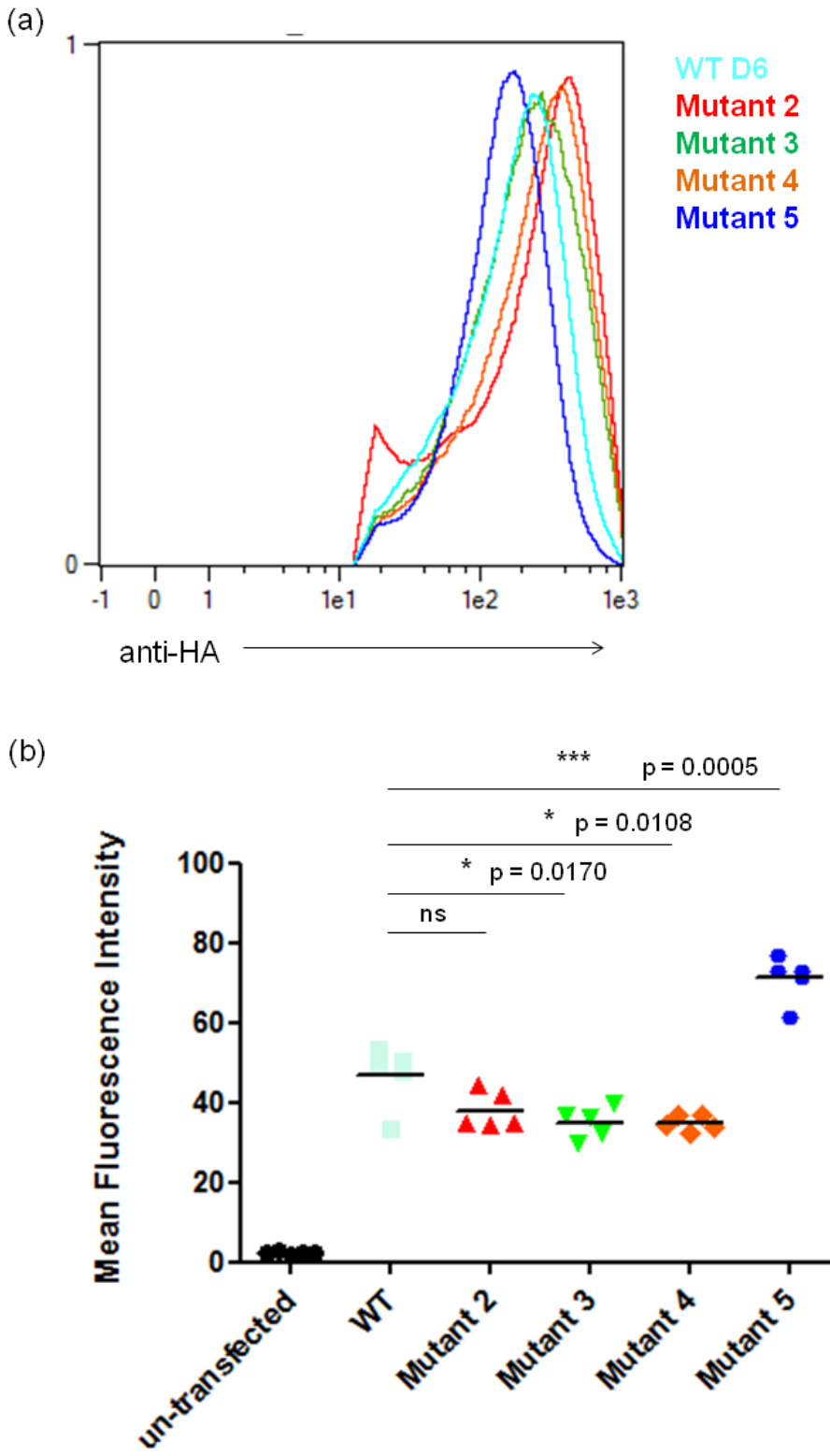


Figure 6-18: Ligand uptake capability is enhanced in Mutant 5.
(a) Flow cytometry profile of each cell line (indicated in different colours). Each cell line was assessed for D6 expression using a mouse anti-HA (biotin) antibody or a mouse (biotin) isotype control and a streptavidin conjugated to R-phycoerythin (PE) secondary. **(b)** Dot plot indicating the results of multiple chemokine uptake experiments using 0.6µg/ml of AF-CCL22. Cells were analysed by flow cytometry and the MFI values for each experiment are plotted. Statistical analysis was performed using two-tailed unpaired t tests. N=5 per sample.

6.8.3 The presence of a single tyrosine residue rescues D6 activity

As demonstrated in previous experiments, Mutant 1 cannot be post-translationally sulphated because all the tyrosine residues on its *N*-terminus have been mutated to phenylalanine. Mutants 6, 7, 8 and 9 each have only one tyrosine residue remaining (see Table 6-2 for details), and were generated to find out if the presence of a single tyrosine residue in any of the four possible positions was sufficient to rescue ligand binding capability in D6. To investigate this, similar chemokine uptake experiments were performed as before with AF-CCL22. The results of the chemokine uptake assays, as well as flow cytometry data showing the level of cell surface D6 expression of each mutant, are displayed in **Figure 6-19**.

The D6 expression of each mutant was measured by flow cytometry, and histograms representing each mutant's MFI are shown in the flow cytometry profile in **Figure 6-19 (a).** This data demonstrated that Mutant 1, and Mutants 6 – 9 had similar levels of cell surface D6 expression at the time of experimentation and could therefore be compared. It was clear from the chemokine uptake assay (**Figure 6-19 (b)**) that the introduction of a single tyrosine residue in any position is sufficient to partially rescue ligand binding by D6. As well as the highly significant increase in uptake between Mutant 1 and each of the single tyrosine knock-ins, there are also significant differences in chemokine uptake capabilities between Mutant 6 and Mutant 8, Mutant 7 and Mutant 9, and Mutant 8 and Mutant 9, as highlighted on the dot plot in **(b).** These data suggest that sulphation of tyrosines in position 1 and position 4 of the conserved motif may be more important than sulphation of tyrosines in position 2 and 3, although no single tyrosine residue is indispensible for rescuing the ligand binding capability of D6. Comparison of Mutants 6 – 9 with WT D6, shown in Figure 5E of Hewit et al, 2014, demonstrated that the single tyrosine knock-ins did not fully restore D6 chemokine uptake capabilities, indicating a requirement for multiple N-terminal tyrosine residues for full D6 function (Hewit et al., 2014)

In summary, the presence of a single tyrosine in any of the four possible positions on the N-terminus of D6 is sufficient to partially rescue the ligand binding and uptake capability of D6.

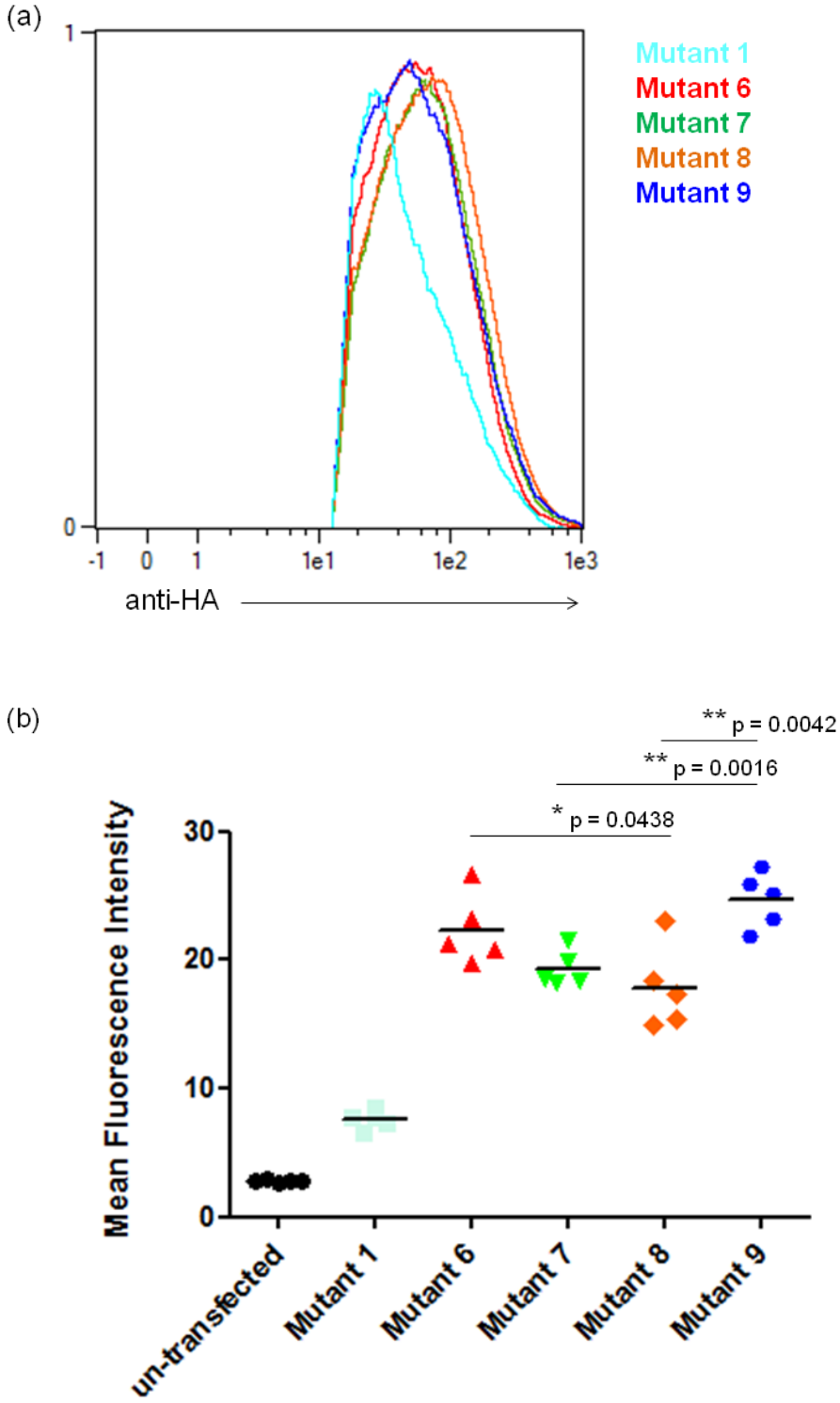


Figure 6-19: The presence of a single tyrosine residue partially rescues D6 activity. **(a)** Flow cytometry profiles of each cell line (represented in different colours). Each cell line was assessed for D6 expression using a mouse anti-HA (biotin) antibody or a mouse (biotin) isotype control and a streptavidin conjugated to R-phycoerythin (PE) secondary. **(b)** Dot plot indicating the results of multiple chemokine uptake experiments using 0.6µg/ml of AF-CCL22. Cells were analysed by flow cytometry and the MFI values for each experiment are plotted. Statistical analysis was performed using two-tailed unpaired t tests. N=5 per sample. Mutant 1 was found to be statistically different to Mutant 6, 7, 8 and 9, $p<0.0001$ for each. p values for the other t tests are indicated. For a comparison of Mutant 6 – 9 with Mutant 1 and WT D6 see (Hewit et al., 2014).

6.9 Summary of Chapter 6

This chapter describes experiments designed to investigate (i) the role of sulphation of the *N*-terminus for ligand binding by the atypical chemokine receptor D6, (ii) the processes involved in post-translational sulphation, including the expression of enzymes critical to this process and the inhibition of the sulphation pathway, (iii) how mutation of the conserved tyrosine motif on the *N*-terminus of D6 affects ligand binding and uptake capability of this receptor.

D6 and the other atypical chemokine receptors are biochemically distinct from typical chemokine receptors, therefore these experiments were important to find out if D6 shared this conserved structural feature of tyrosine sulphation. The experiments in this chapter also led on from the findings presented in Chapter 4 which described a synthetically derived *N*-terminal D6 peptide which was able to bind ligand only in a chemically sulphated form. The main findings that are presented in this chapter are:

- Post-translational sulphation of a conserved tyrosine motif on the *N*-terminus of D6 is an important requirement for optimal ligand binding and uptake; however no single tyrosine residue present on the *N*-terminus of D6 is essential for ligand binding.
- Mutation of all tyrosine residues to phenylalanine inhibits post-translational sulphation and in turn, the chemokine uptake and degradation capability of D6 is greatly reduced.
- The re-introduction of a single tyrosine residue in any of the four possible positions on the *N*-terminus is sufficient to partially rescue the ligand binding and uptake capability of D6.
- Both TPST-1 and TPST-2 are required to retain the ligand uptake capability of D6, suggesting both enzymes are capable of D6 sulphation.

Such findings shed new light on the significance of the D6 *N*-terminus for D6 function and specifically the importance of post-translational sulphation for binding and uptake of inflammatory CC chemokines.

Chapter 7

Discussion

7.1 Introduction

The atypical chemokine receptor, D6 is biologically distinct from classical chemokine receptors and has been shown to be a key regulator of chemokine-driven inflammatory responses. Despite the pivotal findings that D6 is a highly efficient binder, internaliser and scavenger of inflammatory CC chemokines (Fra et al., 2003, Weber et al., 2004), and that D6 is fundamentally important for the resolution of inflammatory responses (Codullo et al., 2011, Jamieson et al., 2005, Martinez de la Torre et al., 2005, Nibbs et al., 2007), little is known about the structure/function relationships that exist within and around D6 that contribute to binding of ligand.

Many conventional chemokine receptors have been shown to rely on GAGs for the sequestration and subsequent presentation of chemokines (Handel et al., 2005, Proudfoot, 2006). In contrast, different biochemical features have been identified on conventional chemokine receptors themselves that are required for or involved in ligand binding. Important among these is the *N*-terminus and in particular tyrosine sulphation at this region (Farzan et al., 1999, Choe et al., 2005, Veldkamp et al., 2006, Simpson et al., 2009, Tan et al., 2013a, Zhu et al., 2011).

Therefore in an attempt to determine whether certain biochemical features that contribute to chemokine interactions with conventional chemokine receptors are also important for the function of the atypical chemokine receptor, D6, we have developed and utilized many molecular and cell based assays to analyse structural moieties that contribute to chemokine internalisation by D6, and therefore D6 function. The importance of proteoglycans for chemokine presentation to D6 was investigated with use of a proteoglycan-deficient cell line and the establishment, and optimisation of, novel assays to measure D6 activity. Various aspects of the *N*-terminus of D6 have been investigated, including its chemokine binding ability and its tendency to be ‘shed’ from the full length D6 protein. Importantly, for the first time, we have synthesized a peptide consisting of the first 35 amino acids of the D6 *N*-terminus, and tested its ability to bind and neutralise the activity of D6 ligands. Additionally, the action of proteases in cleaving the D6 *N*-terminus has been explored. Finally, the functional significance of tyrosine sulphation at the D6 *N*-terminus has been investigated.

In this chapter the main findings of this thesis will be summarized and discussed, and possible future directions highlighted.

7.2 Discussion of Chapter 3

7.2.1 The use of CHO cells

The role of GAGs for D6 function was investigated by utilising the CHO 745 cell line which is deficient in the initiation of GAG chain formation on proteoglycan core proteins (Esko et al., 1985). Wild type CHO cells (CHO K1) express heparan sulphate, which is the most ubiquitous class of GAG and comprises 50-90% of total endothelial proteoglycans (Proudfoot, 2006). Heparan sulphate has also been shown to be expressed on LECs (Rutkowski et al., 2006) and trophoblasts (Van Sinderen et al., 2013) which are naturally D6-expressing cells. Therefore CHO cells were thought to be a good surrogate for the primary cells that normally express D6 *in vivo*, as these cells, e.g. LECs, are often problematic and lose D6 expression quickly *in vitro* (McKimmie et al., 2013).

7.2.2 Chemokine presentation to D6 is facilitated by GAGs

The main finding presented by Chapter 3 was that D6, expressed on GAG-free cells, binds and internalises the inflammatory CC chemokines CCL2 and CCL22 less efficiently than cells expressing GAGs (McKimmie et al., 2013). This finding was not unexpected because previous studies utilizing CHO 745 transfected cells for the study of conventional chemokine receptors have shown that GAGs improve the sensitivity of cells to chemokine stimulation (Ali et al., 2000). This study in particular focussed on the receptors CCR1 and CCR5 and showed that CCL3 stimulation of these receptors was less efficient when they were expressed on CHO 745 as opposed to CHO K1 cells. The effect observed in this study was chemokine-specific however, as CCR1 and CCR5 stimulation by CCL4 or CCL5 did not seem to be affected by the absence of GAGs on the cell surface. More recently, the differences in chemokine binding patterns between CHO K1 and CHO 745 cell lines have been compared using fluorescence microscopy (Kawamura et al., 2014). This study produced images of both cell types after transfection of fluorescently tagged CCR7 and CXCR4, and after the addition of their fluorescently labelled ligands, CCL21

and CXCL12 respectively. The images generated show that when fluorescent chemokine is added to CHO K1 cells, small punctuate staining can be observed, which is not present on CHO 745 cells. This pattern of staining may be the result of chemokine oligomerisation, which is known to be facilitated by GAGs, and has been shown to be important for the activation of certain chemokine receptors both *in vitro* and *in vivo* (Proudfoot et al., 2003). CHO 745 hD6 cells are not incapable of CCL2 or CCL22 uptake (see Figure 3-8), suggesting that oligomerisation of CCL2 or CCL22 is not essential for D6-mediated binding and internalisation of these chemokines *in vitro*. This is consistent with previous studies on CCL2 which showed that it is the monomeric form of CCL2 which is believed to bind to CCR2 to induce cell migration *in vitro* (Paavola et al., 1998), however oligomerisation of CCL2 seems to be required for *in vivo* activation of CCR2 (Proudfoot et al., 2003, Handel et al., 2008). Whether this is also the case with D6, has yet to be elucidated.

Chemokine-binding to GAGs has also been shown to protect them from degradation by proteases in their surrounding environment; thereby increasing their lifespan and increasing the time they have to exert their biological effects (Wagner et al., 1998, Sadir et al., 2004, Adage et al., 2012). Similarly, in the current study there was a subtle reduction in chemokine levels in the media of un-transfected CHO 745 cells compared with un-transfected CHO K1 cells (Figure 3-11 (d) and (e)). These data add further significance to the lower amount of chemokine present in media from CHO K1 hD6 compared with CHO 745 hD6 after 24 hours (Figure 3-11 (c)), and emphasize that D6-mediated scavenging of chemokine from the media by CHO K1 hD6 was still more efficient than CHO 745 hD6, despite the lack of protection from protease-mediated degradation of chemokine in GAG-deficient CHO 745 cells.

7.2.3 How do these findings relate to current knowledge of D6 and GAG function?

Current knowledge of D6 function suggests a requirement for D6 expression on LECs in order to support the selective presentation of CCR7 ligands on LEC surfaces in inflammatory chemokine-rich contexts (McKimmie et al., 2013). The data presented in Chapter 3 suggest that *cis*-presentation of ligand by GAGs is important for D6-dependent internalisation and scavenging of inflammatory chemokines, therefore suggesting a requirement for GAGs in this context (see **Figure 7-1**). In support of this, it has been

shown previously that the expression of heparan sulphate based GAGs appears to be a hallmark of mature lymphatic vessels (Rutkowski et al., 2006), which is suggestive of their functional importance. In addition, GAG-binding of many chemokines has been shown to be required for chemokine-driven cell migration *in vivo*, in the presence of physiological flow in blood and lymph vasculature (Proudfoot et al., 2003, Johnson et al., 2005, Wang et al., 2005). Our data show that even when flow forces are not operative, GAGs still contribute to sequestration of chemokine and subsequent presentation to D6. Therefore it seems likely that the effect of GAGs on D6 function *in vivo* may be even more pronounced than that indicated by the *in vitro* data presented in this thesis.

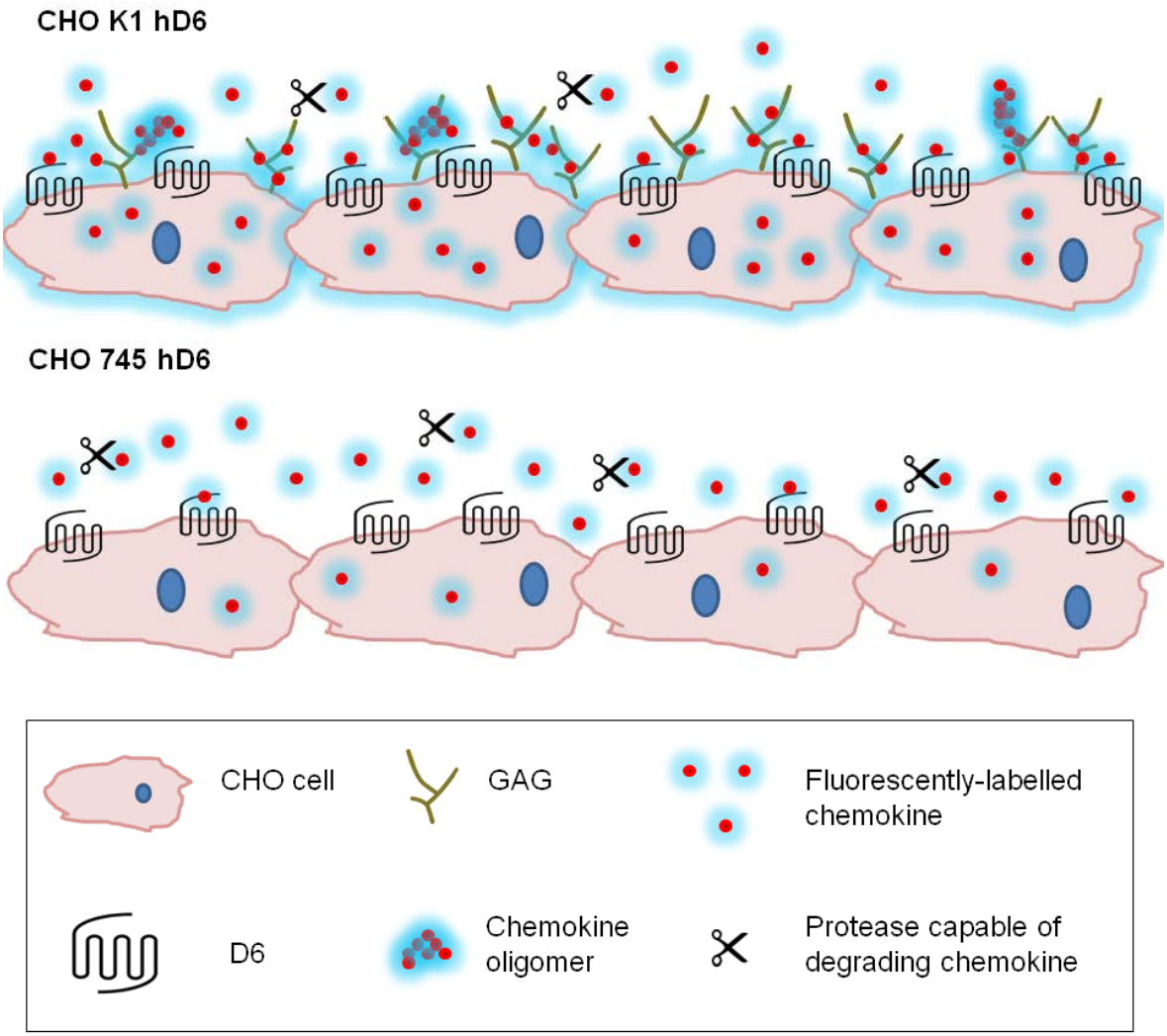


Figure 7-1: Consequences of GAG deficiency for D6 binding and internalisation of chemokines.
D6 expressed on GAG-deficient CHO cells (CHO 745 hD6) is hampered in its ability to bind and internalise ligand. This is thought to be a result of the loss of GAG-mediated chemokine presentation to D6, and may also be partly a consequence of the inhibition of chemokine oligomerisation. Protease-mediated degradation of chemokines is also elevated in the media of CHO 745 cells, which is thought to be another consequence of the absence of GAGs.

7.3 Discussion of Chapters 4, 5 and 6

Among the conventional chemokine receptors, a number of regions are known to be involved in ligand binding. Probably the most important amongst these is the extracellular *N*-terminus, and specifically, clusters of sulphated tyrosines in this region. Chapters 4, 5 and 6 of this thesis investigated the *N*-terminus of D6, and its importance for ligand binding. The main findings presented in these chapters were:

- Sulphated tyrosine residues at the *N*-terminus of D6 are required for optimal chemokine binding and internalisation by D6.
- A sulphated peptide representing the first 35 amino acids of the D6 *N*-terminus is capable of neutralising D6 ligands *in vitro*.
- The bacterial protease, staphopain A, can cleave the *N*-terminus of D6 and suppress its ligand binding, and internalisation, activity.

The potential significance of these findings is discussed in the following sections:

7.3.1 A conserved tyrosine cluster on the *N*-terminus of D6 is a key determinant for ligand binding

Analysis of the amino acid sequences of D6 from many different mammalian species highlighted a highly conserved cluster of tyrosine residues within the *N*-terminus (see Figure 6-1). The nature of this motif in D6 is suggestive of post-translational sulphation, a common biochemical modification among many of the conventional chemokine receptors, and similar to chemokine receptors known to be sulphated such as CCR5, CCR2 and CXCR4, the tyrosine motif in D6 is found in the vicinity of acidic residues (aspartic and glutamic acid). The conserved cysteine found in the *N*-terminal of almost all chemokine receptors is also present in D6, and notably all these chemokine receptors bear a sulphated tyrosine located approximately nine residues before the conserved cysteine (Szpakowska et al., 2012). Tyrosine sulphation is thought to be important for binding interactions between chemokine receptors and their ligands because many chemokines have a positively charged pocket at their surface with which a negatively charged sulphate would interact (Hemmerich et al., 1999, Love et al., 2012, Gozansky et al., 2005). Sulphated tyrosines on chemokine receptors have also been proposed to keep the *N*-terminus in an ‘open’ configuration, as the negative charges of sulphate groups repel each other and prevent the

collapse of this domain, thereby keeping an optimal confirmation for ligand binding (Szpakowska et al., 2012). The results presented in Chapter 4 and Chapter 6 both indicate that ligand binding by D6 is dependent on receptor sulphation. This was demonstrated by both chlorate, and siRNA-mediated, inhibition of sulphation, and tyrosine to phenylalanine site-directed mutagenesis in this area, and was further confirmed by the use of peptides derived from the *N*-terminus of D6, which showed that a sulphated D6 *N*-terminal peptide was able to neutralise the activity of D6 ligands *in vitro*, however a non-sulphated version could not (Hewit et al., 2014). Site-directed mutagenesis studies, in particular, highlighted the requirement for at least one tyrosine residue to restore ligand binding function which was almost completely knocked-out by the complete tyrosine to phenylalanine mutant (Figure 6-19), with the first and fourth tyrosine appearing optimal. Previous comparison of the *N*-termini of chemokine receptors have highlighted a conserved tyrosine approximately 9 residues before the predicted disulphide bond (described in more detail in section 1.2.1.2 of the Introduction). This conserved tyrosine is also present in D6 (YYYD**Y**), suggesting that it is of functional significance. Surprisingly, mutation of the second tyrosine (which is only conserved in higher order primates) resulted in significantly enhanced ligand binding and internalisation of CCL22 (Figure 6-15). This may represent an evolutionary adaptation in humans and higher order primates which reduces the affinity of D6 for CCL22. However whether this is also the case with the other D6 ligands has yet to be tested. Site-directed mutagenesis where all, or all but one, tyrosines were mutated to phenylalanine also seemed to decrease trafficking of the receptor to the cell surface over time (Figures 6-13 and 6-17). This effect was not observed with growth of HEK D6 cells in sodium chlorate (Figure 6-2 (c)), suggesting that it may be a consequence of loss of tyrosine residues, rather than loss of sulphation.

Tyrosine sulphation in mammals is dependent on the action of two tyrosyl-protein sulphotransferases (TPST-1 and TPST-1) (Stone et al., 2009). Previous comparison of these enzymes showed that they have different tissue expression patterns and play distinct but overlapping roles, as well as exhibiting differences in substrate specificity (Mishiro et al., 2006). The results presented in Chapter 6 from studies of TPST-1 and TPST-2 revealed that both enzymes are involved in the post-translational sulphation of D6 (Figure 6-10).Taking this into account, it is possible that the different TPST's act on different tyrosines of the D6 *N*-terminus, therefore explaining why inactivating only TPST-1 or only TPST-2 did not significantly decrease ligand binding by D6, but inactivating both enzymes did. This hypothesis is also in agreement with the site-directed mutagenesis data presented later in Chapter 6 which showed that the most prominent decrease in ligand binding by D6

occurred in mutant 1, where all tyrosines were mutated to phenylalanine, thus inhibiting sulphation of the receptor (Section 6-5).

7.3.2 Shedding and cleavage of the D6 *N*-terminus

Previous reports from this laboratory analysing the biochemistry of D6 have highlighted the existence of, not only the full length D6 receptor, but also a truncated form which cannot be detected by D6 antibodies which recognise the *N*-terminus (Blackburn et al., 2004). It was proposed that this truncated version of D6 may represent shedding of the *N*-terminus from the full length receptor. Shedding of a peptide that is capable of acting as a motile blocker of inflammatory CC chemokines may be advantageous in certain cases, and studies with the synthetic D6-N peptide support such a function for this peptide.

Unexpectedly, sequence analysis of the *N*-terminus of chemokine receptors has shown that the D6 *N*-terminus is more similar to the CXC receptors than to the inflammatory CC receptors (Szpakowska et al., 2012). Several reports of CXC chemokine receptor cleavage by mammalian (Hartl et al., 2007, Levesque et al., 2003, Valenzuela-Fernandez et al., 2002) or bacterial proteases (Laarman et al., 2012) prompted us to examine whether such proteases can also cleave the D6 *N*-terminus. Western blots presented in Chapter 5, provide evidence of D6 truncation by the bacterial protease staphopain A (Figure 5-8), which is released by *Staphylococcus aureus*. Importantly, truncation was also shown to decrease the ability of D6 to bind, and internalise, CCL22 (Figure 5-10), further supporting loss of the *N*-terminus. Unfortunately, attempts to identify the released *N*-terminal peptide by immunoprecipitation were unsuccessful, suggesting that the concentration of the released peptide was too low to detect by this method, or that the peptide was further degraded in the culture medium. Previous studies on the biochemistry of D6 have shown that it is glycosylated on its *N*-terminus (Blackburn et al., 2004). Modifications such as post-translational glycosylation have been shown to stabilise peptides and inhibit their degradation (Ludwig et al., 2000), making the possibility of degradation less likely. Assuming that the action of staphopain A can release an intact D6 *N*-terminal peptide, why would this be beneficial to *S. aureus*? Cleavage of the CXCR2 *N*-terminus demonstrated by Laarman et al makes sense as this will prevent the migration of CXCR2-expressing neutrophils to the site of infection. Thus we propose that cleavage of D6 and release of an *N*-terminal peptide capable of blocking the action of inflammatory CC chemokines would also be beneficial to a bacterial pathogen trying to escape the inflammatory response

because this would result in a decreased recruitment of myelomoncytic inflammatory leukocytes to fight the infection. **Figure 7-2** illustrates this hypothesis.
Alternatively, if the D6 peptide is degraded, cleavage and inactivation of full length D6 may still be beneficial to *S. aureus.* Recent studies investigating the many virulence factors of *S. aureus* have shown that elusive subpopulations of this bacterial pathogen are able to survive inside human macrophages (Kubica et al., 2008, Thwaites and Gant, 2011) and can actually exert cyto-protective effects by up-regulating anti-apoptotic factors (Koziel et al., 2009). Koziel et al hypothesize that this ability may contribute to the survival and systematic dissemination of the *S. aureus* infection. Taking this into account, cleavage of D6 by staphopain A would limit inflammatory CC chemokine scavenging thereby attracting more macrophages into the area of infection. This would give *S. aureus* more opportunity to infect and persist within macrophages in order to be disseminated later at sites distant from the primary infection, see **Figure 7-3**. Given that *S. aureus* has evolved to be a highly human-specific pathogen, an ideal mouse model for staphylococcal disease does not exist (Spaan et al., 2013), therefore it was concluded that testing these different hypotheses *in vivo* would provide little meaningful data.

Comparison of the CXCR2 *N*-terminus with the D6 *N*-terminus by BLAST analysis found no significant similarities, however the sequence of residues on CXCR2 cleaved by staphopain A was found to be LD↓A, with only the leucine residue (L) proven to be essential (Laarman et al., 2012). The D6 *N*-terminus has the sequence LDE at positions 28, 29 and 30 which could be a potential cleavage site and would result in a shed peptide containing the sulphated tyrosine motif shown to be important for ligand binding. Further analysis is required to confirm or reject this hypothesis.

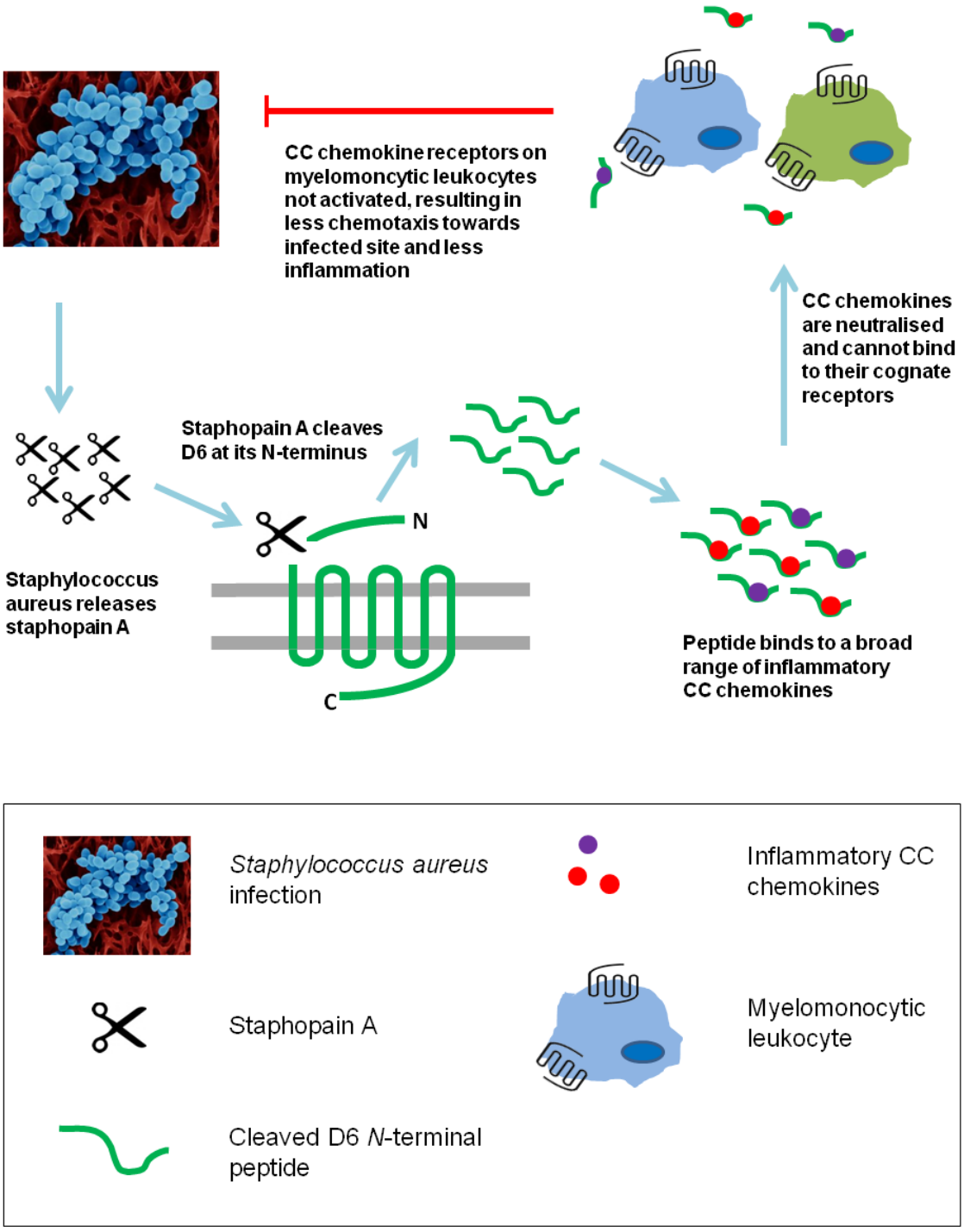


Figure 7-2: Proposed functional consequences for *N*-terminal cleavage of D6 by the bacterial protease staphopain A

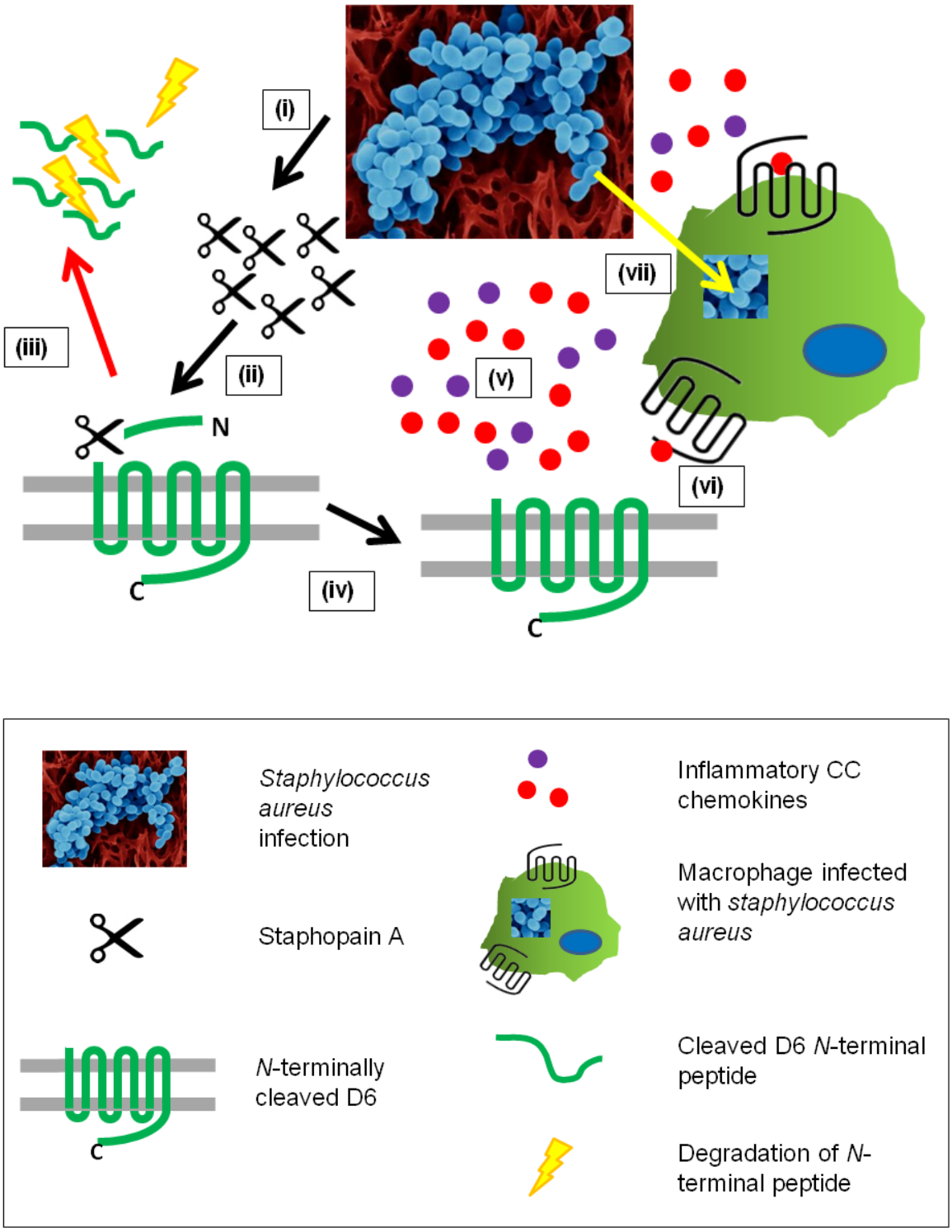


Figure 7-3: Alternative hypothesis on the effect of D6 cleavage for virulence of *S. aureus*. **(i)** *S. aureus* releases staphopain A. **(ii)** Staphopain A cleaves D6. **(iii)** The released D6 *N*-terminal peptide is degraded. **(iv)** The cleaved D6 receptor is unable to scavenge inflammatory CC chemokines. **(v)** Inflammatory CC chemokines build up at the site of infection. **(vi)** Macrophages expressing receptors such as CCR1, CCR2 and CCR5 travel towards the site of infection. **(vii)** *S. aureus* is phagocytosed by macrophages but can survive and persist intra-cellularly, for dissemination and subsequent infection at a later time point.

7.3.3 A sulphated peptide representative of the D6 *N*-terminus has therapeutic potential

Despite the initial cloning of the first chemokine receptor over 20 years ago, there are still no chemokine receptor directed therapeutics currently licensed for the treatment of chronic inflammatory diseases. This is most likely a consequence of the great degree of overlap between the functions of both the inflammatory chemokines and their receptors (Schall and Proudfoot, 2011). An appealing alternative to receptor inhibition may be to neutralize the chemokine ligands involved in the inflammatory response; however the overall effect of a specific blocker against a single chemokine would most likely be lost because of redundancy in the system. A molecule that has the ability to block multiple different inflammatory chemokines would be a great advantage in this regard, and this is the strategy adopted by a number of viral species in order to escape the inflammatory response (Webb and Alcami, 2005).

The atypical chemokine receptor D6 has the unique ability to bind and scavenge all inflammatory CC chemokines, and the *N*-terminus of this receptor was thought to be, and later confirmed to be (Chapter 6), required for ligand binding. For this reason, Chapter 4 focussed on the investigation of sulphated and non-sulphated D6-derived peptides, and specifically their ability to bind D6 ligands and neutralise their *in vitro* activity. The sulphated D6-N peptide demonstrated binding specificity towards the inflammatory CC chemokines CCL2 and CCL22 (section 4.4.2), and was shown to inhibit the binding of CCL2 to full length D6 (Figure 4-12) and also to its cognate receptor; CCR2 (Figure 4-13). These results suggested a potential therapeutic benefit of sulphated D6-N as a non-immunogenic, inflammatory CC chemokine scavenger which could dampen the inflammatory response during chronic inflammatory diseases. On the other hand, because D6-N (s) is likely to be a broad-based inflammatory CC chemokine scavenger, this may have implications with regards to its therapeutic usefulness. Previous studies investigating inflammatory diseases such as RA and asthma have shown that target specificity is crucially important for treatment (Schall and Proudfoot, 2011, Pease and Horuk, 2014). Additionally, different viruses have been shown to release chemokine binding proteins that are capable of binding inflammatory chemokines and inhibiting their action in order to evade the immune system (Deruaz et al., 2008, Epperson et al., 2012). Therefore if D6-N (s) were to be used therapeutically, this may have implications for the inflammatory

response against infection. Therefore care would have to be taken e.g. to ensure immunocompromised patients are not put at risk as a result of treatment with D6-N (s). The change in chemokine-neutralising ability between the old and new batches of sulphated D6-N peptide was a consequence of differentially sulphated peptides between batches (Figure 4-15) most likely caused by the uncontrollable nature of the chemical sulphation process. This has also been shown to be the case in other studies utilizing *N*-terminally derived peptides from receptors with multiple tyrosines (Choe et al., 2005). These studies suggest that artificial *in vitro* sulphation of *N*-terminal peptides derived from receptors bearing multiple tyrosines, such as D6, give rise to products with a variety of sulphation patterns that differentially affect the binding to chemokines.

Whilst the result with the new batch of sulphated D6-N was disappointing, it demonstrated that the pattern and degree of tyrosine sulphation on the D6-N peptide is critical for the ability to bind its ligands. It also highlighted the importance of study towards finding a naturally 'shed' D6 *N*-terminal peptide, as this would contain the native sulphation pattern of D6-N and could therefore be exploited when designing potential therapeutics against inflammatory CC chemokines for the treatment of chronic inflammatory pathologies.

7.4 Future directions and considerations

7.4.1 GAG studies

The results presented in Chapter 3 highlight a number of avenues for further investigation. Previous studies in this field have highlighted large discrepancies between *in vitro* and *in vivo* data for certain chemokines, which is thought to be largely attributable to the physiological flow forces experienced in the vasculature *in vivo*. Therefore to gain a more thorough insight into the relationship between GAGs and D6, *in vivo* models should also be considered. In previous studies investigating the role of GAGs on lymphatic endothelium, loss-of-function genetic approaches were developed, whereby heparan sulphate biosynthesis was conditionally knocked-out on LECs to study the role of lymphatic endothelial heparan sulphate in cancer metastasis (Yin et al., 2010). The use of this model to study the requirement for GAG-mediated presentation of chemokine to D6 may be possible, however GAGs are also required for the presentation of CCL21 to CCR7 on the lymphatic vasculature, therefore the exact consequences of this conditional knock-out for D6 may be difficult to untangle using this model.

The application of primary LECs *in vitro*, and the use of glycosidase enzymes to remove GAGs from the surface of these cells, was also considered to study the relationship between GAGs and D6. This method has been used previously in order to study the re-generation of lymphatic vasculature (Rutkowski et al., 2006). The sensitivity of primary LECs in tissue culture, as well as their tendency to lose D6 expression after a small number of passages, were serious limitations to their use for these purposes, and therefore the use of a GAG-free CHO cell line was deemed to be a more manageable approach to investigations in this area.

As mentioned previously, oligomerisation of CCL2 has been shown to be important for its activation of CCR2 *in vivo*, however, in contrast, monomeric CCL2 mutants that are unable to form dimers have the same chemotactic properties as wildtype CCL2 *in vitro* (Proudfoot et al., 2003, Handel et al., 2008). It has yet to be discovered whether oligomerisation of certain chemokines is required for optimal D6 binding and internalisation, however the results presented in Chapter 3 suggest that this is a possibility. If the decrease in D6 activity observed on CHO 745 cells was an indirect result of the prevention of chemokine oligomerisation on GAG-free cells, this could be confirmed with use of monomeric CC-chemokine mutants such as the CCL2 mutant used by Handel et al, which has a mutation in a residue that comprises the core of the dimerization interface (proline to alanine mutation in position 8 of the *N*-terminal of CCL2). In this scenario we would expect D6 activity to be reduced in cells where the mutant chemokine was used compared with wildtype chemokine.

Finally, the CHO K1 hD6 and CHO 745 hD6 clones generated for this study are currently being used by our laboratory to develop fluorescence microscopy techniques for the study of D6. As a knock-on effect of this future research, more extensive insight into the functional relationship between GAGs and D6 should be gained that was not possible in the course of this study.

7.4.2 D6 *N*-terminal peptide studies

The results presented in Chapters 4, 5 and 6 provide evidence of the importance of *N*-terminal tyrosine residues for D6 function. However they also highlight some unanswered questions and opportunities for further investigation. The inability of the new batch of

sulphated D6-N to bind chemokines emphasizes a requirement to uncover the optimum tyrosine sulphation pattern of D6 to allow the design of successful therapeutics based on this motif. The mass spectrum of the new batch of D6-N showed that it is a mixture of mostly di- and tri-sulphated peptides (Figure 4-15). Separation of each type of peptide based on their degree of sulphation may be possible using reverse-phase chromatography and this would help to determine the optimum sulphation pattern required for chemokine binding. This approach was initiated but unfortunately could not be followed up because of time constraints.

Providing the successful re-synthesis of a D6-N (s) peptide capable of binding chemokines, many avenues for further investigation could be followed up, most importantly the testing of the D6-N (s) peptide *in vivo* for its ability to neutralise inflammatory CC chemokines during induced inflammatory pathologies.

From a biochemical stand-point, critical information would undoubtedly be gained from the crystallization of a D6-N – chemokine complex, such as the binding sites and the role of sulphate groups in the D6-N (s) – chemokine interaction. The determination of a three-dimensional structure would importantly provide a more complete understanding of the nature of the interactions between the full length D6 receptor and its ligands, as well as facilitating rational drug design. In addition, it has been notoriously difficult to obtain crystal structures of chemokine receptors, however obtaining a crystal structure of the *N*-terminal peptide should, in theory, be easier and less time consuming.

7.4.3 Further functional characterisation of the D6 *N*-terminus

The current study attempted to elucidate the functional significance of tyrosine sulphation on the D6 *N*-terminus for ligand binding. Interestingly it was noted that mutagenesis of three or more of the tyrosine residues to phenylalanine resulted in the expression of less D6 on the cell surface after a few days in tissue culture (Figure 6-17 (b)), whereas inhibiting sulphation did not have any effect on D6 surface expression (Figure 6-2 (c)). These results imply that tyrosine residues themselves may also be required for optimal trafficking of D6 to the cell surface or recycling of the receptor after internalisation, and this may also be an additional function common to the tyrosine residues on other conventional or atypical chemokine receptors. Further investigation is required to test this hypothesis.

7.5 Conclusions

The aim of this thesis was to investigate the biochemical properties of the D6 *N*-terminus that make it the principle site for binding inflammatory CC chemokines. Post-translational tyrosine sulphation has been previously shown to be important for ligand binding in many conventional chemokine receptors, and GAGs have been highlighted for their role in ligand acquisition and presentation to chemokine receptors, therefore the roles for sulphation and GAGs for ligand binding to D6 were focussed on.

The results presented provide evidence that GAGs are involved in the presentation of ligand to D6 and, in their absence, D6-mediated internalisation and degradation of chemokines is significantly reduced. Confirmation of the importance of *N*-terminal tyrosine residues for D6 function was established through extensive site-directed mutagenesis studies. Furthermore, sulphation was implicated as a prerequisite for optimal ligand binding by means of sulphation inhibition studies, either by siRNA knock-down of TPST-1 and TPST-2, or inhibition of the universal sulphate donor PAPS with the use of sodium chlorate. Support for the importance of the *N*-terminus of D6 was further provided with indications that it is a cleavage target of staphopain A, a protease released by the bacterial pathogen *Staphylococcus aureus.* Finally, a sulphated peptide derived from the *N*-terminus of D6 was shown, for the first time, to be capable of binding CCL2 *in vitro* and preventing binding to both D6, and its cognate receptor CCR2; however the positioning and degree of sulphation of this peptide was shown to be crucial to its ability in this regard. These results give further validation to the proposal that a strategically sulphated version of D6-N may have the potential to be used therapeutically as a non-immunogenic, broad-based chemokine scavenger that is likely to be beneficial in the treatment of chronic inflammatory conditions. The experiments presented in this thesis also demonstrate the importance of studies investigating the biochemical and structural features of proteins and how such investigations facilitate a more in-depth understanding of their function.

Appendix 1: Publications arising from this work

The following publications are included in this appendix:

MCKIMMIE, C. S., SINGH, M. D., **HEWIT, K**., LOPEZ-FRANCO, O., LE BROCQ, M., ROSE-JOHN, S., LEE, K. M., BAKER, A. H., WHEAT, R., BLACKBOURN, D. J., NIBBS, R. J. B. & GRAHAM, G. J. (2013) An analysis of the function and expression of D6 on lymphatic endothelial cells. *Blood,* 121**,** 3768-77.

HEWIT, K. D., FRASER, A., NIBBS, R. J. & GRAHAM, G. J. (2014) The N-terminal region of the atypical chemokine receptor ACKR2 is a key determinant of ligand binding. *Journal of Biological Chemistry*, 289, 12330-42.

LE BROCQ, M. L., FRASER, A. R., COTTON, G., WOZNICA, K., MCCULLOCH, C. V., **HEWIT, K. D**., MCKIMMIE, C. S., NIBBS, R. J. B., CAMPBELL, J. D. M. & GRAHAM, G. J. (2014) Chemokines as novel and versatile reagents for flow cytometry and cell sorting. *J Immun,* 192, 6120-30.

Bibliography

ADAGE, T., PICCININI, A. M., FALSONE, A., TRINKER, M., ROBINSON, J., GESSLBAUER, B. & KUNGL, A. J. (2012) Structure-based design of decoy chemokines as a way to explore the pharmacological potential of glycosaminoglycans. *British Journal of Pharmacology,* 167**,** 1195-1205.

AHMED, T., UNGO, J., ZHOU, M. & CAMPO, C. (2000) Inhibition of allergic late airway responses by inhaled heparin-derived oligosaccharides. *Journal of Applied Physiology,* 88**,** 1721-1729.

ALCAMI, A. (2003) Viral mimicry of cytokines, chemokines and their receptors. *Nature Reviews Immunology,* 3**,** 36-50.

ALI, S., PALMER, A. C. V., BANERJEE, B., FRITCHLEY, S. J. & KIRBY, J. A. (2000) Examination of the function of RANTES, MIP-1 alpha, and MIP-1 beta following interaction with heparin-like glycosaminoglycans. *Journal of Biological Chemistry,* 275**,** 11721-11727.

ALI, S., ROBERTSON, H., WAIN, J. H., ISAACS, J. D., MALIK, G. & KIRBY, J. A. (2005) A non-glycosaminoglycan-binding variant of CC chemokine ligand 7 (monocyte chemoattractant protein-3) antagonizes chemokine-mediated inflammation. *Journal of Immunology,* 175**,** 1257-1266.

ALIBERTI, J., SOUSA, C. R. E., SCHITO, M., HIENY, S., WELLS, T., HUFFNAGLE, G. B. & SHER, A. (2000) CCR5 provides a signal for microbial induced production of IL-12 by CD8 alpha(+) dendritic cells. *Nature Immunology,* 1**,** 83-87.

ALIBERTI, J., VALENZUELA, J. G., CARRUTHERS, V. B., HIENY, S., ANDERSEN, J., CHAREST, H., REIS E SOUSA, C., FAIRLAMB, A., RIBEIRO, J. M. & SHER, A. (2003) Molecular mimicry of a CCR5 binding-domain in the microbial activation of dendritic cells. *Nature Immunology,* 4**,** 485-490.

ALKHATIB, G., AHUJA, S. S., LIGHT, D., MUMMIDI, S., BERGER, E. A. & AHUJA, S. K. (1997) CC chemokine receptor 5-mediated signaling and HIV-1 co-receptor activity share common structural determinants - Critical residues in the third extracellular loop support HIV-1 fusion. *Journal of Biological Chemistry,* 272**,** 19771-19776.

ALLEN, S. J., CROWN, S. E. & HANDEL, T. M. (2007) Chemokine: Receptor structure, interactions, and antagonism. *Annual Review of Immunology.* Palo Alto, Annual Reviews. Vol 25, 787-820.

ALON, R. (2010) Trapped versus Soluble Chemokines: Functions in Leukocyte Adhesion and Motility. *Immunity,* 33**,** 654-656.

ANTHONY, D. D. & HAQQI, T. M. (1999) Collagen-induced arthritis in mice: An animal model to study the pathogenesis of rheumatoid arthritis. *Clinical and Experimental Rheumatology,* 17**,** 240-244.

ARVANITAKIS, L., GERASRAAKA, E., VARMA, A., GERSHENGORN, M. C. & CESARMAN, E. (1997) Human herpesvirus KSHV encodes a constitutively active G-protein-coupled receptor linked to cell proliferation. *Nature,* 385**,** 347-350.

ATCHISON, R. E., GOSLING, J., MONTECLARO, F. S., FRANCI, C., DIGILIO, L., CHARO, I. F. & GOLDSMITH, M. A. (1996) Multiple extracellular elements of CCR5 and HIV-1 entry: Dissociation from response to chemokines. *Science,* 274**,** 1924-1926.

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. I., ROSENKILDE, M. M., ROT, A., SOZZANI, S., THELEN, M., YOSHIE, O. & ZLOTNIK, A. (2014) International Union of Pharmacology. LXXXIX.

Update on the Extended Family of Chemokine Receptors and Introducing a New Nomenclature for Atypical Chemokine Receptors. *Pharmacological Reviews,* 66**,** 1-79.

BAGGIOLINI, M., DEWALD, B. & MOSER, B. (1994) INTERLEUKIN-8 AND RELATED CHEMOTACTIC CYTOKINES - CXC AND CC CHEMOKINES. *Advances in Immunology, Vol 55,* 55**,** 97-179.

BAIS, C., SANTOMASSO, B., COSO, O., ARVANITAKIS, L., RAAKA, E. G., GUTKIND, J. S., ASCH, A. S., CESARMAN, E., GERHENGORN, M. C. & MESRI, E. A. (1998) G-protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus is a viral oncogene and angiogenesis activator. *Nature,* 391**,** 86-89.

BALDWIN, H. M., PALLAS, K., KING, V., JAMIESON, T., MCKIMMIE, C. S., NIBBS, R. J. B., CARBALLIDO, J. M., JARITZ, M., ROT, A. & GRAHAM, G. J. (2013) Microarray Analyses Demonstrate the Involvement of Type I Interferons in Psoriasiform Pathology Development in D6-deficient Mice. *Journal of Biological Chemistry,* 288**,** 36473-36483.

BALKWILL, F. & MANTOVANI, A. (2001) Inflammation and cancer: back to Virchow? *Lancet,* 357**,** 539-545.

BALKWILL, F. R. (2012) The chemokine system and cancer. *Journal of Pathology,* 226**,** 148-157.

BALTUS, T., WEBER, K. S. C., JOHNSON, Z., PROUDFOOT, A. E. I. & WEBER, C. (2003) Oligomerization of RANTES is required for CCR1-mediated arrest but not CCR5-mediated transmigration of leukocytes on inflamed endothelium. *Blood,* 102**,** 1985-1988.

BAO, L., ZHU, Y., ZHU, H. & LINDGREN, J. U. (2005) Decreased IgG production but increased MIP-1 beta expression in collagen-induced arthritis in C-C chemokine receptor 5-deficient mice. *Cytokine,* 31**,** 64-71.

BAO, X. F., MOSEMAN, E. A., SAITO, H., PETRYANIK, B., THIRIOT, A., HATAKEYAMA, S., ITO, Y., KAWASHIMA, H., YAMAGUCHI, Y., LOWE, J. B., VON ANDRIAN, U. H. & FUKUDA, M. (2010) Endothelial Heparan Sulfate Controls Chemokine Presentation in Recruitment of Lymphocytes and Dendritic Cells to Lymph Nodes. *Immunity,* 33**,** 817-829.

BARRESINOUSSI, F., CHERMANN, J. C., REY, F., NUGEYRE, M. T., CHAMARET, S., GRUEST, J., DAUGUET, C., AXLERBLIN, C., VEZINETBRUN, F., ROUZIOUX, C., ROZENBAUM, W. & MONTAGNIER, L. (1983) isolation of a t-lymphotropic retrovirus from a patient at risk for acquired immune-deficiency syndrome (aids). *Science,* 220**,** 868-871.

BAZAN, J. F., BACON, K. B., HARDIMAN, G., WANG, W., SOO, K., ROSSI, D., GREAVES, D. R., ZLOTNIK, A. & SCHALL, T. J. (1997) A new class of membrane-bound chemokine with a CX(3)C motif. *Nature,* 385**,** 640-644.

BAZZAN, E., SAETTA, M., TURATO, G., BORRONI, E. M., CANCELLIERI, C., BARALDO, S., SAVINO, B., CALABRESE, F., BALLARIN, A., BALESTRO, E., MANTOVANI, A., COSIO, M. G., BONECCHI, R. & LOCATI, M. (2013) Expression of the Atypical Chemokine Receptor D6 in Human Alveolar Macrophages in COPD. *Chest,* 143**,** 98-106.

BELPERIO, J. A., KEANE, M. P., ARENBERG, D. A., ADDISON, C. L., EHLERT, J. E., BURDICK, M. D. & STRIETER, R. M. (2000) CXC chemokines in angiogenesis. *Journal of Leukocyte Biology,* 68**,** 1-8.

BENNETT, L. D., FOX, J. M. & SIGNORET, N. (2011) Mechanisms regulating chemokine receptor activity. *Immunology,* 134**,** 246-256.

BERAHOVICH, R. D., MIAO, Z. H., WANG, Y., PREMACK, B., HOWARD, M. C. & SCHALL, T. J. (2005) Proteolytic activation of alternative CCR1 ligands in inflammation. *Journal of Immunology,* 174**,** 7341-7351.

BERGER, E. A., DOMS, R. W., FENYO, E. M., KORBER, B. T. M., LITTMAN, D. R., MOORE, J. P., SATTENTAU, Q. J., SCHUITEMAKER, H., SODROSKI, J. & WEISS, R. A. (1998) A new classification for HIV-1. *Nature,* 391**,** 240-240.

BERGER, E. A., MURPHY, P. M. & FARBER, J. M. (1999) Chemokine receptors as HIV-1 coreceptors: Roles in viral entry, tropism, and disease. *Annual Review of Immunology,* 17**,** 657-700.

BERKOWITZ, R. D., ALEXANDER, S., BARE, C., LINQUIST-STEPPS, V., BOGAN, M., MORENO, M. E., GIBSON, L., WIEDER, E. D., KOSEK, J., STODDART, C. A. & MCCUNE, J. M. (1998) CCR5- and CXCR4-utilizing strains of human immunodeficiency virus type 1 exhibit differential tropism and pathogenesis in vivo. *Journal of Virology,* 72**,** 10108-10117.

BLACKBURN, P. E., SIMPSON, C. V., NIBBS, R. J. B., O'HARA, M., BOOTH, R., POULOS, J., ISAACS, N. W. & GRAHAM, G. J. (2004) Purification and biochemical characterization of the D6 chemokine receptor. *Biochemical Journal,* 379**,** 263-272.

BLANPAIN, C., DORANZ, B. J., VAKILI, J., RUCKER, J., GOVAERTS, C., BAIK, S. S. W., LORTHIOIR, O., MIGEOTTE, I., LIBERT, F., BALEUX, F., VASSART, G., DOMS, R. W. & PARMENTIER, M. (1999a) Multiple charged and aromatic residues in CCR5 amino-terminal domain are involved in high affinity binding of both chemokines and HIV-1 Env protein. *Journal of Biological Chemistry,* 274**,** 34719-34727.

BLANPAIN, C., LEE, B., VAKILI, J., DORANZ, B. J., GOVAERTS, C., MIGEOTTE, I., SHARRON, M., DUPRIEZ, V., VASSART, G., DOMS, R. W. & PARMENTIER, M. (1999b) Extracellular cysteines of CCR5 are required for chemokine binding, but dispensable for HIV-1 coreceptor activity. *Journal of Biological Chemistry,* 274**,** 18902-18908.

BLANPAIN, C., WITTAMER, V., VANDERWINDEN, J. M., BOOM, A., RENNEBOOG, B., LEE, B., LE POUL, E., EL ASMAR, L., GOVAERTS, C., VASSART, G., DOMS, R. W. & PARMENTIER, M. (2001) Palmitoylation of CCR5 is critical for receptor trafficking and efficient activation of intracellular signaling pathways. *Journal of Biological Chemistry,* 276**,** 23795-23804.

BONECCHI, R., BORRONI, E. M., ANSELMO, A., DONI, A., SAVINO, B., MIROLO, M., FABBRI, M., JALA, V. R., HARIBABU, B., MANTOVANI, A. & LOCATI, M. (2008) Regulation of D6 chemokine scavenging activity by ligand- and Rab11-dependent surface up-regulation. *Blood,* 112**,** 493-503.

BONECCHI, R., LOCATI, M., GALLIERA, E., VULCANO, M., SIRONI, M., FRA, A. M., GOBBI, M., VECCHI, A., SOZZANI, S., HARIBABU, B., VAN DAMME, J. & MANTOVANI, A. (2004) Differential recognition and scavenging of native and truncated macrophage-derived chemokine (macrophage-derived chemokine/CC chemokine ligand 22) by the D6 decoy receptor. *Journal of Immunology,* 172**,** 4972-4976.

BONINI, J. A., MARTIN, S. K., DRALYUK, F., ROE, M. W., PHILIPSON, L. H. & STEINER, D. F. (1997) Cloning, expression, and chromosomal mapping of a novel human CC-chemokine receptor (CCR10) that displays high-affinity binding for MCP-1 and MCP-3. *DNA and Cell Biology,* 16**,** 1249-1256.

BORDON, Y., HANSELL, C. A. H., SESTER, D. P., CLARKE, M., MOWAT, A. M. & NIBBS, R. J. B. (2009) The Atypical Chemokine Receptor D6 Contributes to the Development of Experimental Colitis. *Journal of Immunology,* 182**,** 5032-5040.

BORGHEI, A., OUYANG, Y. B., WESTMUCKETT, A. D., MARCELLO, M. R., LANDEL, C. P., EVANS, J. P. & MOORE, K. L. (2006) Targeted disruption of tyrosylprotein sulfotransferase-2, an enzyme that catalyzes post-translational protein tyrosine O-sulfation, causes male infertility. *Journal of Biological Chemistry,* 281**,** 9423-9431.

BORRONI, E. M., CANCELLIERI, C., VACCHINI, A., BENUREAU, Y., LAGANE, B., BACHELERIE, F., ARENZANA-SEISDEDOS, F., MIZUNO, K., MANTOVANI, A., BONECCHI, R. & LOCATI, M. (2013) beta-arrestin-dependent activation of the cofilin pathway is required for the scavenging activity of the atypical chemokine receptor D6. *Science signaling,* 6**,** ra30.1-11, S1-3.

BORRONI, E. M., MANTOVANI, A., LOCATI, M. & BONECCHI, R. (2010) Chemokine receptors intracellular trafficking. *Pharmacology & Therapeutics,* 127**,** 1-8.

BROXMEYER, H. E., ORSCHELL, C. M., CLAPP, D. W., HANGOC, G., COOPER, S., PLETT, P. A., LILES, W. C., LI, X. X., GRAHAM-EVANS, B., CAMPBELL, T. B., CALANDRA, G., BRIDGER, G., DALE, D. C. & SROUR, E. F. (2005) Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *Journal of Experimental Medicine,* 201**,** 1307-1318.

BRUHL, H., CIHAK, J., SCHNEIDER, M. A., PLACHY, J., RUPP, T., WENZEL, I., SHAKARAMI, M., MILZ, S., ELLWART, J. W., STANGASSINGER, M., SCHLONDORFF, D. & MACK, M. (2004) Dual role of CCR2 during initiation and progression of collagen-induced arthritis: Evidence for regulatory activity of CCR2(+) T cells. *Journal of Immunology,* 172**,** 890-898.

BRUHL, H., WAGNER, K., KELLNER, H., SCHATTENKIRCHNER, M., SCHLONDORFF, D. & MACK, M. (2001) Surface expression of CC- and CXC-chemokine receptors on leucocyte subsets in inflammatory joint diseases. *Clinical and Experimental Immunology,* 126**,** 551-559.

BULENGER, S., MARULLO, S. & BOUVIER, M. (2005) Emerging role of homo- and heterodimerization in G-protein-coupled receptor biosynthesis and maturation. *Trends in Pharmacological Sciences,* 26**,** 131-137.

BUNTING, M. D., COMERFORD, I., SEACH, N., HAMMETT, M. V., ASQUITH, D. L., KORNER, H., BOYD, R. L., NIBBS, R. J. B. & MCCOLL, S. R. (2013) CCX-CKR deficiency alters thymic stroma impairing thymocyte development and promoting autoimmunity. *Blood,* 121**,** 118-128.

BURNS, J. M., SUMMERS, B. C., WANG, Y., MELIKIAN, A., BERAHOVICH, R., MIAO, Z. H., PENFOLD, M. E. T., SUNSHINE, M. J., LITTMAN, D. R., KUO, C. J., WEI, K., MCMASTER, B. E., WRIGHT, K., HOWARD, M. C. & SCHALL, T. J. (2006) A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *Journal of Experimental Medicine,* 203**,** 2201-2213.

CAMPANELLA, G. S. V., LEE, E. M. J., SUN, J. & LUSTER, A. D. (2003) CXCR3 and heparin binding sites of the chemokine IP-10 (CXCL10). *Journal of Biological Chemistry,* 278**,** 17066-17074.

CHABOT, D. J., CHEN, H., DIMITROV, D. S. & BRODER, C. C. (2000) N-linked glycosylation of CXCR4 masks coreceptor function for CCR5-dependent human immunodeficiency virus type 1 isolates. *Journal of Virology,* 74**,** 4404-4413.

CHAKERA, A., SEEBER, R. M., JOHN, A. E., EIDNE, K. A. & GREAVES, D. R. (2008) The Duffy antigen/receptor for chemokines exists in an oligomeric form in living cells and functionally antagonizes CCR5 signaling through hetero-oligomerization. *Molecular Pharmacology,* 73**,** 1362-1370.

CHARO, I. F. & RANSOHOFF, R. M. (2006) Mechanisms of disease - The many roles of chemokines and chemokine receptors in inflammation. *New England Journal of Medicine,* 354**,** 610-621.

CHOE, H., MOORE, M. J., OWENS, C. M., WRIGHT, P. L., VASILIEVA, N., LI, W., SINGH, A. P., SHAKRI, R., CHITNIS, C. E. & FARZAN, M. (2005) Sulphated tyrosines mediate association of chemokines and Plasmodium vivax Duffy binding

protein with the Duffy antigen/receptor for chemokines (DARC). *Molecular Microbiology,* 55**,** 1413-1422.

CLAING, A., LAPORTE, S. A., CARON, M. G. & LEFKOWITZ, R. J. (2002) Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins. *Progress in Neurobiology,* 66**,** 61-79.

CLARKLEWIS, I., DEWALD, B., GEISER, T., MOSER, B. & BAGGIOLINI, M. (1993) platelet factor-iv binds to interleukin-8 receptors and activates neutrophils when its n-terminus is modified with GLU-LEU-ARG. *Proceedings of the National Academy of Sciences of the United States of America,* 90**,** 3574-3577.

CLARKLEWIS, I., KIM, K. S., RAJARATHNAM, K., GONG, J. H., DEWALD, B., MOSER, B., BAGGIOLINI, M. & SYKES, B. D. (1995) structure-activity-relationships of chemokines. *Journal of Leukocyte Biology,* 57**,** 703-711.

COCCHI, F., DEVICO, A. L., GARZINODEMO, A., ARYA, S. K., GALLO, R. C. & LUSSO, P. (1995) identification of rantes, mip-1-alpha, and mip-1-beta as the major hiv-suppressive factors produced by cd8(+) t-cells. *Science,* 270**,** 1811-1815.

COCHAIN, C., AUVYNET, C., POUPEL, L., VILAR, J., DUMEAU, E., RICHART, A., RECALDE, A., ZOUGGARI, Y., YIN, K., BRUNEVAL, P., RENAULT, G., MARCHIOL, C., BONNIN, P., LEVY, B., BONECCHI, R., LOCATI, M., COMBADIERE, C. & SILVESTRE, J. S. (2012) The Chemokine Decoy Receptor D6 Prevents Excessive Inflammation and Adverse Ventricular Remodeling After Myocardial Infarction. *Arteriosclerosis Thrombosis and Vascular Biology,* 32**,** 2206-+.

CODULLO, V., BALDWIN, H. M., SINGH, M. D., FRASER, A. R., WILSON, C., GILMOUR, A., HUEBER, A. J., BONINO, C., MCINNES, I. B., MONTECUCCO, C. & GRAHAM, G. J. (2011) An investigation of the inflammatory cytokine and chemokine network in systemic sclerosis. *Annals of the Rheumatic Diseases,* 70**,** 1115-1121.

COLLINS, C. B., MCNAMEE, E. N., WERMERS, J. D., LEBSACK, M. D. P. & RIVERA-NIEVES, J. (2010) Chemokine decoy receptor D6 in inflammatory bowel disease (IBD) and IBD-associated colon cancer. *Gut,* 59**,** 151-152.

COLVIN, R. A., CAMPANELLA, G. S. V., MANICE, L. A. & LUSTER, A. D. (2006) CXCR3 requires tyrosine sulfation for ligand binding and a second extracellular loop arginine residue for ligand-induced chemotaxis. *Molecular and Cellular Biology,* 26**,** 5838-5849.

COMERFORD, I., HARATA-LEE, Y., BUNTING, M. D., GREGOR, C., KARA, E. E. & MCCOLL, S. R. (2013) A myriad of functions and complex regulation of the CCR7/CCL19/CCL21 chemokine axis in the adaptive immune system. *Cytokine & Growth Factor Reviews,* 24**,** 269-283.

COMERFORD, I., MILASTA, S., MORROW, V., MILLIGAN, G. & NIBBS, R. (2006) The chemokine receptor CCX-CKR mediates effective scavenging of CCL19 in vitro. *European Journal of Immunology,* 36**,** 1904-1916.

COMERFORD, I. & NIBBS, R. J. B. (2005) Post-translational control of chemokines: a role for,decoy receptors? *Immunology Letters,* 96**,** 163-174.

COMERFORD, I., NIBBS, R. J. B., LITCHFIELD, W., BUNTING, M., HARATA-LEE, Y., HAYLOCK-JACOBS, S., FORROW, S., KORNER, H. & MCCOLL, S. R. (2010) The atypical chemokine receptor CCX-CKR scavenges homeostatic chemokines in circulation and tissues and suppresses Th17 responses. *Blood,* 116**,** 4130-4140.

CONNOR, R. I., SHERIDAN, K. E., CERADINI, D., CHOE, S. & LANDAU, N. R. (1997) Change in coreceptor use correlates with disease progression in HIV-1-infected individuals. *Journal of Experimental Medicine,* 185**,** 621-628.

CORMIER, E. G., PERSUH, M., THOMPSON, D. A. D., LIN, S. W., SAKMAR, T. P., OLSON, W. C. & DRAGIC, T. (2000) Specific interaction of CCR5 amino-

terminal domain peptides containing sulfotyrosines with HIV-1 envelope glycoprotein gp120. *Proceedings of the National Academy of Sciences of the United States of America,* 97**,** 5762-5767.

COSTIN, J. M. (2007) Cytopathic mechanisms of HIV-I. *Virology Journal,* 4**,** 22.

COTTON, M. & CLAING, A. (2009) G protein-coupled receptors stimulation and the control of cell migration. *Cellular Signalling,* 21**,** 1045-1053.

COUCHMAN, J. R. & PATAKI, C. A. (2012) An Introduction to Proteoglycans and Their Localization. *Journal of Histochemistry & Cytochemistry,* 60**,** 885-897.

COUSSENS, L. M. & WERB, Z. (2002) Inflammation and cancer. *Nature,* 420**,** 860-867.

DAIRAGHI, D. J., ZHANG, P., WANG, Y., SEITZ, L. C., JOHNSON, D. A., MIAO, S., ERTL, L. S., ZENG, Y., POWERS, J. P., PENNELL, A. M., BEKKER, P., SCHALL, T. J. & JAEN, J. C. (2011) Pharmacokinetic and Pharmacodynamic Evaluation of the Novel CCR1 Antagonist CCX354 in Healthy Human Subjects: Implications for Selection of Clinical Dose. *Clinical Pharmacology & Therapeutics,* 89**,** 726-734.

DANAN, L. M., YU, Z. H., HOFFHINES, A. J., MOORE, K. L. & LEARY, J. A. (2008) Mass Spectrometric Kinetic Analysis of Human Tyrosylprotein Sulfotransferase-1 and-2. *Journal of the American Society for Mass Spectrometry,* 19**,** 1459-1466.

DANIELS, G. D., ZOU, J., CHARLEMAGNE, J., PARTULA, S., CUNNINGHAM, C. & SECOMBES, C. J. (1999) Cloning of two chemokine receptor homologs (CXC-R4 and CC-R7) in rainbow trout Oncorhynchus mykiss. *Journal of Leukocyte Biology,* 65**,** 684-690.

DAWSON, T. C., LENTSCH, A. B., WANG, Z. X., COWHIG, J. E., ROT, A., MAEDA, N. & PEIPER, S. C. (2000) Exaggerated response to endotoxin in mice lacking the Duffy antigen/receptor for chemokines (DARC). *Blood,* 96**,** 1681-1684.

DE RODA HUSMAN, A.-M., KOOT, M., CORNELISSEN, M., KEET, I. P. M., BROUWER, M., BROERSEN, S. M., BAKKER, M., ROOS, M. T. L., PRINS, M., DE WOLF, F., COUTINHO, R. A., MIEDEMA, F., GOUDSMIT, J. & SCHUITEMAKER, H. (1997) Association between CCR5 genotype and the clinical course of HIV-1 infection. *Annals of Internal Medicine,* 127**,** 882-890.

DERUAZ, M., FRAUENSCHUH, A., ALESSANDRI, A. L., DIAS, J. M., COELHO, F. M., RUSSO, R. C., FERREIRA, B. R., GRAHAM, G. J., SHAW, J. P., WELLS, T. N. C., TEIXEIRA, M. M., POWER, C. A. & PROUDFOOT, A. E. I. (2008) Ticks produce highly selective chemokine binding proteins with antiinflammatory activity. *Journal of Experimental Medicine,* 205**,** 2019-2031.

DI LIBERTO, D., LOCATI, M., CACCAMO, N., VECCHI, A., MERAVIGLIA, S., SALERNO, A., SIRECI, G., NEBULONI, M., CACERES, N., CARDONA, P. J., DIELI, F. & MANTOVANI, A. (2008) Role of the chemokine decoy receptor D6 in balancing inflammation, immune activation, and antimicrobial resistance in Mycobacterium tuberculosis infection. *Journal of Experimental Medicine,* 205**,** 2075-2084.

DIMITROV, D. S. (1997) How do viruses enter cells? The HIV coreceptors teach us a lesson of complexity. *Cell,* 91**,** 721-730.

DING, Y. Z., SHIMADA, Y., MAEDA, M., KAWABE, A., KAGANOI, J., KOMOTO, I., HASHIMOTO, Y., MIYAKE, M., HASHIDA, H. & IMAMURA, M. (2003) Association of CC chemokine receptor 7 with lymph node metastasis of esophageal squamous cell carcinoma. *Clinical Cancer Research,* 9**,** 3406-3412.

DORR, P., WESTBY, M., DOBBS, S., GRIFFIN, P., IRVINE, B., MACARTNEY, M., MORI, J., RICKETT, G., SMITH-BURCHNELL, C., NAPIER, C., WEBSTER, R., ARMOUR, D., PRICE, D., STAMMEN, B., WOOD, A. & PERROS, M. (2005) Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human

immunodeficiency virus type 1 activity. *Antimicrobial Agents and Chemotherapy,* 49**,** 4721-4732.

DRURY, L. J., ZIAREK, J. J., GRAVEL, S., VELDKAMP, C. T., TAKEKOSHI, T., HWANG, S. T., HEVEKER, N., VOLKMAN, B. F. & DWINELL, M. B. (2011) Monomeric and dimeric CXCL12 inhibit metastasis through distinct CXCR4 interactions and signaling pathways. *Proceedings of the National Academy of Sciences of the United States of America,* 108**,** 17655-17660.

DUMA, L., HAUSSINGER, D., ROGOWSKI, M., LUSSO, P. & GRZESIEK, S. (2007) Recognition of RANTES by extracellular parts of the CCR5 receptor. *Journal of Molecular Biology,* 365**,** 1063-1075.

EL-ASMAR, L., SPRINGAEL, J. Y., BALLET, S., ANDRIEU, E. U., VASSART, G. & PARMENTIER, M. (2005) Evidence for negative binding cooperativity within CCR5-CCR2b heterodimers. *Molecular Pharmacology,* 67**,** 460-469.

EPPERSON, M. L., LEE, C. A. & FREMONT, D. H. (2012) Subversion of cytokine networks by virally encoded decoy receptors. *Immunological Reviews,* 250**,** 199-215.

ESKO, J. D., STEWART, T. E. & TAYLOR, W. H. (1985) Animal-cell mutants defective in glycosaminoglycan biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America,* 82**,** 3197-3201.

FARZAN, M., MIRZABEKOV, T., KOLCHINSKY, P., WYATT, R., CAYABYAB, M., GERARD, N. P., GERARD, C., SODROSKI, J. & CHOE, H. (1999) Tyrosine sulfation of the amino terminus of CCR5 facilitates HIV-1 entry. *Cell,* 96**,** 667-676.

FARZAN, M., VASILIEVA, N., SCHNITZLER, C. E., CHUNG, S., ROBINSON, J., GERARD, N. P., GERARD, C., CHOE, H. & SODROSKI, J. (2000) A tyrosine-sulfated peptide based on the N terminus of CCR5 interacts with a CD4-enhanced epitope of the HIV-1 gp120 envelope glycoprotein and inhibits HIV-1 entry. *Journal of Biological Chemistry,* 275**,** 33516-33521.

FATKENHEUER, G., POZNIAK, A. L., JOHNSON, M. A., PLETTENBERG, A., STASZEWSKI, S., HOEPELMAN, A. I. M., 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. & VAN DER RYST, E. (2005) Efficacy of short-term monotherapy with maraviroc, a new CCR5 antagonist, in patients infected with HIV-1. *Nature Medicine,* 11**,** 1170-1172.

FENG, L. Y., OU, Z. L., WU, F. Y., SHEN, Z. Z. & SHAO, Z. M. (2009) Involvement of a Novel Chemokine Decoy Receptor CCX-CKR in Breast Cancer Growth, Metastasis and Patient Survival. *Clinical Cancer Research,* 15**,** 2962-2970.

FERNANDO, H., CHIN, C., ROSGEN, J. & RAJARATHNAM, K. (2004) Dimer dissociation is essential for interleukin-8 (IL-8) binding to CXCR1 receptor. *Journal of Biological Chemistry,* 279**,** 36175-36178.

FIRESTEIN, G. S. (2003) Evolving concepts of rheumatoid arthritis. *Nature,* 423**,** 356-361.

FONG, A. M., ALAM, S. M., IMAI, T., HARIBABU, B. & PATEL, D. D. (2002) CX(3)CR1 tyrosine sulfation enhances fractalkine-induced cell adhesion. *Journal of Biological Chemistry,* 277**,** 19418-19423.

FONG, A. M., ROBINSON, L. A., STEEBER, D. A., TEDDER, T. F., YOSHIE, O., IMAI, T. & PATEL, D. D. (1998) Fractalkine and CX(3)CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. *Journal of Experimental Medicine,* 188**,** 1413-1419.

FORSTER, R., DAVALOS-MISSLITZ, A. C. & ROT, A. (2008) CCR7 and its ligands: balancing immunity and tolerance. *Nature Reviews Immunology,* 8**,** 362-371.

FRA, A. M., LOCATI, M., OTERO, K., SIRONI, M., SIGNORELLI, P., MASSARDI, M. L., GOBBI, M., VECCHI, A., SOZZANI, S. & MANTOVANI, A. (2003) Cutting

edge: Scavenging of inflammatory CC chemokines by the promiscuous putatively silent chemokine receptor D6(1). *Journal of Immunology,* 170**,** 2279-2282.

FUKUMA, N., AKIMITSU, N., HAMAMOTO, H., KUSUHARA, H., SUGIYAMA, Y. & SEKIMIZU, K. (2003) A role of the Duffy antigen for the maintenance of plasma chemokine concentrations. *Biochemical and Biophysical Research Communications,* 303**,** 137-139.

GALLIERA, E., JALA, V. R., TRENT, J. O., BONECCHI, R., SIGNORELLI, P., LEFKOWITZ, R. J., MANTOVANI, A., LOCATI, M. & HARIBABU, B. (2004) beta-arrestin-dependent constitutive internalization of the human chemokine decoy receptor D6. *Journal of Biological Chemistry,* 279**,** 25590-25597.

GALLO, R. C. & MONTAGNIER, L. (2003) Retrospective: The discovery of HIV as the cause of AIDS. *New England Journal of Medicine,* 349**,** 2283-2285.

GALZI, J. L., HACHET-HAAS, M., BONNET, D., DAUBEUF, F., LECAT, S., HIBERT, M., HAIECH, J. & FROSSARD, N. (2010) Neutralizing endogenous chemokines with small molecules Principles and potential therapeutic applications. *Pharmacology & Therapeutics,* 126**,** 39-55.

GAO, P., ZHOU, X. Y., YASHIRO-OHTANI, Y., YANG, Y. F., SUGIMOTO, N., ONO, S., NAKANISHI, T., OBIKA, S., IMANISHI, T., EGAWA, T., NAGASAWA, T., FUJIWARA, H. & HAMAOKA, T. (2003) The unique target specificity of a nonpeptide chemokine receptor antagonist: selective blockade of two Th1 chemokine receptors CCR5 and CXCR3. *Journal of Leukocyte Biology,* 73**,** 273-280.

GARDNER, L., PATTERSON, A. M., ASHTON, B. A., STONE, M. A. & MIDDLETON, J. (2004) The human Duffy antigen binds selected inflammatory but not homeostatic chemokines. *Biochemical and Biophysical Research Communications,* 321**,** 306-312.

GARLANDA, C., MAINA, V., MARTINEZ DE LA TORRE, Y., NEBULONI, M. & LOCATI, M. (2008) Inflammatory reaction and implantation: the new entries PTX3 and D6. *Placenta,* 29 Suppl B**,** 129-34.

GARRED, P., MADSEN, H. O., PETERSEN, J., MARQUART, H., HANSEN, T. M., SORENSEN, S. F., VOLCK, B., SVEJGAARD, A. & ANDERSEN, V. (1998) CC chemokine receptor 5 polymorphism in rheumatoid arthritis. *Journal of Rheumatology,* 25**,** 1462-1465.

GERARD, C. & ROLLINS, B. J. (2001) Chemokines and disease. *Nature Immunology,* 2**,** 108-115.

GERRITS, H., SCHENAU, D., BAKKER, N. E. C., VAN DISSELDORP, A. J. M., STRIK, A., HERMENS, L. S., KOENEN, T. B., KRAJNC-FRANKEN, M. A. M. & GOSSEN, J. A. (2008) Early postnatal lethality and cardiovascular defects in CXCR7-deficient mice. *Genesis,* 46**,** 235-245.

GILLITZER, R., WOLFF, K., TONG, D., MULLER, C., YOSHIMURA, T., HARTMANN, A. A., STINGL, G. & BERGER, R. (1993) MCP-1 messenger-RNA expression in basal keratinocytes of psoriatic lesions. *Journal of Investigative Dermatology,* 101**,** 127-131.

GLUSHAKOVA, S., GRIVEL, J. C., FITZGERALD, W., SYLWESTER, A., ZIMMERBERG, J. & MARGOLIS, L. B. (1998) Evidence for the HIV-1 phenotype switch as a causal factor in acquired immunodeficiency. *Nature Medicine,* 4**,** 346-349.

GOMEZ-REINO, J. J., PABLOS, J. L., CARREIRA, P. E., SANTIAGO, B., SERRANO, L., VICARIO, J. L., BALSA, A., FIGUEROA, M. & DE JUAN, M. D. (1999) Association of rheumatoid arthritis with a functional chemokine receptor, CCR5. *Arthritis and Rheumatism,* 42**,** 989-992.

GONG, J. H. & CLARKLEWIS, I. (1995) Antagonists of monocyte chemoattractant protein-1 identified by modification of functionally critical NH2-terminal residues. *Journal of Experimental Medicine,* 181**,** 631-640.

GONG, J. H., RATKAY, L. G., WATERFIELD, J. D. & CLARKLEWIS, I. (1997) An antagonist of monocyte chemoattractant protein 1 (MCP-1) inhibits arthritis in the MRL-lpr mouse model. *Journal of Experimental Medicine,* 186**,** 131-137.

GONG, J. H., UGUCCIONI, M., DEWALD, B., BAGGIOLINI, M. & CLARKLEWIS, I. (1996) RANTES and MCP-3 antagonists bind multiple chemokine receptors. *Journal of Biological Chemistry,* 271**,** 10521-10527.

GONZALEZ, E., KULKARNI, H., BOLIVAR, H., MANGANO, A., SANCHEZ, R., CATANO, G., NIBBS, R. J., FREEDMAN, B. I., QUINONES, M. P., BAMSHAD, M. J., MURTHY, K. K., ROVIN, B. H., BRADLEY, W., CLARK, R. A., ANDERSON, S. A., O'CONNELL, R. J., AGAN, B. K., AHUJA, S. S., BOLOGNA, R., SEN, L., DOLAN, M. J. & AHUJA, S. K. (2005) The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. *Science,* 307**,** 1434-1440.

GORRY, P. R., DUNFEE, R. L., MEFFORD, M. E., KUNSTMAN, K., MORGAN, T., MOORE, J. P., MASCOLA, J. R., AGOPIAN, K., HOLM, G. H., MEHLE, A., TAYLOR, J., FARZAN, M., WANG, H., ELLERY, P., WILLEY, S. J., CLAPHAM, P. R., WOLINSKY, S. M., CROWE, S. M. & GABUZDA, D. (2007) Changes in the V3 region of gp 120 contribute to unusually broad coreceptor usage of an HIV-1 isolate from a CCR5 Delta 32 heterozygote. *Virology,* 362**,** 163-178.

GOZANSKY, E. K., LOUIS, J. M., CAFFREY, M. C. & CLORE, G. M. (2005) Mapping the binding of the tail of the CXCR4 receptor n-terminal extracellular to stromal cell-derived factor-1 alpha. *Journal of Molecular Biology,* 345**,** 651-658.

GRAHAM, G. J. (2009) D6 and the atypical chemokine receptor family: Novel regulators of immune and inflammatory processes. *European Journal of Immunology,* 39**,** 342-351.

GRAHAM, G. J. & LOCATI, M. (2013) Regulation of the immune and inflammatory responses by the 'atypical' chemokine receptor D6. *Journal of Pathology,* 229**,** 168-175.

GRAHAM, G. J., LOCATI, M., MANTOVANI, A., ROT, A. & THELEN, M. (2012) The biochemistry and biology of the atypical chemokine receptors. *Immunology Letters,* 145**,** 30-38.

GRAHAM, G. J., MACKENZIE, J., LOWE, S., TSANG, M. L. S., WEATHERBEE, J. A., ISSACSON, A., MEDICHERLA, J., FANG, F., WILKINSON, P. C. & PRAGNELL, I. B. (1994) Aggregation of the chemokine MIP-1-alpha is a dynamic and reversible phenomenon - biochemical and biological analyses. *Journal of Biological Chemistry,* 269**,** 4974-4978.

GRAHAM, G. J. & MCKIMMIE, C. S. (2006) Chemokine scavenging by D6: a movable feast? *Trends in Immunology,* 27**,** 381-386.

GRAHAM, G. J., WILKINSON, P. C., NIBBS, R. J. B., LOWE, S., KOLSET, S. O., PARKER, A., FRESHNEY, M. G., TSANG, M. L. S. & PRAGNELL, I. B. (1996) Uncoupling of stem cell inhibition from monocyte chemoattraction in MIP-1 alpha by mutagenesis of the proteoglycan binding site. *Embo Journal,* 15**,** 6506-6515.

GRAHAM, G. J., WRIGHT, E. G., HEWICK, R., WOLPE, S. D., WILKIE, N. M., DONALDSON, D., LORIMORE, S. & PRAGNELL, I. B. (1990) Identification and characterization of an inhibitor of hematopoietic stem-cell proliferation. *Nature,* 344**,** 442-444.

GRAY, L., STERJOVSKI, J., CHURCHILL, M., ELLERY, P., NASR, N., LEWIN, S. R., CROWE, S. M., WESSELINGH, S. L., CUNNINGHAM, A. L. & GORRY, P. R. (2005) Uncoupling coreceptor usage of human immunodeficiency virus type 1 (HIV-1) from macrophage tropism reveals biological properties of CCR5-restricted

HIV-1 isolates from patients with acquired immunodeficiency syndrome. *Virology,* 337**,** 384-398.

GREENE, C. M. & MCELVANEY, N. G. (2009) Proteases and antiproteases in chronic neutrophilic lung disease - relevance to drug discovery. *British Journal of Pharmacology,* 158**,** 1048-1058.

GUAN, E., WANG, J. H., RODERIQUEZ, G. & NORCROSS, M. A. (2002) Natural truncation of the chemokine MIP-1 beta/CCL4 affects receptor specificity but not Anti-HIV-1 activity. *Journal of Biological Chemistry,* 277**,** 32348-32352.

GUNTHER, K., LEIER, J., HENNING, G., DIMMLER, A., WEISSBACH, R., HOHENBERGER, W. & FORSTER, R. (2005) Prediction of lymph node metastasis in colorectal carcinoma by expression of chemokine receptor CCR7. *International Journal of Cancer,* 116**,** 726-733.

GUPTA, S. K., PILLARISETTI, K., GRAY, S. L. & STADEL, J. M. (1998) Molecular cloning of a novel chemokine receptor-like gene from early stage chick embryos. *Biochemistry and Molecular Biology International,* 44**,** 673-681.

GUTIERREZ, J., KREMER, L., ZABALLOS, A., GOYA, I., MARTINEZ, C. & MARQUEZ, G. (2004) Analysis of post-translational CCR8 modifications and their influence on receptor activity. *Journal of Biological Chemistry,* 279**,** 14726-14733.

HAAS, C. S., MARTINEZ, R. J., ATTIA, N., HAINES, G. K., CAMPBELL, P. L. & KOCH, A. E. (2005) Chemokine receptor expression in rat adjuvant-induced arthritis. *Arthritis and Rheumatism,* 52**,** 3718-3730.

HAESSLER, U., PISANO, M., WU, M. M. & SWARTZ, M. A. (2011) Dendritic cell chemotaxis in 3D under defined chemokine gradients reveals differential response to ligands CCL21 and CCL19. *Proceedings of the National Academy of Sciences of the United States of America,* 108**,** 5614-5619.

HAN, K. H., GREEN, S. R., TANGIRALA, R. K., TANAKA, S. & QUEHENBERGER, O. (1999) Role of the first extracellular loop in the functional activation of CCR2 - The first extracellular loop contains distinct domains necessary for both agonist binding and transmembrane signaling. *Journal of Biological Chemistry,* 274**,** 32055-32062.

HANAHAN, D. & WEINBERG, R. A. (2000) The hallmarks of cancer. *Cell,* 100**,** 57-70.

HANDEL, T. M., JOHNSON, Z., CROWN, S. E., LAU, E. K., SWEENEY, M. & PROUDFOOT, A. E. (2005) Regulation of protein function by glycosaminoglycans - as exemplified by chemokines. *Annual Review of Biochemistry,* 74**,** 385-410.

HANDEL, T. M., JOHNSON, Z., RODRIGUES, D. H., DOS SANTOS, A. C., CIRILLO, R., MUZIO, V., RIVA, S., MACK, M., DERUAZ, M., BORLAT, F., VITTE, P. A., WELLS, T. N. C., TEIXEIRA, M. M. & PROUDFOOT, A. E. I. (2008) An engineered monomer of CCL2 has anti-inflammatory properties emphasizing the importance of oligomerization for chemokine activity in vivo. *Journal of Leukocyte Biology,* 84**,** 1101-1108.

HANSELL, C. A. H., SCHIERING, C., KINSTRIE, R., FORD, L., BORDON, Y., MCINNES, I. B., GOODYEAR, C. S. & NIBBS, R. J. B. (2011) Universal expression and dual function of the atypical chemokine receptor D6 on innate-like B cells in mice. *Blood,* 117**,** 5413-5424.

HARTL, D., LATZIN, P., HORDIJK, P., MARCOS, V., RUDOLPH, C., WOISCHNIK, M., KRAUSS-ETSCHMANN, S., KOLLER, B., REINHARDT, D., ROSCHER, A. A., ROOS, D. & GRIESE, M. (2007) Cleavage of CXCR1 on neutrophils disables bacterial killing in cystic fibrosis lung disease. *Nature Medicine,* 13**,** 1423-1430.

HARTLEY, O., KLASSE, P. J., SATTENTAU, Q. J. & MOORE, J. P. (2005) V3: HIV's switch-hitter. *Aids Research and Human Retroviruses,* 21**,** 171-189.

HASKELL, C. A., CLEARY, M. D. & CHARO, I. F. (1999) Molecular uncoupling of fractalkine-mediated cell adhesion and signal transduction - Rapid flow arrest of CX(3)CR1-expressing cells is independent of G-protein activation. *Journal of Biological Chemistry,* 274**,** 10053-10058.

HELBIG, G., CHRISTOPHERSON, K. W., BHAT-NAKSHATRI, P., KUMAR, S., KISHIMOTO, H., MILLER, K. D., BROXMEYER, H. E. & NAKSHATRI, H. (2003) NF-kappa B promotes breast cancer cell migration and metastasis by inducing the expression of the chemokine receptor CXCR4. *Journal of Biological Chemistry,* 278**,** 21631-21638.

HEMMERICH, S., PAAVOLA, C., BLOOM, A., BHAKTA, S., FREEDMAN, R., GRUNBERGER, D., KRSTENANSKY, J., LEE, S., MCCARLEY, D., MULKINS, M., WONG, B., PEASE, J., MIZOUE, L., MIRZADEGAN, T., POLSKY, I., THOMPSON, K., HANDEL, T. M. & JARNAGIN, K. (1999) Identification of residues in the monocyte chemotactic protein-1 that contact the MCP-1 receptor, CCR2. *Biochemistry,* 38**,** 13013-13025.

HENDRIX, C. W., COLLIER, A. C., LEDERMAN, M. M., SCHOLS, D., POLLARD, R. B., BROWN, S., JACKSON, J. B., COOMBS, R. W., GLESHY, M. J., FLEXNER, C. W., BRIDGER, G. J., BADEL, K., MACFARLAND, R. T., HENSON, G. W., CALANDRA, G. & GRP, A. H. S. (2004) Safety, pharmacokinetics, and antiviral activity of AMD3100, a selective CXCR4 receptor inhibitor, in HIV-1 infection. *Jaids-Journal of Acquired Immune Deficiency Syndromes,* 37**,** 1253-1262.

HEWIT, K. D., FRASER, A., NIBBS, R. J. & GRAHAM, G. J. (2014) The N-terminal region of the atypical chemokine receptor ACKR2 is a key determinant of ligand binding. *Journal of Biological Chemistry,* 289, 12330-12342.

HIROTA, K., YOSHITOMI, H., HASHIMOTO, M., MAEDA, S., TERADAIRA, S., SUGIMOTO, N., YAMAGUCHI, T., NOMURA, T., ITO, H., NAKAMURA, T., SAKAGUCHI, N. & SAKAGUCHI, S. (2007) Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. *Journal of Experimental Medicine,* 204**,** 2803-2812.

HOFFHINES, A. J., DAMOC, E., BRIDGES, K. G., LEARY, J. A. & MOORE, K. L. (2006) Detection and purification of tyrosine-sulfated proteins using a novel anti-sulfotyrosine monoclonal antibody. *Journal of Biological Chemistry,* 281**,** 37877-37887.

HOLST, P. J., LUTTICHAU, H. R., SCHWARTZ, T. W. & ROSENKILDE, M. M. (2003) Virally encoded chemokines and chemokine receptors in the role of viral infections. *Contributions to microbiology,* 10**,** 232-52.

HOMEY, B., ALENIUS, H., MULLER, A., SOTO, H., BOWMAN, E. P., YUAN, W., MCEVOY, L., LAUERMA, A. I., ASSMANN, T., BUNEMANN, E., LEHTO, M., WOLFF, H., YEN, D., MARXHAUSEN, H., TO, W., SEDGWICK, J., RUZICKA, T., LEHMANN, P. & ZLOTNIK, A. (2002) CCL27-CCR10 interactions regulate T cell-mediated skin inflammation. *Nature Medicine,* 8**,** 157-165.

HOMEY, B., DIEU-NOSJEAN, M. C., WIESENBORN, A., MASSACRIER, C., PIN, J. J., OLDHAM, E., CATRON, D., BUCHANAN, M. E., MULLER, A., MALEFYT, R. D., DENG, G., OROZCO, R., RUZICKA, T., LEHMANN, P., LEBECQUE, S., CAUX, C. & ZLOTNIK, A. (2000) Up-regulation of macrophage inflammatory protein-3 alpha/CCL20 and CC chemokine receptor 6 in psoriasis. *Journal of Immunology,* 164**,** 6621-6632.

HOPKINS, A. L. & GROOM, C. R. (2002) The druggable genome. *Nature Reviews Drug Discovery,* 1**,** 727-730.

HORUK, R. (2009) OPINION Chemokine receptor antagonists: overcoming developmental hurdles. *Nature Reviews Drug Discovery,* 8**,** 23-33.

HORUK, R., CHITNIS, C. E., DARBONNE, W. C., COLBY, T. J., RYBICKI, A., HADLEY, T. J. & MILLER, L. H. (1993) A receptor for the malarial parasite plasmodium-vivax - the erythrocyte chemokine receptor. *Science,* 261**,** 1182-1184.

HOU, T., LIANG, D., XU, L., HUANG, X., HUANG, Y. & ZHANG, Y. (2013) Atypical chemokine receptors predict lymph node metastasis and prognosis in patients with cervical squamous cell cancer. *Gynecologic oncology,* 130**,** 181-7.

HOWARD, O. M. Z., DONG, H. F., SU, S. B., CASPI, R. R., CHEN, X., PLOTZ, P. & OPPENHEIM, J. J. (2005) Autoantigens signal through chemokine receptors: uveitis antigens induce CXCR3- and CXCR5-expressing lymphocytes and immature dendritic cells to migrate. *Blood,* 105**,** 4207-4214.

HUISING, M. O., STET, R. J. M., KRUISWIJK, C. P., SAVELKOUL, H. F. J. & VERBURG-VAN KEMENADE, B. M. L. (2003) Molecular evolution of CXC chemokines: extant CXC chemokines originate from the CNS. *Trends in Immunology,* 24**,** 307-313.

ISSAFRAS, H., ANGERS, S., BULENGER, S., BLANPAIN, C., PARMENTIER, M., LABBE-JULLIE, C., BOUVIER, M. & MARULLO, S. (2002) Constitutive agonist-independent CCR5 oligomerization and antibody-mediated clustering occurring at physiological levels of receptors. *Journal of Biological Chemistry,* 277**,** 34666-34673.

JAMIESON, T., COOK, D. N., NIBBS, R. J. B., ROT, A., NIXON, C., MCLEAN, P., ALCAMI, A., LIRA, S. A., WIEKOWSKI, M. & GRAHAM, G. J. (2005) The chemokine receptor D6 limits the inflammatory response in vivo. *Nature Immunology,* 6**,** 403-411.

JILMA-STOHLAWETZ, P., HOMONCIK, M., DRUCKER, C., MARSIK, C., ROT, A., MAYR, W. R., SEIBOLD, B. & JILMA, B. (2001) Fy phenotype and gender determine plasma levels of monocyte chemotactic protein. *Transfusion,* 41**,** 378-381.

JOHN, S., SMITH, S., MORRISON, J. F., SYMMONS, D., WORTHINGTON, J., SILMAN, A. & BARTON, A. (2003) Genetic variation in CCR5 does not predict clinical outcome in inflammatory arthritis. *Arthritis and Rheumatism,* 48**,** 3615-3616.

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. C. & PROUDFOOT, A. E. I. (2004) Interference with heparin binding and oligomerization creates a novel anti-inflammatory strategy targeting the chemokine system. *Journal of Immunology,* 173**,** 5776-5785.

JOHNSON, Z., PROUDFOOT, A. E. & 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 Reviews,* 16**,** 625-636.

JOYCE, J. A. & POLLARD, J. W. (2009) Microenvironmental regulation of metastasis. *Nature Reviews Cancer,* 9**,** 239-252.

KATADA, T. (2012) The Inhibitory G Protein G(i) Identified as Pertussis Toxin-Catalyzed ADP-Ribosylation. *Biological & Pharmaceutical Bulletin,* 35**,** 2103-2111.

KAWAMURA, T., STEPHENS, B., QIN, L., YIN, X., DORES, M. R., SMITH, T. H., GRIMSEY, N., ABAGYAN, R., TREJO, J., KUFAREVA, I., FUSTER, M. M., SALANGA, C. L. & HANDEL, T. M. (2014) A General Method for Site Specific Fluorescent Labeling of Recombinant Chemokines. *Plos One,* 9**,** 10..

KEHOE, J. W. & BERTOZZI, C. R. (2000) Tyrosine sulfation: a modulator of extracellular protein-protein interactions. *Chemistry & Biology,* 7**,** R57-R61.

KOCH, A. E., KUNKEL, S. L., HARLOW, L. A., JOHNSON, B., EVANOFF, H. L., HAINES, G. K., BURDICK, M. D., POPE, R. M. & STRIETER, R. M. (1992)

Enhanced production of monocyte chemoattractant protein-1 in rheumatoid-arthritis. *Journal of Clinical Investigation,* 90**,** 772-779.

KOCHI, Y., OKADA, Y., SUZUKI, A., IKARI, K., TERAO, C., TAKAHASHI, A., YAMAZAKI, K., HOSONO, N., MYOUZEN, K., TSUNODA, T., KAMATANI, N., FURUICHI, T., IKEGAWA, S., OHMURA, K., MIMORI, T., MATSUDA, F., IWAMOTO, T., MOMOHARA, S., YAMANAKA, H., YAMADA, R., KUBO, M., NAKAMURA, Y. & YAMAMOTO, K. (2010) A regulatory variant in CCR6 is associated with rheumatoid arthritis susceptibility. *Nature Genetics,* 42**,** 515-U63.

KOENEN, R. R., VON HUNDELSHAUSEN, P., NESMELOVA, I. V., ZERNECKE, A., LIEHN, E. A., SARABI, A., KRAMP, B. K., PICCININI, A. M., PALUDAN, S. R., KOWALSKA, M. A., KUNGL, A. J., HACKENG, T. M., MAYO, K. H. & WEBER, C. (2009) Disrupting functional interactions between platelet chemokines inhibits atherosclerosis in hyperlipidemic mice. *Nature Medicine,* 15**,** 97-103.

KOLTSOVA, E. & LEY, K. (2009) Tyrosine Sulfation of Leukocyte Adhesion Molecules and Chemokine Receptors Promotes Atherosclerosis. *Arteriosclerosis Thrombosis and Vascular Biology,* 29**,** 1709-1711.

KOOPMANN, W. & KRANGEL, M. S. (1997) Identification of a glycosaminoglycan-binding site in chemokine macrophage inflammatory protein-1 alpha. *Journal of Biological Chemistry,* 272**,** 10103-10109.

KOZIEL, J., MACIAG-GUDOWSKA, A., MIKOLAJCZYK, T., BZOWSKA, M., STURDEVANT, D. E., WHITNEY, A. R., SHAW, L. N., DELEO, F. R. & POTEMPA, J. (2009) Phagocytosis of Staphylococcus aureus by Macrophages Exerts Cytoprotective Effects Manifested by the Upregulation of Antiapoptotic Factors. *Plos One,* 4**,** 14.

KRIEGOVA, E., TSYRULNYK, A., ARAKELYAN, A., MRAZEK, F., ORDELTOVA, M., PETZMANN, S., ZATLOUKAL, J., KOLEK, V., DU BOIS, R. M., POPPER, H. & PETREK, M. (2006) Expression of CCX CKR in pulmonary sarcoidosis. *Inflammation Research,* 55**,** 441-445.

KRUEGER, J. G. (2002) The immunologic basis for the treatment of psoriasis with new biologic agents. *Journal of the American Academy of Dermatology,* 46**,** 1-23.

KUBICA, M., GUZIK, K., KOZIEL, J., ZAREBSKI, M., RICHTER, W., GAJKOWSKA, B., GOLDA, A., MACIAG-GUDOWSKA, A., BRIX, K., SHAW, L., FOSTER, T. & POTEMPA, J. (2008) A Potential New Pathway for Staphylococcus aureus Dissemination: The Silent Survival of S. aureus Phagocytosed by Human Monocyte-Derived Macrophages. *Plos One,* 3**,** 16.

KUCIA, M., RECA, R., MIEKUS, K., WANZECK, J., WOJAKOWSKI, W., JANOWSKA-WIECZOREK, A., RATAJCZAK, J. & RATAJCZAK, M. Z. (2005) Trafficking of normal stem cells and metastasis of cancer stem cells involve similar mechanisms: Pivotal role of the SDF-1-CXCR4 axis. *Stem Cells,* 23**,** 879-894.

KUSCHERT, G. S. V., COULIN, F., POWER, C. A., PROUDFOOT, A. E. I., HUBBARD, R. E., HOOGEWERF, A. J. & WELLS, T. N. C. (1999) Glycosaminoglycans interact selectively with chemokines and modulate receptor binding and cellular responses. *Biochemistry,* 38**,** 12959-12968.

LAARMAN, A. J., MIJNHEER, G., MOOTZ, J. M., VAN ROOIJEN, W. J. M., RUYKEN, M., MALONE, C. L., HEEZIUS, E. C., WARD, R., MILLIGAN, G., VAN STRIJP, J. A. G., DE HAAS, C. J. C., HORSWILL, A. R., VAN KESSEL, K. P. M. & ROOIJAKKERS, S. H. M. (2012) Staphylococcus aureus Staphopain A inhibits CXCR2-dependent neutrophil activation and chemotaxis. *Embo Journal,* 31**,** 3607-3619.

LAINE, R. A. (1994) A calculation of all possible oligosaccharide isomers both branched and linear yields $1.05x10^{(12)}$ structures for a reducing hexasaccharide - the isomer-

barrier to development of single-method saccharide sequencing or synthesis systems. *Glycobiology,* 4**,** 759-767.

LAPIDOT, T. & PETIT, I. (2002) Current understanding of stem cell mobilization: The roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. *Experimental Hematology,* 30**,** 973-981.

LAU, E. K., PAAVOLA, C. D., JOHNSON, Z., GAUDRY, J. P., GERETTI, E., BORLAT, F., KUNGL, A. J., PROUDFOOT, A. E. & HANDEL, T. M. (2004) Identification of the glycosaminoglycan binding site of the CC chemokine, MCP-1 - Implications for structure and function in vivo. *Journal of Biological Chemistry,* 279**,** 22294-22305.

LAX, P., LIMATOLA, C., FUCILE, S., TRETTEL, F., DI BARTOLOMEO, S., RENZI, M., RAGOZZINO, D. & EUSEBI, F. (2002) Chemokine receptor CXCR2 regulates the functional properties of AMPA-type glutamate receptor GluR 1 in HEK cells. *Journal of Neuroimmunology,* 129**,** 66-73.

LEE, B., SHARRON, M., BLANPAIN, C., DORANZ, B. J., VAKILI, J., SETOH, P., BERG, E., LIU, G., GUY, H. R., DURELL, S. R., PARMENTIER, M., CHANG, C. N., PRICE, K., TSANG, M. & DOMS, R. W. (1999) Epitope mapping of CCR5 reveals multiple conformational states and distinct but overlapping structures involved in chemokine and coreceptor function. *Journal of Biological Chemistry,* 274**,** 9617-9626.

LEE, K. M., MCKIMMIE, C. S., GILCHRIST, D. S., PALLAS, K. J., NIBBS, R. J., GARSIDE, P., MCDONALD, V., JENKINS, C., RANSOHOFF, R., LIU, L. P., MILLING, S., CEROVIC, V. & GRAHAM, G. J. (2011) D6 facilitates cellular migration and fluid flow to lymph nodes by suppressing lymphatic congestion. *Blood,* 118**,** 6220-6229.

LEE, K. M., NIBBS, R. J. B. & GRAHAM, G. J. (2013a) D6: the 'crowd controller' at the immune gateway. *Trends in Immunology,* 34**,** 7-12.

LEE, Y. H., BAE, S. C. & SONG, G. G. (2013b) Association between the chemokine receptor 5 delta32 polymorphism and rheumatoid arthritis: a meta-analysis. *Modern Rheumatology,* 23**,** 304-310.

LEI, Y. & TAKAHAMA, Y. (2012) XCL1 and XCR1 in the immune system. *Microbes and Infection,* 14**,** 262-267.

LEVER, R. & PAGE, C. R. (2002) Novel drug development opportunities for heparin. *Nature Reviews Drug Discovery,* 1**,** 140-148.

LEVESQUE, J. P., HENDY, J., TAKAMATSU, Y., SIMMONS, P. J. & BENDALL, L. J. (2003) Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by GCSF or cyclophosphamide. *Journal of Clinical Investigation,* 111**,** 187-196.

LEVESQUE, J. P., LIU, F., SIMMONS, P. J., BETSUYAKU, T., SENIOR, R. M., PHAM, C. & LINK, D. C. (2004) Characterization of hematopoietic progenitor mobilization in protease-deficient mice. *Blood,* 104**,** 65-72.

LEVOYE, A., BALABANIAN, K., BALEUX, F., BACHELERIE, F. & LAGANE, B. (2009) CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling. *Blood,* 113**,** 6085-6093.

LIDER, O., BAHARAV, E., MEKORI, Y. A., MILLER, T., NAPARSTEK, Y., VLODAVSKY, I. & COHEN, I. R. (1989) Suppression of experimental autoimmune-diseases and prolongation of allograft survival by treatment of animals with low-doses of heparins. *Journal of Clinical Investigation,* 83**,** 752-756.

LINDNER, E., NORDANG, G. B. N., MELUM, E., FLATO, B., SELVAAG, A. M., THORSBY, E., KVIEN, T. K., FORRE, O. T. & LIE, B. A. (2007) Lack of association between the chemokine receptor 5 polymorphism CCR5delta32 in rheumatoid arthritis and juvenile idiopathic arthritis. *Bmc Medical Genetics,* 8**,** 5.

LIU, J., LOUIE, S., HSU, W., YU, K. M., NICHOLAS, H. B. & ROSENQUIST, G. L. (2008) Tyrosine sulfation is prevalent in human chemokine receptors important in lung disease. *American Journal of Respiratory Cell and Molecular Biology,* 38**,** 738-743.

LIU, R., PAXTON, W. A., CHOE, S., CERADINI, D., MARTIN, S. R., HORUK, R., MACDONALD, M. E., STUHLMANN, H., KOUP, R. A. & LANDAU, N. R. (1996) Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell,* 86**,** 367-377.

LOCATI, M., TORRE, Y. M. D. L., GALLIERA, E., BONECCHI, R., BODDULURI, H., VAGO, G., VECCHI, A. & MANTOVANI, A. (2005) Silent chemoattractant receptors: D6 as a decoy and scavenger receptor for inflammatory CC chemokines. *Cytokine & Growth Factor Reviews,* 16**,** 679-686.

LOOS, T., MORTIER, A., GOUWY, M., RONSSE, I., PUT, W., LENAERTS, J. P., VAN DAMME, J. & 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**,** 2648-2656.

LOVE, M., SANDBERG, J. L., ZIAREK, J. J., GERARDEN, K. P., RODE, R. R., JENSEN, D. R., MCCASLIN, D. R., PETERSON, F. C. & VELDKAMP, C. T. (2012) Solution Structure of CCL21 and Identification of a Putative CCR7 Binding Site. *Biochemistry,* 51**,** 733-735.

LUDWIG, A., EHLERT, J. E., FLAD, H. D. & BRANDT, E. (2000) Identification of distinct surface-expressed and intracellular CXC-chemokine receptor 2 glycoforms in neutrophils: N-glycosylation is essential for maintenance of receptor surface expression. *Journal of Immunology,* 165**,** 1044-1052.

LUKACS, N. W. & HARRISON, J. K. (2007) *The birth and maturation of chemokines and their receptors*, Humana Press Inc.

LUKER, K. E., STEELE, J. M., MIHALKO, L. A., RAY, P. & LUKER, G. D. (2010) Constitutive and chemokine-dependent internalization and recycling of CXCR7 in breast cancer cells to degrade chemokine ligands. *Oncogene,* 29**,** 4599-4610.

LUROSS, J. A. & WILLIAMS, N. A. (2001) The genetic and immunopathological processes underlying collagen-induced arthritis. *Immunology,* 103**,** 407-416.

LUSTER, A. D. (1998) Chemokines - Chemotactic cytokines that mediate inflammation. *New England Journal of Medicine,* 338**,** 436-445.

LUTHER, S. A., BIDGOL, A., HARGREAVES, D. C., SCHMIDT, A., XU, Y., PANIYADI, J., MATLOUBIAN, M. & CYSTER, J. G. (2002) Differing activities of homeostatic chemokines CCL19, CCL21, and CXCL12 in lymphocyte and dendritic cell recruitment and lymphoid neogenesis. *Journal of Immunology,* 169**,** 424-433.

LUTTICHAU, H. R., STINE, J., BOESEN, T. P., JOHNSEN, A. H., CHANTRY, D., GERSTOFT, J. & SCHWARTZ, T. W. (2000) A highly selective CC chemokine receptor (CCR)8 antagonist encoded by the poxvirus molluscum contagiosum. *Journal of Experimental Medicine,* 191**,** 171-179.

MA, Q., JONES, D., BORGHESANI, P. R., SEGAL, R. A., NAGASAWA, T., KISHIMOTO, T., BRONSON, R. T. & SPRINGER, T. A. (1998) Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America,* 95**,** 9448-9453.

MADIGAN, J., FREEMAN, D. J., MENZIES, F., FORROW, S., NELSON, S. M., YOUNG, A., SHARKEY, A., MOFFET, A., GRAHAM, G. J., GREER, I. A., ROT, A. & NIBBS, R. J. B. (2010) Chemokine Scavenger D6 Is Expressed by Trophoblasts and Aids the Survival of Mouse Embryos Transferred into Allogeneic Recipients. *Journal of Immunology,* 184**,** 3202-3212.

MANTOVANI, A. (1999) The chemokine system: redundancy for robust outputs. *Immunology Today,* 20**,** 254-257.

MANTOVANI, A., BONECCHI, R. & LOCATI, M. (2006) Tuning inflammation and immunity by chemokine sequestration: decoys and more. *Nature Reviews Immunology,* 6**,** 907-918.

MARTINEZ DE LA TORRE, Y., LOCATI, M., BURACCHI, C., DUPOR, J., COOK, D. N., BONECCHI, R., NEBULONI, M., RUKAVINA, D., VAGO, L., VECCHI, A., LIRA, S. A. & MANTOVANI, A. (2005) Increased inflammation in mice deficient for the chemokine decoy receptor D6. *European Journal of Immunology,* 35**,** 1342-6.

MASHINO, K., SADANAGA, N., YAMAGUCHI, H., TANAKA, F., OHTA, M., SHIBUTA, K., INOUE, H. & MORI, M. (2002) Expression of chemokine receptor CCR7 is associated with lymph node metastasis of gastric carcinoma. *Cancer Research,* 62**,** 2937-2941.

MATLOUBIAN, M., DAVID, A., ENGEL, S., RYAN, J. E. & CYSTER, J. G. (2000) A transmembrane CXC chemokine is a ligand for HIV-coreceptor Bonzo. *Nature Immunology,* 1**,** 298-304.

MCCULLOCH, C. V., MORROW, V., MILASTA, S., COMERFORD, I., MILLIGAN, G., GRAHAM, G. J., ISAACS, N. W. & NIBBS, R. J. B. (2008) Multiple roles for the C-terminal tail of the chemokine scavenger D6. *Journal of Biological Chemistry,* 283**,** 7972-7982.

MCKIMMIE, C. S., FRASER, A. R., HANSELL, C., GUTIERREZ, L., PHILIPSEN, S., CONNELL, L., ROT, A., KUROWSKA-STOLARSKA, M., CARRENO, P., PRUENSTER, M., CHU, C. C., LOMBARDI, G., HALSEY, C., MCINNES, L. B., LIEW, F. Y., NIBBS, R. J. & GRAHAM, G. J. (2008) Hemopoietic cell expression of the chemokine decoy receptor D6 is dynamic and regulated by GATA1. *Journal of Immunology,* 181**,** 3353-3363.

MCKIMMIE, C. S. & GRAHAM, G. J. (2006) Leucocyte expression of the chemokine scavenger D6. *Biochemical Society Transactions,* 34**,** 1002-1004.

MCKIMMIE, C. S., SINGH, M. D., HEWIT, K., LOPEZ-FRANCO, O., LE BROCQ, M., ROSE-JOHN, S., LEE, K. M., BAKER, A. H., WHEAT, R., BLACKBOURN, D. J., NIBBS, R. J. B. & GRAHAM, G. J. (2013) An analysis of the function and expression of D6 on lymphatic endothelial cells. *Blood,* 121**,** 3768-77.

MCLEAN, K. A., HOLST, P. J., MARTINI, L., SCHWARTZ, T. W. & ROSENKILDE, M. M. (2004) Similar activation of signal transduction pathways by the herpesvirus-encoded chemokine receptors US28 and ORF74. *Virology,* 325**,** 241-251.

MCQUIBBAN, G. A., GONG, J. H., TAM, E. M., MCCULLOCH, C. A. G., CLARK-LEWIS, I. & OVERALL, C. M. (2000) Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. *Science,* 289**,** 1202-1206.

MENARD, D., BARNADAS, C., BOUCHIER, C., HENRY-HALLDIN, C., GRAY, L. R., RATSIMBASOA, A., THONIER, V., CAROD, J. F., DOMARLE, O., COLIN, Y., BERTRAND, O., PICOT, J., KING, C. L., GRIMBERG, B. T., MERCEREAU-PUIJALON, O. & ZIMMERMAN, P. A. (2010) Plasmodium vivax clinical malaria is commonly observed in Duffy-negative Malagasy people. *Proceedings of the National Academy of Sciences of the United States of America,* 107**,** 5967-5971.

MIAO, Z., LUKER, K. E., SUMMERS, B. C., BERAHOVICH, R., BHOJANI, M. S., REHEMTULLA, A., KLEER, C. G., ESSNER, J. J., NASEVICIUS, A., LUKER, G. D., HOWARD, M. C. & SCHALL, T. J. (2007) CXCR7 (RDC1) promotes breast and lung tumor growth in vivo and is expressed on tumor-associated vasculature. *Proceedings of the National Academy of Sciences of the United States of America,* 104**,** 15735-15740.

MILLER, L. H., MASON, S. J., DVORAK, J. A., MCGINNISS, M. H. & ROTHMAN, I. K. (1975) Erythrocyte receptors for (plasmodium-knowlesi) malaria - duffy blood-group determinants. *Science,* 189**,** 561-563.

MISHIRO, E., SAKAKIBARA, Y., LIU, M. C. & SUIKO, M. (2006) Differential enzymatic characteristics and tissue-specific expression of human TPST-1 and TPST-2. *Journal of Biochemistry,* 140**,** 731-737.

MITHAL, D. S., BANISADR, G. & MILLER, R. J. (2012) CXCL12 Signaling in the Development of the Nervous System. *Journal of Neuroimmune Pharmacology,* 7**,** 820-834.

MIZOUE, L. S., BAZAN, J. F., JOHNSON, E. C. & HANDEL, T. M. (1999) Solution structure and dynamics of the CX3C chemokine domain of fractalkine and its interaction with an N-terminal fragment of CX(3)CR1. *Biochemistry,* 38**,** 1402-1414.

MOLYNEAUX, K. A., ZINSZNER, H., KUNWAR, P. S., SCHAIBLE, K., STEBLER, J., SUNSHINE, M. J., O'BRIEN, W., RAZ, E., LITTMAN, D., WYLIE, C. & LEHMANN, R. (2003) The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival. *Development,* 130**,** 4279-4286.

MONIGATTI, F., HEKKING, B. & STEEN, H. (2006) Protein sulfation analysis - A primer. *Biochimica Et Biophysica Acta-Proteins and Proteomics,* 1764**,** 1904-1913.

MONTECLARO, F. S. & CHARO, I. F. (1996) The amino-terminal extracellular domain of the MCP-1 receptor, but not the RANTES/MIP-1 alpha receptor, confers chemokine selectivity - Evidence for a two-step mechanism for MCP-1 receptor activation. *Journal of Biological Chemistry,* 271**,** 19084-19092.

MONTECLARO, F. S. & CHARO, I. F. (1997) The amino-terminal domain of CCR2 is both necessary and sufficient for high affinity binding of monocyte chemoattractant protein 1 - Receptor activation by a pseudo-tethered ligand. *Journal of Biological Chemistry,* 272**,** 23186-23190.

MOORE, J. P., KITCHEN, S. G., PUGACH, P. & ZACK, J. A. (2004) The CCR5 and CXCR4 coreceptors - Central to understanding the transmission and pathogenesis of human immunodeficiency virus type 1 infection. *Aids Research and Human Retroviruses,* 20**,** 111-126.

MORTIER, A., GOUWY, M., VAN DAMME, J. & PROOST, P. (2011) Effect of posttranslational processing on the in vitro and in vivo activity of chemokines. *Experimental Cell Research,* 317**,** 642-654.

MORTIER, A., LOOS, T., GOUWY, M., RONSSE, I., VAN DAMME, J. & PROOST, P. (2010) Posttranslational Modification of the NH2-terminal Region of CXCL5 by Proteases or Peptidylarginine Deiminases (PAD) Differently Affects Its Biological Activity. *Journal of Biological Chemistry,* 285**,** 29750-29759.

MOSER, B., WOLF, M., WALZ, A. & LOETSCHER, P. (2004) Chemokines: multiple levels of leukocyte migration control. *Trends in Immunology,* 25**,** 75-84.

MULLER, A., HOMEY, B., SOTO, H., GE, N. F., CATRON, D., BUCHANAN, M. E., MCCLANAHAN, T., MURPHY, E., YUAN, W., WAGNER, S. N., BARRERA, J. L., MOHAR, A., VERASTEGUI, E. & ZLOTNIK, A. (2001) Involvement of chemokine receptors in breast cancer metastasis. *Nature,* 410**,** 50-56.

MURAKAMI, T., MAKI, W., CARDONES, A. R., FANG, H., KYI, A. T., NESTLE, F. O. & HWANG, S. T. (2002) Expression of CXC chemokine receptor-4 enhances the pulmonary metastatic potential of murine B16 melanoma cells. *Cancer Research,* 62**,** 7328-7334.

MURPHY, P. M., BAGGIOLINI, M., CHARO, I. F., HEBERT, C. A., HORUK, R., MATSUSHIMA, K., MILLER, L. H., OPPENHEIM, J. J. & POWER, C. A. (2000) International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacological Reviews,* 52**,** 145-176.

NAGASAWA, T., HIROTA, S., TACHIBANA, K., TAKAKURA, N., NISHIKAWA, S., KITAMURA, Y., YOSHIDA, N., KIKUTANI, H. & KISHIMOTO, T. (1996) Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature,* 382**,** 635-638.

NAGASAWA, T., TACHIBANA, K. & KISHIMOTO, T. (1998) A novel CXC chemokine PBSF/SDF-1 and its receptor CXCR4: their functions in development, hematopoiesis and HIV infection. *Seminars in Immunology,* 10**,** 179-185.

NAKAMURA, K., WILLIAMS, I. R. & KUPPER, T. S. (1995) Keratinocyte-derived monocyte chemoattractant protein-1 (MCP-1) - analysis in a transgenic model demonstrates MCP-1 can recruit dendritic and langerhans cells to skin. *Journal of Investigative Dermatology,* 105**,** 635-643.

NAUMANN, U., CAMERONI, E., PRUENSTER, M., MAHABALESHWAR, H., RAZ, E., ZERWES, H. G., ROT, A. & THELEN, M. (2010) CXCR7 Functions as a Scavenger for CXCL12 and CXCL11. *Plos One,* 5**,** 11.

NEEL, N. F., SCHUTYSER, E., SAI, J., FAN, G. H. & RICHMOND, A. (2005) Chemokine receptor internalization and intracellular trafficking. *Cytokine & Growth Factor Reviews,* 16**,** 637-658.

NEGUS, R. P. M., STAMP, G. W. H., HADLEY, J. & BALKWILL, F. R. (1997) Quantitative assessment of the leukocyte infiltrate in ovarian cancer and its relationship to the expression of C-C chemokines. *American Journal of Pathology,* 150**,** 1723-1734.

NEIL, S. J. D., AASA-CHAPMAN, M. M. I., CLAPHAM, P. R., NIBBS, R. J., MCKNIGHT, A. & WEISS, R. A. (2005) The promiscuous CC chemokine receptor D6 is a functional coreceptor for primary isolates of human immunodeficiency virus type 1 (HIV-1) and HIV-2 on astrocytes. *Journal of Virology,* 79**,** 9618-9624.

NIBBS, R., GRAHAM, G. & ROT, A. (2003) Chemokines on the move: control by the chemokine "interceptors" Duffy blood group antigen and D6. *Seminars in Immunology,* 15**,** 287-294.

NIBBS, R. J. B., GILCHRIST, D. S., KING, V., FERRA, A., FORROW, S., HUNTER, K. D. & GRAHAM, G. J. (2007) The atypical chemokine receptor D6 suppresses the development of chemically induced skin tumours. *Journal of Clinical Investigation,* 117**,** 1884-1892.

NIBBS, R. J. B. & GRAHAM, G. J. (2013) Immune regulation by atypical chemokine receptors. *Nature Reviews Immunology,* 13**,** 815-829.

NIBBS, R. J. B., KRIEHUBER, E., PONATH, P. D., PARENT, D., QIN, S. X., CAMPBELL, J. D. M., HENDERSON, A., KERJASCHKI, D., MAURER, D., GRAHAM, G. J. & ROT, A. (2001) The beta-chemokine receptor D6 is expressed by lymphatic endothelium and a subset of vascular tumors. *American Journal of Pathology,* 158**,** 867-877.

NIBBS, R. J. B., WYLIE, S. M., YANG, J. Y., LANDAU, N. R. & GRAHAM, G. J. (1997) Cloning and characterization of a novel promiscuous human beta-chemokine receptor D6. *Journal of Biological Chemistry,* 272**,** 32078-32083.

NIBBS, R. J. B., YANG, J. Y., LANDAU, N. R., MAO, J. H. & GRAHAM, G. J. (1999) LD78 beta, a non-allelic variant of human MIP-1 alpha (LD78 alpha), has enhanced receptor interactions and potent HIV suppressive activity. *Journal of Biological Chemistry,* 274**,** 17478-17483.

NICKOLOFF, B. J., XIN, H., NESTLE, F. O. & QIN, J. Z. (2007) The cytokine and chemokine network in psoriasis. *Clinics in Dermatology,* 25**,** 568-573.

NOMIYAMA, H., OSADA, N. & YOSHIE, O. (2013) Systematic classification of vertebrate chemokines based on conserved synteny and evolutionary history. *Genes to Cells,* 18**,** 1-16.

O'HAYRE, M., SALANGA, C. L., HANDEL, T. M. & HAMEL, D. J. (2010) Emerging concepts and approaches for chemokine-receptor drug discovery. *Expert Opinion on Drug Discovery,* 5**,** 1109-1122.

OGATA, H., TAKEYA, M., YOSHIMURA, T., TAKAGI, K. & TAKAHASHI, K. (1997) The role of monocyte chemoattractant protein-1 (MCP-1) in the pathogenesis of collagen-induced arthritis in rats. *Journal of Pathology,* 182**,** 106-114.

OLDHAM, W. M. & HAMM, H. E. (2008) Heterotrimeric G protein activation by G-protein-coupled receptors. *Nature Reviews Molecular Cell Biology,* 9**,** 60-71.

ORAVECZ, T., PALL, M., RODERIQUEZ, G., GORRELL, M. D., DITTO, M., NGUYEN, N. Y., BOYKINS, R., UNSWORTH, E. & NORCROSS, M. A. (1997) Regulation of the receptor specificity and function of the chemokine RANTES (regulated on activation, normal T cell expressed and secreted) by dipeptidyl peptidase IV (CD26)-mediated cleavage. *Journal of Experimental Medicine,* 186**,** 1865-1872.

OUYANG, Y. B., CRAWLEY, J. T. B., ASTON, C. E. & MOORE, K. L. (2002) Reduced body weight and increased postimplantation fetal death in tyrosylprotein sulfotransferase-1-deficient mice. *Journal of Biological Chemistry,* 277**,** 23781-23787.

PAAVOLA, C. D., HEMMERICH, S., GRUNBERGER, D., POLSKY, I., BLOOM, A., FREEDMAN, R., MULKINS, M., BHAKTA, S., MCCARLEY, D., WIESENT, L., WONG, B., JARNAGIN, K. & HANDEL, T. M. (1998) Monomeric monocyte chemoattractant protein-1 binds and activates the MCP-1 receptor CCR2B. *Journal of Biological Chemistry,* 273**,** 33157-33165.

PARK, S. H., DAS, B. B., CASAGRANDE, F., TIAN, Y., NOTHNAGEL, H. J., CHU, M. N., KIEFER, H., MAIER, K., DE ANGELIS, A. A., MARASSI, F. M. & OPELLA, S. J. (2012) Structure of the chemokine receptor CXCR1 in phospholipid bilayers. *Nature,* 491**,** 779-+.

PARKS, W. C., WILSON, C. L. & LOPEZ-BOADO, Y. S. (2004) Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nature Reviews Immunology,* 4**,** 617-629.

PARRY, C. M., SIMAS, J. P., SMITH, V. P., STEWART, C. A., MINSON, A. C., EFSTATHIOU, S. & ALCAMI, A. (2000) A broad spectrum secreted chemokine binding protein encoded by a herpesvirus. *Journal of Experimental Medicine,* 191**,** 573-578.

PARSONAGE, G., FILER, A. D., HAWORTH, O., NASH, G. B., RAINGER, G. E., SALMON, M. & BUCKLEY, C. D. (2005) A stromal address code defined by fibroblasts. *Trends in Immunology,* 26**,** 150-156.

PEETERS, M. C., VAN WESTEN, G. J. P., LI, Q. & IJZERMAN, A. P. (2011) Importance of the extracellular loops in G protein-coupled receptors for ligand recognition and receptor activation. *Trends in Pharmacological Sciences,* 32**,** 35-42.

PERCHERANCIER, Y., BERCHICHE, Y. A., SLIGHT, I., VOLKMER-ENGERT, R., TAMAMURA, H., FUJII, N., BOUVIER, M. & HEVEKER, N. (2005) Bioluminescence resonance energy transfer reveals ligand-induced conformational changes in CXCR4 homo- and heterodimers. *Journal of Biological Chemistry,* 280**,** 9895-9903.

PERCHERANCIER, Y., PLANCHENAULT, T., VALENZUELA-FERNANDEZ, A., VIRELIZIER, J. L., ARENZANA-SEISDEDOS, F. & BACHELERIE, F. (2001) Palmitoylation-dependent control of degradation, life span, and membrane expression of the CCR5 receptor. *Journal of Biological Chemistry,* 276**,** 31936-31944.

PERERA, G. K., DI MEGLIO, P. & NESTLE, F. O. (2012) Psoriasis. IN ABBAS, A. K., GALLI, S. J. & HOWLEY, P. M. (Eds.) *Annual Review of Pathology: Mechanisms of Disease, Vol 7.* Palo Alto, Annual Reviews.

PLATERZYBERK, C., HOOGEWERF, A. J., PROUDFOOT, A. E. I., POWER, C. A. & WELLS, T. N. C. (1997) Effect of a CC chemokine receptor antagonist on collagen induced arthritis in DBA/1 mice. *Immunology Letters,* 57**,** 117-120.

PRAHALAD, S. (2006) Negative association between the chemokine receptor CCR5-Delta 32 polymorphism and rheumatoid arthritis: a meta-analysis. *Genes and Immunity,* 7**,** 264-268.

PREOBRAZHENSKY, A. A., DRAGAN, S., KAWANO, T., GAVRILIN, M. A., GULINA, I. V., CHAKRAVARTY, L. & KOLATTUKUDY, P. E. (2000) Monocyte chemotactic protein-1 receptor CCR2B is a glycoprotein that has tyrosine sulfation in a conserved extracellular N-terminal region. *Journal of Immunology,* 165**,** 5295-5303.

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. & VAN DAMME, J. (2008) Citrullination of CXCL8 by peptidylarginine deiminase alters receptor usage, prevents proteolysis, and dampens tissue inflammation. *Journal of Experimental Medicine,* 205**,** 2085-2097.

PROUDFOOT, A. E. I. (2002) Chemokine receptors: Multifaceted therapeutic targets. *Nature Reviews Immunology,* 2**,** 106-115.

PROUDFOOT, A. E. I. (2006) The biological relevance of chemokine-proteoglycan interactions. *Biochemical Society Transactions,* 34**,** 422-426.

PROUDFOOT, A. E. I., FRITCHLEY, S., BORLAT, F., SHAW, J. P., VILBOIS, F., ZWAHLEN, C., TRKOLA, A., MARCHANT, D., CLAPHAM, P. R. & WELLS, T. N. C. (2001) The BBXB motif of RANTES is the principal site for heparin binding and controls receptor selectivity. *Journal of Biological Chemistry,* 276**,** 10620-10626.

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. (2003) Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proceedings of the National Academy of Sciences of the United States of America,* 100**,** 1885-1890.

PRUENSTER, M., MUDDE, L., BOMBOSI, P., DIMITROVA, S., ZSAK, M., MIDDLETON, J., RICHMOND, A., GRAHAM, G. J., SEGERER, S., NIBBS, R. J. B. & ROT, A. (2009) The Duffy antigen receptor for chemokines transports chemokines and supports their promigratory activity. *Nature Immunology,* 10**,** 101-108.

QANBAR, R. & BOUVIER, M. (2003) Role of palmitoylation/depalmitoylation reactions in G-protein-coupled receptor function. *Pharmacology & Therapeutics,* 97**,** 1-33.

QIN, S. X., ROTTMAN, J. B., MYERS, P., KASSAM, N., WEINBLATT, M., LOETSCHER, M., KOCH, A. E., MOSER, B. & MACKAY, C. R. (1998) The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *Journal of Clinical Investigation,* 101**,** 746-754.

QUINONES, M. P., AHUJA, S. K., JIMENEZ, F., SCHAEFER, J., GARAVITO, E., RAO, A., CHENAUX, G., REDDICK, R. L., KUZIEL, W. A. & AHUJA, S. S. (2004) Experimental arthritis in CC chemokine receptor 2-null mice closely mimics severe human rheumatoid arthritis. *Journal of Clinical Investigation,* 113**,** 856-866.

RAJAGOPAL, S., KIM, J., AHN, S., CRAIG, S., LAM, C. M., GERARD, N. P., GERARD, C. & LEFKOWITZ, R. J. (2010) beta-arrestin- but not G protein-mediated signaling by the "decoy" receptor CXCR7. *Proceedings of the National Academy of Sciences of the United States of America,* 107**,** 628-632.

RAJAGOPALAN, L. & RAJARATHNAM, K. (2006) Structural basis of chemokine receptor function - A model for binding affinity and ligand selectivity. *Bioscience Reports,* 26**,** 325-339.

RATAJCZAK, M. Z., ZUBA-SURMA, E., KUCIA, M., RECA, R., WOJAKOWSKI, W. & RATAJCZAK, J. (2006) The pleiotropic effects of the SDF-1-CXCR4 axis in organogenesis, regeneration and tumorigenesis. *Leukemia,* 20**,** 1915-1924.

RAVINDRAN, A., SAWANT, K. V., SARMIENTO, J., NAVARRO, J. & RAJARATHNAM, K. (2013) Chemokine CXCL1 Dimer Is a Potent Agonist for the CXCR2 Receptor. *Journal of Biological Chemistry,* 288**,** 12244-12252.

REGOES, R. R. & BONHOEFFER, S. (2005) The HIV coreceptor switch: a population dynamical perspective. *Trends in Microbiology,* 13**,** 269-277.

RIBEIRO, S. & HORUK, R. (2007) *Pharmaceutical targeting of chemokine receptors,* Humana Press Inc.

RODRIGUEZ-FRADE, J. M., DEL REAL, G., SERRANO, A., HERNANZ-FALCON, P., SORIANO, S. F., VILA-CORO, A. J., DE ANA, A. M., LUCAS, P., PRIETO, I., MARTINEZ-A, C. & MELLADO, M. (2004) Blocking HIV-1 infection via CCR5 and CXCR4 receptors by acting in trans on the CCR2 chemokine receptor. *Embo Journal,* 23**,** 66-76.

RODRIGUEZ-FRADE, J. M., VILA-CORO, A. J., DE ANA, A. M., ALBAR, J. P., MARTINEZ-A, C. & MELLADO, M. (1999) The chemokine monocyte chemoattractant protein-1 induces functional responses through dimerization of its receptor CCR2. *Proceedings of the National Academy of Sciences of the United States of America,* 96**,** 3628-3633.

ROLLINS, B. J. (1997) Chemokines. *Blood,* 90**,** 909-928.

ROSE, J. J., FOLEY, J. F., MURPHY, P. M. & VENKATESAN, S. (2004) On the mechanism and significance of ligand-induced internalization of human neutrophil chemokine receptors CXCR1 and CXCR2. *Journal of Biological Chemistry,* 279**,** 24372-24386.

ROSENKILDE, M. M. (2005) Virus-encoded chemokine receptors - putative novel antiviral drug targets. *Neuropharmacology,* 48**,** 1-13.

ROSENKILDE, M. M., KLEDAL, T. N., BRAUNER-OSBORNE, H. & SCHWARTZ, T. W. (1999) Agonists and inverse agonists for the herpesvirus 8-encoded constitutively active seven-transmembrane oncogene product, ORF-74. *Journal of Biological Chemistry,* 274**,** 956-961.

ROSSI, D. & ZLOTNIK, A. (2000) The biology of chemokines and their receptors. *Annual Review of Immunology,* 18**,** 217-243.

ROT, A. (1993) Neutrophil attractant activation protein-1 (interleukin-8) induces invitro neutrophil migration by haptotactic mechanism. *European Journal of Immunology,* 23**,** 303-306.

ROT, A. (2005) Contribution of Duffy antigen to chemokine function. *Cytokine & Growth Factor Reviews,* 16**,** 687-694.

ROT, A., MCKIMMIE, C., BURT, C. L., PALLAS, K. J., JAMIESON, T., PRUENSTER, M., HORUK, R., NIBBS, R. J. B. & GRAHAM, G. J. (2013) Cell-autonomous regulation of neutrophil migration by the D6 chemokine decoy receptor. *Journal of immunology,* 190**,** 6450-6.

ROT, A. & VON ANDRIAN, U. H. (2004) Chemokines in innate and adaptive host defense: Basic chemokinese grammar for immune cells. *Annual Review of Immunology,* 22**,** 891-928.

RUTKOWSKI, J. M., BOARDMAN, K. C. & SWARTZ, M. A. (2006) Characterization of lymphangiogenesis in a model of adult skin regeneration. *American Journal of Physiology-Heart and Circulatory Physiology,* 291**,** H1402-H1410.

SADIR, R., IMBERTY, A., BALEUX, F. & LORTAT-JACOB, H. (2004) Heparan sulfate/heparin oligosaccharides protect stromal cell-derived factor-1 (SDF-

1)/CXCL12 against proteolysis induced by CD26/dipeptidyl peptidase IV. *Journal of Biological Chemistry,* 279**,** 43854-43860.

SAFAIYAN, F., KOLSET, S. O., PRYDZ, K., GOTTFRIDSSON, E., LINDAHL, U. & SALMIVIRTA, M. (1999) Selective effects of sodium chlorate treatment on the sulfation of heparan sulfate. *Journal of Biological Chemistry,* 274**,** 36267-36273.

SAVINO, B., BORRONI, E. M., TORRES, N. M., PROOST, P., STRUYF, S., MORTIER, A., MANTOVANI, A., LOCATI, M. & BONECCHI, R. (2009) Recognition Versus Adaptive Up-regulation and Degradation of CC Chemokines by the Chemokine Decoy Receptor D6 Are Determined by Their N-terminal Sequence. *Journal of Biological Chemistry,* 284**,** 26207-26215.

SCHALL, T. J. & PROUDFOOT, A. E. I. (2011) Overcoming hurdles in developing successful drugs targeting chemokine receptors. *Nature Reviews Immunology,* 11**,** 355-363.

SCHIOPPA, T., URANCHIMEG, B., SACCANI, A., BISWAS, S. K., DONI, A., RAPISARDA, A., BERNASCONI, S., SACCANI, S., NEBULONI, M., VAGO, L., MANTOVANI, A., MELILLO, G. & SICA, A. (2003) Regulation of the chemokine receptor CXCR4 by hypoxia. *Journal of Experimental Medicine,* 198**,** 1391-1402.

SCHNABEL, R. B., BAUMERT, J., BARBALIC, M., DUPUIS, J., ELLINOR, P. T., DURDA, P., DEHGHAN, A., BIS, J. C., ILLIG, T., MORRISON, A. C., JENNY, N. S., KEANEY, J. F., GIEGER, C., TILLEY, C., YAMAMOTO, J. F., KHUSEYINOVA, N., HEISS, G., DOYLE, M., BLANKENBERG, S., HERDER, C., WALSTON, J. D., ZHU, Y. Y., VASAN, R. S., KLOPP, N., BOERWINKLE, E., LARSON, M. G., PSATY, B. M., PETERS, A., BALLANTYNE, C. M., WITTEMAN, J. C. M., HOOGEVEEN, R. C., BENJAMIN, E. J., KOENIG, W. & TRACY, R. P. (2010) Duffy antigen receptor for chemokines (Darc) polymorphism regulates circulating concentrations of monocyte chemoattractant protein-1 and other inflammatory mediators. *Blood,* 115**,** 5289-5299.

SHULMAN, N. R. (1952) Studies on the inhibition of proteolytic enzymes by serum .1. The mechanism of the inhibition of trypsin, plasmin, and chymotrypsin by serum using fibrin tagged with I^{131} as a substrate. *Journal of Experimental Medicine,* 95**,** 571-591.

SCHURGERS, E., BILLIAU, A. & MATTHYS, P. (2011) Collagen-Induced Arthritis as an Animal Model for Rheumatoid Arthritis: Focus on Interferon-gamma. *Journal of Interferon and Cytokine Research,* 31**,** 917-926.

SCOTTON, C., MILLIKEN, D., WILSON, J., RAJU, S. & BALKWILL, F. (2001a) Analysis of CC chemokine and chemokine receptor expression in solid ovarian tumours. *British Journal of Cancer,* 85**,** 891-897.

SCOTTON, C. J., WILSON, J. L., MILLIKEN, D., STAMP, G. & BALKWILLL, F. R. (2001b) Epithelial cancer cell migration: A role for chemokine receptors? *Cancer Research,* 61**,** 4961-4965.

SEIBERT, C., CADENE, M., SANFIZ, A., CHAIT, B. T. & SAKMAR, T. P. (2002) Tyrosine sulfation of CCR5 N-terminal peptide by tyrosylprotein sulfotransferases 1 and 2 follows a discrete pattern and temporal sequence. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 11031-11036.

SEIBERT, C. & SAKMAR, T. P. (2008) Toward a framework for sulfoproteomics: Synthesis and characterization of sulfotyrosine-containing peptides. *Biopolymers,* 90**,** 459-477

SHANKARAPPA, R., MARGOLICK, J. B., GANGE, S. J., RODRIGO, A. G., UPCHURCH, D., FARZADEGAN, H., GUPTA, P., RINALDO, C. R., LEARN, G. H., HE, X., HUANG, X. L. & MULLINS, J. I. (1999) Consistent viral evolutionary changes associated with the progression of human immunodeficiency virus type 1 infection. *Journal of Virology,* 73**,** 10489-10502.

SHEN, H., SCHUSTER, R., STRINGER, K. F., WALTZ, S. E. & LENTSCH, A. B. (2006) The Duffy antigen/receptor for chemokines (DARC) regulates prostate tumor growth. *Faseb Journal,* 20**,** 59-64.

SHERRY, B., TEKAMPOLSON, P., GALLEGOS, C., BAUER, D., DAVATELIS, G., WOLPE, S. D., MASIARZ, F., COIT, D. & CERAMI, A. (1988) Resolution of the 2 components of macrophage inflammatory protein-1, and cloning and characterization of one of those components, macrophage inflammatory protein-1-beta. *Journal of Experimental Medicine,* 168**,** 2251-2259.

SHERRY, D. M., MURRAY, A. R., KANAN, Y., ARBOGAST, K. L., HAMILTON, R. A., FLIESLER, S. J., BURNS, M. E., MOORE, K. L. & AL-UBAIDI, M. R. (2010) Lack of protein-tyrosine sulfation disrupts photoreceptor outer segment morphogenesis, retinal function and retinal anatomy. *European Journal of Neuroscience,* 32**,** 1461-1472.

SIERRO, F., BIBEN, C., MARTINEZ-MUNOZ, L., MELLADO, M., RANSOHOFF, R. M., LI, M., WOEHL, B., LEUNG, H., GROOM, J., BATTEN, M., HARVEY, R. P., MARTINEZ-A, C., MACKAY, C. R. & MACKAY, F. (2007) Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. *Proceedings of the National Academy of Sciences of the United States of America,* 104**,** 14759-14764.

SIGNORET, N., HEWLETT, L., WAVRE, S., PELCHEN-MATTHEWS, A., OPPERMANN, M. & MARSH, M. (2005) Agonist-induced endocytosis of CC chemokine receptor 5 is clathrin dependent. *Molecular Biology of the Cell,* 16**,** 902-917.

SIMPSON, L. S., ZHU, J. Z., WIDLANSKI, T. S. & STONE, M. J. (2009) Regulation of Chemokine Recognition by Site-Specific Tyrosine Sulfation of Receptor Peptides. *Chemistry & Biology,* 16**,** 153-161.

SINGH, M. D., KING, V., BALDWIN, H., BURDEN, D., THORRAT, A., HOLMES, S., MCINNES, I. B., NICOLL, R., SHAMS, K., PALLAS, K., JAMIESON, T., LEE, K. M., CARBALLIDO, J. M., ROT, A. & GRAHAM, G. J. (2012) Elevated Expression of the Chemokine-Scavenging Receptor D6 Is Associated with Impaired Lesion Development in Psoriasis. *American Journal of Pathology,* 181**,** 1158-1164.

SORIANO, S. F., SERRANO, A., HERNANZ-FALCON, P., DE ANA, A. M., MONTERRUBIO, M., MARTINEZ, C., RODRIGUEZ-FRADE, J. M. & MELLADO, M. (2003) Chemokines integrate JAK/STAT and G-protein pathways during chemotaxis and calcium flux responses. *European Journal of Immunology,* 33**,** 1328-1333.

SOULIE, C., CALVEZ, V. & MARCELIN, A. G. (2012) Coreceptor usage in different reservoirs. *Current Opinion in Hiv and Aids,* 7**,** 450-455.

SPAAN, A. N., SUREWAARD, B. G. J., NIJLAND, R. & VAN STRIJP, J. A. G. (2013) Neutrophils Versus Staphylococcus aureus: A Biological Tug of War. IN GOTTESMAN, S. (Ed.) *Annual Review of Microbiology, Vol 67.* Palo Alto, Annual Reviews.

SPARMANN, A. & BAR-SAGI, D. (2004) Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. *Cancer Cell,* 6**,** 447-458.

SPRINGAEL, J. Y., URIZAR, E., COSTAGLIOLA, S., VASSART, G. & PARMENTIER, M. (2007) Allosteric properties of G protein-coupled receptor oligomers. *Pharmacology & Therapeutics,* 115**,** 410-418.

STIEVANO, L., PIOVAN, E. & AMADORI, A. (2004) C and CX3C chemokines: Cell sources and physiopathological implications. *Critical Reviews in Immunology,* 24**,** 205-228.

STONE, M. J., CHUANG, S., HOU, X., SHOHAM, M. & ZHU, J. Z. (2009) Tyrosine sulfation: an increasingly recognised post-translational modification of secreted proteins. *New Biotechnology,* 25**,** 299-317.

STRIETER, R. M., BURDICK, M. D., GOMPERTS, B. N., BELPERIO, J. A. & KEANE, M. P. (2005) CXC chemokines in angiogenesis. *Cytokine & Growth Factor Reviews,* 16**,** 593-609.

STRIETER, R. M., POLVERINI, P. J., KUNKEL, S. L., ARENBERG, D. A., BURDICK, M. D., KASPER, J., DZUIBA, J., VANDAMME, J., WALZ, A., MARRIOTT, D., CHAN, S. Y., ROCZNIAK, S. & SHANAFELT, A. B. (1995) The functional-role of the ELR motif in CXC chemokine-mediated angiogenesis. *Journal of Biological Chemistry,* 270**,** 27348-27357.

STRUYF, S., PROOST, P. & VAN DAMME, J. (2003) Regulation of the immune response by the interaction of chemokines and proteases. *Advances in Immunology, Vol 81.* San Diego, Elsevier Academic Press Inc.

SUZUKI, A., YAMADA, R., CHANG, X. T., TOKUHIRO, S., SAWADA, T., SUZUKI, M., NAGASAKI, M., NAKAYAMA-HAMADA, M., KAWAIDA, R., ONO, M., OHTSUKI, M., FURUKAWA, H., YOSHINO, S., YUKIOKA, M., TOHMA, S., MATSUBARA, T., WAKITANI, S., TESHIMA, R., NISHIOKA, Y., SEKINE, A., IIDA, A., TAKAHASHI, A., TSUNODA, T., NAKAMURA, Y. & YAMAMOTO, K. (2003) Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. *Nature Genetics,* 34**,** 395-402.

SZEKANECZ, Z., KOCH, A. E. & TAK, P. P. (2011) Chemokine and chemokine receptor blockade in arthritis, a prototype of immune-mediated inflammatory diseases. *Netherlands Journal of Medicine,* 69**,** 356-366.

SZEKANECZ, Z., VEGVARI, A., SZABO, Z. & KOCH, A. E. (2010) Chemokines and chemokine receptors in arthritis. *Frontiers in bioscience (Scholar edition),* 2**,** 153-67.

SZPAKOWSKA, M., FIEVEZ, V., ARUMUGAN, K., VAN NULAND, N., SCHMIT, J. C. & CHEVIGNE, A. (2012) Function, diversity and therapeutic potential of the N-terminal domain of human chemokine receptors. *Biochemical Pharmacology,* 84**,** 1366-1380.

TAKASHIMA, A. & FALLER, D. V. (2013) Targeting the RAS oncogene. *Expert Opinion on Therapeutic Targets,* 17**,** 507-531.

TAKEKOSHI, T., ZIAREK, J. J., VOLKMAN, B. F. & HWANG, S. T. (2012) A Locked, Dimeric CXCL12 Variant Effectively Inhibits Pulmonary Metastasis of CXCR4-Expressing Melanoma Cells Due to Enhanced Serum Stability. *Molecular Cancer Therapeutics,* 11**,** 2516-2525.

TALMADGE, J. E. (2011) Immune cell infiltration of primary and metastatic lesions: Mechanisms and clinical impact. *Seminars in Cancer Biology,* 21**,** 131-138.

TAN, J. H. Y., CANALS, M., LUDEMAN, J. P., WEDDERBURN, J., BOSTON, C., BUTLER, S. J., CARRICK, A. M., PARODY, T. R., TALESKI, D., CHRISTOPOULOS, A., PAYNE, R. J. & STONE, M. J. (2012) Design and Receptor Interactions of Obligate Dimeric Mutant of Chemokine Monocyte Chemoattractant Protein-1 (MCP-1). *Journal of Biological Chemistry,* 287**,** 14692-14702.

TAN, J. H. Y., LUDEMAN, J. P., WEDDERBURN, J., CANALS, M., HALL, P., BUTLER, S. J., TALESKI, D., CHRISTOPOULOS, A., HICKEY, M. J., PAYNE, R. J. & STONE, M. J. (2013a) Tyrosine Sulfation of Chemokine Receptor CCR2 Enhances Interactions with Both Monomeric and Dimeric Forms of the Chemokine Monocyte Chemoattractant Protein-1 (MCP-1). *Journal of Biological Chemistry,* 288**,** 10024-10034.

TAN, Q. X., ZHU, Y., LI, J., CHEN, Z. X., HAN, G. W., KUFAREVA, I., LI, T. T., MA, L. M., FENALTI, G., ZHANG, W. R., XIE, X., YANG, H. Y., JIANG, H. L., CHEREZOV, V., LIU, H., STEVENS, R. C., ZHAO, Q. & WU, B. L. (2013b) Structure of the CCR5 Chemokine Receptor-HIV Entry Inhibitor Maraviroc Complex. *Science,* 341**,** 1387-1390.

THELEN, M. (2001) Dancing to the tune of chemokines. *Nature Immunology,* 2**,** 129-134.

THELEN, M., MUNOZ, L. M., RODRIGUEZ-FRADE, J. M. & MELLADO, M. (2010) Chemokine receptor oligomerization: functional considerations. *Current Opinion in Pharmacology,* 10**,** 38-43.

THORNTON, S., DUWEL, L. E., BOIVIN, G. P., MA, Y. H. & HIRSCH, R. (1999) Association of the course of collagen-induced arthritis with distinct patterns of cytokine and chemokine messenger RNA expression. *Arthritis and Rheumatism,* 42**,** 1109-1118.

THWAITES, G. E. & GANT, V. (2011) Are bloodstream leukocytes Trojan Horses for the metastasis of Staphylococcus aureus? *Nature Reviews Microbiology,* 9**,** 215-222.

TSOU, C. L., HASKELL, C. A. & CHARO, I. F. (2001) Tumor necrosis factor-alpha-converting enzyme mediates the inducible cleavage of fractalkine. *Journal of Biological Chemistry,* 276**,** 44622-44626.

TURNBULL, J., POWELL, A. & GUIMOND, S. (2001) Heparan sulfate: decoding a dynamic multifunctional cell regulator. *Trends in Cell Biology,* 11**,** 75-82.

ULLUM, H., LEPRI, A. C., VICTOR, J., ALADDIN, H., PHILLIPS, A. N., GERSTOFT, J., SKINHOJ, P. & PEDERSEN, B. K. (1998) Production of beta-chemokines in human immunodeficiency virus (HIV) infection: Evidence that high levels of macrophage in inflammatory protein-1 beta are associated with a decreased risk of HIV disease progression. *Journal of Infectious Diseases,* 177**,** 331-336.

ULVMAR, M. H., HUB, E. & ROT, A. (2011) Atypical chemokine receptors. *Experimental Cell Research,* 317**,** 556-568.

UMEHARA, H., BLOOM, E. T., OKAZAKI, T., NAGANO, Y., YOSHIE, O. & IMAI, T. (2004) Fractalkine in vascular biology - From basic research to clinical disease. *Arteriosclerosis Thrombosis and Vascular Biology,* 24**,** 34-40.

UNUTMAZ, D. & LITTMAN, D. R. (1997) Expression pattern of HIV-1 coreceptors on T cells: Implications for viral transmission and lymphocyte homing. *Proceedings of the National Academy of Sciences of the United States of America,* 94**,** 1615-1618.

VALENTIN, G., HAAS, P. & GILMOUR, D. (2007) The chemokine SDF1a coordinates tissue migration through the spatially restricted activation of Cxcr7 and Cxcr4b. *Current Biology,* 17**,** 1026-1031.

VALENZUELA-FERNANDEZ, A., PLANCHENAULT, T., BALEUX, F., STAROPOLI, I., LE-BARILLEC, K., LEDUC, D., DELAUNAY, T., LAZARINI, F., VIRELIZIER, J. L., CHIGNARD, M., PIDARD, D. & ARENZANA-SEISDEDOS, F. (2002) Leukocyte elastase negatively regulates stromal cell-derived factor-1 (SDF-1)/CXCR4 binding and functions by amino-terminal processing of SDF-1 and CXCR4. *Journal of Biological Chemistry,* 277**,** 15677-15689.

VAN DEN STEEN, P. E., PROOST, P., WUYTS, A., VAN DAMME, J. & OPDENAKKER, G. (2000) Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. *Blood,* 96**,** 2673-2681.

VAN SINDEREN, M., CUMAN, C., WINSHIP, A., MENKHORST, E. & DIMITRIADIS, E. (2013) The chrondroitin sulfate proteoglycan (CSPG4) regulates human trophoblast function. *Placenta,* 34**,** 907-912.

VELDKAMP, C. T., SEIBERT, C., PETERSON, F. C., SAKMAR, T. P. & VOLKMAN, B. F. (2006) Recognition of a CXCR4 sulfotyrosine by the chemokine stromal cell-

derived factor-1 alpha (SDF-1 alpha/CXCL12). *Journal of Molecular Biology,* 359**,** 1400-1409.

VESTERGAARD, C., JUST, H., NIELSEN, J. B., THESTRUP-PEDERSEN, K. & DELEURAN, M. (2004) Expression of CCR2 on monocytes and macrophages in chronically inflamed skin in atopic dermatitis and psoriasis. *Acta Dermato-Venereologica,* 84**,** 353-358.

VETRANO, S., BORRONI, E. M., SARUKHAN, A., SAVINO, B., BONECCHI, R., CORREALE, C., ARENA, V., FANTINI, M., RONCALLI, M., MALESCI, A., MANTOVANI, A., LOCATI, M. & DANESE, S. (2010) The lymphatic system controls intestinal inflammation and inflammation-associated colon cancer through the chemokine decoy receptor D6. *Gut,* 59**,** 197-206.

VIERBOOM, M. P. M., ZAVODNY, P. J., CHOU, C. C., TAGAT, J. R., PUGLIESE-SIVO, C., STRIZKI, J., STEENSMA, R. W., MCCOMBIE, S. W., CELEBI-PAUL, L., REMARQUE, E., JONKER, M., NARULA, S. K. & T HART, B. (2005) Inhibition of the development of collagen-induced arthritis in rhesus monkeys by a small molecular weight antagonist of CCR5. *Arthritis and Rheumatism,* 52**,** 627-636.

VINET, J., VAN ZWAM, M., DIJKSTRA, I. M., BROUWER, N., VAN WEERING, H. R. J., WATTS, A., MEIJER, M., FOKKENS, M. R., KANNAN, V., VERZIJL, D., VISCHER, H. F., SMIT, M. J., LEURS, R., BIBER, K. & BODDEKE, H. (2013) Inhibition of CXCR3-mediated chemotaxis by the human chemokine receptor-like protein CCX-CKR. *British Journal of Pharmacology,* 168**,** 1375-1387.

VOLEJNIKOVA, S., LASKARI, M., MARKS, S. C. & GRAVES, D. T. (1997) Monocyte recruitment and expression of monocyte chemoattractant protein-1 are developmentally regulated in remodeling bone in the mouse. *American Journal of Pathology,* 150**,** 1711-1721.

VON STEBUT, E., METZ, M., MILON, G., KNOP, J. & MAURER, M. (2003) Early macrophage influx to sites of cutaneous granuloma formation is dependent on MIP-1 alpha/beta released from neutrophils recruited by mast cell-derived TNF alpha. *Blood,* 101**,** 210-215.

WAGNER, L., YANG, O. O., GARCIA-ZEPEDA, E. A., GE, Y. M., KALAMS, S. A., WALKER, B. D., PASTERNACK, M. S. & LUSTER, A. D. (1998) beta-chemokines are released from HIV-1-specific cytolytic T-cell granules complexed to proteoglycans. *Nature,* 391**,** 908-911.

WANG, J., OU, Z. L., HOU, Y. F., LUO, J. M., SHEN, Z. Z., DING, J. & SHAO, Z. M. (2006) Enhanced expression of Duffy antigen receptor for chemokines by breast cancer cells attenuates growth and metastasis potential. *Oncogene,* 25**,** 7201-7211.

WANG, L. C., FUSTER, M., SRIRAMARAO, P. & ESKO, J. D. (2005) Endothelial heparan sulfate deficiency impairs L-selectin- and chemokine-mediated neutrophil trafficking during inflammatory responses. *Nature Immunology,* 6**,** 902-910.

WATTS, A. O., VERKAAR, F., VAN DER LEE, M. M. C., TIMMERMAN, C. A. W., KUIJER, M., VAN OFFENBEEK, J., VAN LITH, L., SMIT, M. J., LEURS, R., ZAMAN, G. J. R. & VISCHER, H. F. (2013) beta-Arrestin Recruitment and G Protein Signaling by the Atypical Human Chemokine Decoy Receptor CCX-CKR. *Journal of Biological Chemistry,* 288**,** 7169-7181.

WEBB, L. M. C. & ALCAMI, A. (2005) Virally encoded chemokine binding proteins. *Mini-Reviews in Medicinal Chemistry,* 5**,** 833-848.

WEBER, M., BLAIR, E., SIMPSON, C. V., O'HARA, M., BLACKBURN, P. E., ROT, A., GRAHAM, G. J. & NIBBS, R. J. B. (2004) The chemokine receptor D6 constitutively traffics to and from the cell surface to internalize and degrade chemokine. *Molecular Biology of the Cell,* 15**,** 2492-2508.

WEBER, M., HAUSCHILD, R., SCHWARZ, J., MOUSSION, C., DE VRIES, I., LEGLER, D. F., LUTHER, S. A., BOLLENBACH, T. & SIXT, M. (2013)

Interstitial Dendritic Cell Guidance by Haptotactic Chemokine Gradients. *Science,* 339**,** 328-332.

WESTMUCKETT, A. D., HOFFHINES, A. J., BORGHEI, A. & MOORE, K. L. (2008) Early postnatal pulmonary failure and primary hypothyroidism in mice with combined TPST-1 and TPST-2 deficiency. *General and Comparative Endocrinology,* 156**,** 145-153.

WESTMUCKETT, A. D. & MOORE, K. L. (2009) Lack of Tyrosylprotein Sulfotransferase Activity in Hematopoietic Cells Drastically Attenuates Atherosclerosis in Ldlr(-/-) Mice. *Arteriosclerosis Thrombosis and Vascular Biology,* 29**,** 1730-1736.

WHITEHEAD, G. S., WANG, T., DEGRAFF, L. M., CARD, J. W., LIRA, S. A., GRAHAM, G. J. & COOK, D. N. (2007) The chemokine receptor D6 has opposing effects on allergic inflammation and airway reactivity. *American Journal of Respiratory and Critical Care Medicine,* 175**,** 243-249.

WILEN, C. B., TILTON, J. C. & DOMS, R. W. (2012) Molecular Mechanisms of HIV Entry. IN ROSSMANN, M. G. & RAO, V. B. (Eds.) *Viral Molecular Machines.* Berlin, Springer-Verlag Berlin.

WOLF, M., ALBRECHT, S. & MARKI, C. (2008) Proteolytic processing of chemokines: Implications in physiological and pathological conditions. *International Journal of Biochemistry & Cell Biology,* 40**,** 1185-1198.

WONG, M. M. & FISH, E. N. (2003) Chemokines: attractive mediators of the immune response. *Seminars in Immunology,* 15**,** 5-14.

WU, B. L., CHIEN, E. Y. T., MOL, C. D., FENALTI, G., LIU, W., KATRITCH, V., ABAGYAN, R., BROOUN, A., WELLS, P., BI, F. C., HAMEL, D. J., KUHN, P., HANDEL, T. M., CHEREZOV, V. & STEVENS, R. C. (2010) Structures of the CXCR4 Chemokine GPCR with Small-Molecule and Cyclic Peptide Antagonists. *Science,* 330**,** 1066-1071.

WU, F. Y., OU, Z. L., FENG, L. Y., LUO, J. M., WANG, L. P., SHEN, Z. Z. & SHAO, Z. M. (2008) Chemokine decoy receptor D6 plays a negative role in human breast cancer. *Molecular Cancer Research,* 6**,** 1276-1288.

YANG, Y. F., MUKAI, T., GAO, P., YAMAGUCHI, N., ONO, S., IWAKI, H., OBIKA, S., IMANISHI, T., TSUJIMURA, T., HAMAOKA, T. & FUJIWARA, H. (2002) A non-peptide CCR5 antagonist inhibits collagen-induced arthritis by modulating T cell migration without affecting anti-collagen T cell responses. *European Journal of Immunology,* 32**,** 2124-2132.

YE, J. P., KOHLI, L. L. & STONE, M. J. (2000) Characterization of binding between the chemokine eotaxin and peptides derived from the chemokine receptor CCR3. *Journal of Biological Chemistry,* 275**,** 27250-27257.

YIN, X., TRUTY, J., LAWRENCE, R., JOHNS, S. C., SRINIVASAN, R. S., HANDEL, T. M. & FUSTER, M. M. (2010) A critical role for lymphatic endothelial heparan sulfate in lymph node metastasis. *Molecular Cancer,* 9**,** 20.

YOSHIDA, T., IMAI, T., TAKAGI, S., NISHIMURA, M., ISHIKAWA, I., YAOI, T. & YOSHIE, O. (1996) Structure and expression of two highly related genes encoding SCM-1 human lymphotactin. *Febs Letters,* 395**,** 82-88.

ZENG, X. H., OU, Z. L., YU, K. D., FENG, L. Y., YIN, W. J., LI, J., SHEN, Z. Z. & SHAO, Z. M. (2011) Coexpression of atypical chemokine binders (ACBs) in breast cancer predicts better outcomes. *Breast Cancer Research and Treatment,* 125**,** 715-727.

ZHU, J. Z., MILLARD, C. J., LUDEMAN, J. P., SIMPSON, L. S., CLAYTON, D. J., PAYNE, R. J., WIDLANSKI, T. S. & STONE, M. J. (2011) Tyrosine Sulfation Influences the Chemokine Binding Selectivity of Peptides Derived from Chemokine Receptor CCR3. *Biochemistry,* 50**,** 1524-1534.

ZHU, Z., SUN, Z., WANG, Z., GUO, P., ZHENG, X. & XU, H. (2013) Prognostic impact of atypical chemokine receptor expression in patients with gastric cancer. *The Journal of surgical research,* 183**,** 177-83.

ZIMMERMAN, P. A., FERREIRA, M. U., HOWES, R. E. & MERCEREAU-PUIJALON, O. (2013) Red Blood Cell Polymorphism and Susceptibility to Plasmodium vivax. IN HAY, S. I., PRICE, R. & BAIRD, J. K. (Eds.) *Advances in Parasitology, Vol 81: Epidemiology of Plasmodium Vivax: History, Hiatus and Hubris, Pt B.* San Diego, Elsevier Academic Press Inc.

ZLOTNIK, A. & YOSHIE, O. (2000) Chemokines: A new classification system and their role in immunity. *Immunity,* 12**,** 121-127.

ZLOTNIK, A., YOSHIE, O. & NOMIYAMA, H. (2006) The chemokine and chemokine receptor superfamilies and their molecular evolution. *Genome Biology,* 7**,** 11.

ZOU, P., ISEGAWA, Y., NAKANO, K., HAQUE, M., HORIGUCHI, Y. & YAMANISHI, K. (1999) Human herpesvirus 6 open reading frame U83 encodes a functional chemokine. *Journal of Virology,* 73**,** 5926-5933.

ADAGE, T., PICCININI, A. M., FALSONE, A., TRINKER, M., ROBINSON, J., GESSLBAUER, B. & KUNGL, A. J. (2012) Structure-based design of decoy chemokines as a way to explore the pharmacological potential of glycosaminoglycans. *British Journal of Pharmacology,* 167**,** 1195-1205.

AHMED, T., UNGO, J., ZHOU, M. & CAMPO, C. (2000) Inhibition of allergic late airway responses by inhaled heparin-derived oligosaccharides. *Journal of Applied Physiology,* 88**,** 1721-1729.

ALCAMI, A. (2003) Viral mimicry of cytokines, chemokines and their receptors. *Nature Reviews Immunology,* 3**,** 36-50.

ALI, S., PALMER, A. C. V., BANERJEE, B., FRITCHLEY, S. J. & KIRBY, J. A. (2000) Examination of the function of RANTES, MIP-1 alpha, and MIP-1 beta following interaction with heparin-like glycosaminoglycans. *Journal of Biological Chemistry,* 275**,** 11721-11727.

ALI, S., ROBERTSON, H., WAIN, J. H., ISAACS, J. D., MALIK, G. & KIRBY, J. A. (2005) A non-glycosaminoglycan-binding variant of CC chemokine ligand 7 (monocyte chemoattractant protein-3) antagonizes chemokine-mediated inflammation. *Journal of Immunology,* 175**,** 1257-1266.

ALIBERTI, J., SOUSA, C. R. E., SCHITO, M., HIENY, S., WELLS, T., HUFFNAGLE, G. B. & SHER, A. (2000) CCR5 provides a signal for microbial induced production of IL-12 by CD8 alpha(+) dendritic cells. *Nature Immunology,* 1**,** 83-87.

ALIBERTI, J., VALENZUELA, J. G., CARRUTHERS, V. B., HIENY, S., ANDERSEN, J., CHAREST, H., REIS E SOUSA, C., FAIRLAMB, A., RIBEIRO, J. M. & SHER, A. (2003) Molecular mimicry of a CCR5 binding-domain in the microbial activation of dendritic cells. *Nature Immunology,* 4**,** 485-490.

ALKHATIB, G., AHUJA, S. S., LIGHT, D., MUMMIDI, S., BERGER, E. A. & AHUJA, S. K. (1997) CC chemokine receptor 5-mediated signaling and HIV-1 co-receptor activity share common structural determinants - Critical residues in the third extracellular loop support HIV-1 fusion. *Journal of Biological Chemistry,* 272**,** 19771-19776.

ALLEN, S. J., CROWN, S. E. & HANDEL, T. M. (2007) Chemokine: Receptor structure, interactions, and antagonism. *Annual Review of Immunology.* Palo Alto, Annual Reviews.

ALON, R. (2010) Trapped versus Soluble Chemokines: Functions in Leukocyte Adhesion and Motility. *Immunity,* 33**,** 654-656.

ANTHONY, D. D. & HAQQI, T. M. (1999) Collagen-induced arthritis in mice: An animal model to study the pathogenesis of rheumatoid arthritis. *Clinical and Experimental Rheumatology,* 17**,** 240-244.

ARVANITAKIS, L., GERASRAAKA, E., VARMA, A., GERSHENGORN, M. C. & CESARMAN, E. (1997) Human herpesvirus KSHV encodes a constitutively active G-protein-coupled receptor linked to cell proliferation. *Nature,* 385**,** 347-350.

ATCHISON, R. E., GOSLING, J., MONTECLARO, F. S., FRANCI, C., DIGILIO, L., CHARO, I. F. & GOLDSMITH, M. A. (1996) Multiple extracellular elements of CCR5 and HIV-1 entry: Dissociation from response to chemokines. *Science,* 274**,** 1924-1926.

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. I., ROSENKILDE, M. M., ROT, A., SOZZANI, S., THELEN, M., YOSHIE, O. & ZLOTNIK, A. (2014) International Union of Pharmacology. LXXXIX. Update on the Extended Family of Chemokine Receptors and Introducing a New Nomenclature for Atypical Chemokine Receptors. *Pharmacological Reviews,* 66**,** 1-79.

BAGGIOLINI, M., DEWALD, B. & MOSER, B. (1994) INTERLEUKIN-8 AND RELATED CHEMOTACTIC CYTOKINES - CXC AND CC CHEMOKINES. *Advances in Immunology, Vol 55,* 55**,** 97-179.

BAIS, C., SANTOMASSO, B., COSO, O., ARVANITAKIS, L., RAAKA, E. G., GUTKIND, J. S., ASCH, A. S., CESARMAN, E., GERHENGORN, M. C. & MESRI, E. A. (1998) G-protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus is a viral oncogene and angiogenesis activator. *Nature,* 391**,** 86-89.

BALDWIN, H. M., PALLAS, K., KING, V., JAMIESON, T., MCKIMMIE, C. S., NIBBS, R. J. B., CARBALLIDO, J. M., JARITZ, M., ROT, A. & GRAHAM, G. J. (2013) Microarray Analyses Demonstrate the Involvement of Type I Interferons in Psoriasiform Pathology Development in D6-deficient Mice. *Journal of Biological Chemistry,* 288**,** 36473-36483.

BALKWILL, F. & MANTOVANI, A. (2001) Inflammation and cancer: back to Virchow? *Lancet,* 357**,** 539-545.

BALKWILL, F. R. (2012) The chemokine system and cancer. *Journal of Pathology,* 226**,** 148-157.

BALTUS, T., WEBER, K. S. C., JOHNSON, Z., PROUDFOOT, A. E. I. & WEBER, C. (2003) Oligomerization of RANTES is required for CCR1-mediated arrest but not CCR5-mediated transmigration of leukocytes on inflamed endothelium. *Blood,* 102**,** 1985-1988.

BAO, L., ZHU, Y., ZHU, H. & LINDGREN, J. U. (2005) Decreased IgG production but increased MIP-1 beta expression in collagen-induced arthritis in C-C chemokine receptor 5-deficient mice. *Cytokine,* 31**,** 64-71.

BAO, X. F., MOSEMAN, E. A., SAITO, H., PETRYANIK, B., THIRIOT, A., HATAKEYAMA, S., ITO, Y., KAWASHIMA, H., YAMAGUCHI, Y., LOWE, J. B., VON ANDRIAN, U. H. & FUKUDA, M. (2010) Endothelial Heparan Sulfate Controls Chemokine Presentation in Recruitment of Lymphocytes and Dendritic Cells to Lymph Nodes. *Immunity,* 33**,** 817-829.

BARRESINOUSSI, F., CHERMANN, J. C., REY, F., NUGEYRE, M. T., CHAMARET, S., GRUEST, J., DAUGUET, C., AXLERBLIN, C., VEZINETBRUN, F., ROUZIOUX, C., ROZENBAUM, W. & MONTAGNIER, L. (1983) ISOLATION OF A T-LYMPHOTROPIC RETROVIRUS FROM A PATIENT AT RISK FOR ACQUIRED IMMUNE-DEFICIENCY SYNDROME (AIDS). *Science,* 220**,** 868-871.

BAZAN, J. F., BACON, K. B., HARDIMAN, G., WANG, W., SOO, K., ROSSI, D., GREAVES, D. R., ZLOTNIK, A. & SCHALL, T. J. (1997) A new class of membrane-bound chemokine with a CX(3)C motif. *Nature,* 385**,** 640-644.

BAZZAN, E., SAETTA, M., TURATO, G., BORRONI, E. M., CANCELLIERI, C., BARALDO, S., SAVINO, B., CALABRESE, F., BALLARIN, A., BALESTRO, E., MANTOVANI, A., COSIO, M. G., BONECCHI, R. & LOCATI, M. (2013) Expression of the Atypical Chemokine Receptor D6 in Human Alveolar Macrophages in COPD. *Chest,* 143**,** 98-106.

BELPERIO, J. A., KEANE, M. P., ARENBERG, D. A., ADDISON, C. L., EHLERT, J. E., BURDICK, M. D. & STRIETER, R. M. (2000) CXC chemokines in angiogenesis. *Journal of Leukocyte Biology,* 68**,** 1-8.

BENNETT, L. D., FOX, J. M. & SIGNORET, N. (2011) Mechanisms regulating chemokine receptor activity. *Immunology,* 134**,** 246-256.

BERAHOVICH, R. D., MIAO, Z. H., WANG, Y., PREMACK, B., HOWARD, M. C. & SCHALL, T. J. (2005) Proteolytic activation of alternative CCR1 ligands in inflammation. *Journal of Immunology,* 174**,** 7341-7351.

BERGER, E. A., DOMS, R. W., FENYO, E. M., KORBER, B. T. M., LITTMAN, D. R., MOORE, J. P., SATTENTAU, Q. J., SCHUITEMAKER, H., SODROSKI, J. & WEISS, R. A. (1998) A new classification for HIV-1. *Nature,* 391**,** 240-240.

BERGER, E. A., MURPHY, P. M. & FARBER, J. M. (1999) Chemokine receptors as HIV-1 coreceptors: Roles in viral entry, tropism, and disease. *Annual Review of Immunology,* 17**,** 657-700.

BERKOWITZ, R. D., ALEXANDER, S., BARE, C., LINQUIST-STEPPS, V., BOGAN, M., MORENO, M. E., GIBSON, L., WIEDER, E. D., KOSEK, J., STODDART, C. A. & MCCUNE, J. M. (1998) CCR5- and CXCR4-utilizing strains of human immunodeficiency virus type 1 exhibit differential tropism and pathogenesis in vivo. *Journal of Virology,* 72**,** 10108-10117.

BLACKBURN, P. E., SIMPSON, C. V., NIBBS, R. J. B., O'HARA, M., BOOTH, R., POULOS, J., ISAACS, N. W. & GRAHAM, G. J. (2004) Purification and biochemical characterization of the D6 chemokine receptor. *Biochemical Journal,* 379**,** 263-272.

BLANPAIN, C., DORANZ, B. J., VAKILI, J., RUCKER, J., GOVAERTS, C., BAIK, S. S. W., LORTHIOIR, O., MIGEOTTE, I., LIBERT, F., BALEUX, F., VASSART, G., DOMS, R. W. & PARMENTIER, M. (1999a) Multiple charged and aromatic residues in CCR5 amino-terminal domain are involved in high affinity binding of both chemokines and HIV-1 Env protein. *Journal of Biological Chemistry,* 274**,** 34719-34727.

BLANPAIN, C., LEE, B., VAKILI, J., DORANZ, B. J., GOVAERTS, C., MIGEOTTE, I., SHARRON, M., DUPRIEZ, V., VASSART, G., DOMS, R. W. & PARMENTIER, M. (1999b) Extracellular cysteines of CCR5 are required for chemokine binding, but dispensable for HIV-1 coreceptor activity. *Journal of Biological Chemistry,* 274**,** 18902-18908.

BLANPAIN, C., WITTAMER, V., VANDERWINDEN, J. M., BOOM, A., RENNEBOOG, B., LEE, B., LE POUL, E., EL ASMAR, L., GOVAERTS, C., VASSART, G., DOMS, R. W. & PARMENTIER, M. (2001) Palmitoylation of CCR5 is critical for receptor trafficking and efficient activation of intracellular signaling pathways. *Journal of Biological Chemistry,* 276**,** 23795-23804.

BONECCHI, R., BORRONI, E. M., ANSELMO, A., DONI, A., SAVINO, B., MIROLO, M., FABBRI, M., JALA, V. R., HARIBABU, B., MANTOVANI, A. & LOCATI, M. (2008) Regulation of D6 chemokine scavenging activity by ligand- and Rab11-dependent surface up-regulation. *Blood,* 112**,** 493-503.

BONECCHI, R., LOCATI, M., GALLIERA, E., VULCANO, M., SIRONI, M., FRA, A. M., GOBBI, M., VECCHI, A., SOZZANI, S., HARIBABU, B., VAN DAMME, J.

& MANTOVANI, A. (2004) Differential recognition and scavenging of native and truncated macrophage-derived chemokine (macrophage-derived chemokine/CC chemokine ligand 22) by the D6 decoy receptor. *Journal of Immunology,* 172**,** 4972-4976.

BONINI, J. A., MARTIN, S. K., DRALYUK, F., ROE, M. W., PHILIPSON, L. H. & STEINER, D. F. (1997) Cloning, expression, and chromosomal mapping of a novel human CC-chemokine receptor (CCR10) that displays high-affinity binding for MCP-1 and MCP-3. *DNA and Cell Biology,* 16**,** 1249-1256.

BORDON, Y., HANSELL, C. A. H., SESTER, D. P., CLARKE, M., MOWAT, A. M. & NIBBS, R. J. B. (2009) The Atypical Chemokine Receptor D6 Contributes to the Development of Experimental Colitis. *Journal of Immunology,* 182**,** 5032-5040.

BORGHEI, A., OUYANG, Y. B., WESTMUCKETT, A. D., MARCELLO, M. R., LANDEL, C. P., EVANS, J. P. & MOORE, K. L. (2006) Targeted disruption of tyrosylprotein sulfotransferase-2, an enzyme that catalyzes post-translational protein tyrosine O-sulfation, causes male infertility. *Journal of Biological Chemistry,* 281**,** 9423-9431.

BORRONI, E. M., CANCELLIERI, C., VACCHINI, A., BENUREAU, Y., LAGANE, B., BACHELERIE, F., ARENZANA-SEISDEDOS, F., MIZUNO, K., MANTOVANI, A., BONECCHI, R. & LOCATI, M. (2013) beta-arrestin-dependent activation of the cofilin pathway is required for the scavenging activity of the atypical chemokine receptor D6. *Science signaling,* 6**,** ra30.1-11, S1-3.

BORRONI, E. M., MANTOVANI, A., LOCATI, M. & BONECCHI, R. (2010) Chemokine receptors intracellular trafficking. *Pharmacology & Therapeutics,* 127**,** 1-8.

BROXMEYER, H. E., ORSCHELL, C. M., CLAPP, D. W., HANGOC, G., COOPER, S., PLETT, P. A., LILES, W. C., LI, X. X., GRAHAM-EVANS, B., CAMPBELL, T. B., CALANDRA, G., BRIDGER, G., DALE, D. C. & SROUR, E. F. (2005) Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *Journal of Experimental Medicine,* 201**,** 1307-1318.

BRUHL, H., CIHAK, J., SCHNEIDER, M. A., PLACHY, J., RUPP, T., WENZEL, I., SHAKARAMI, M., MILZ, S., ELLWART, J. W., STANGASSINGER, M., SCHLONDORFF, D. & MACK, M. (2004) Dual role of CCR2 during initiation and progression of collagen-induced arthritis: Evidence for regulatory activity of CCR2(+) T cells. *Journal of Immunology,* 172**,** 890-898.

BRUHL, H., WAGNER, K., KELLNER, H., SCHATTENKIRCHNER, M., SCHLONDORFF, D. & MACK, M. (2001) Surface expression of CC- and CXC-chemokine receptors on leucocyte subsets in inflammatory joint diseases. *Clinical and Experimental Immunology,* 126**,** 551-559.

BULENGER, S., MARULLO, S. & BOUVIER, M. (2005) Emerging role of homo- and heterodimerization in G-protein-coupled receptor biosynthesis and maturation. *Trends in Pharmacological Sciences,* 26**,** 131-137.

BUNTING, M. D., COMERFORD, I., SEACH, N., HAMMETT, M. V., ASQUITH, D. L., KORNER, H., BOYD, R. L., NIBBS, R. J. B. & MCCOLL, S. R. (2013) CCX-CKR deficiency alters thymic stroma impairing thymocyte development and promoting autoimmunity. *Blood,* 121**,** 118-128.

BURKHARDT, A. M., MARAVILLAS-MONTERO, J. L., CARNEVALE, C. D., VILCHES-CISNEROS, N., FLORES, J. P., HEVEZI, P. A. & ZLOTNIK, A. (2014) CXCL17 Is a Major Chemotactic Factor for Lung Macrophages. *Journal of Immunology,* 193**,** 1468-1474.

BURNS, J. M., SUMMERS, B. C., WANG, Y., MELIKIAN, A., BERAHOVICH, R., MIAO, Z. H., PENFOLD, M. E. T., SUNSHINE, M. J., LITTMAN, D. R., KUO, C. J., WEI, K., MCMASTER, B. E., WRIGHT, K., HOWARD, M. C. & SCHALL,

T. J. (2006) A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *Journal of Experimental Medicine,* 203**,** 2201-2213.

CAMPANELLA, G. S. V., LEE, E. M. J., SUN, J. & LUSTER, A. D. (2003) CXCR3 and heparin binding sites of the chemokine IP-10 (CXCL10). *Journal of Biological Chemistry,* 278**,** 17066-17074.

CHABOT, D. J., CHEN, H., DIMITROV, D. S. & BRODER, C. C. (2000) N-linked glycosylation of CXCR4 masks coreceptor function for CCR5-dependent human immunodeficiency virus type 1 isolates. *Journal of Virology,* 74**,** 4404-4413.

CHAKERA, A., SEEBER, R. M., JOHN, A. E., EIDNE, K. A. & GREAVES, D. R. (2008) The Duffy antigen/receptor for chemokines exists in an oligomeric form in living cells and functionally antagonizes CCR5 signaling through hetero-oligomerization. *Molecular Pharmacology,* 73**,** 1362-1370.

CHARO, I. F. & RANSOHOFF, R. M. (2006) Mechanisms of disease - The many roles of chemokines and chemokine receptors in inflammation. *New England Journal of Medicine,* 354**,** 610-621.

CHOE, H., MOORE, M. J., OWENS, C. M., WRIGHT, P. L., VASILIEVA, N., LI, W., SINGH, A. P., SHAKRI, R., CHITNIS, C. E. & FARZAN, M. (2005) Sulphated tyrosines mediate association of chemokines and Plasmodium vivax Duffy binding protein with the Duffy antigen/receptor for chemokines (DARC). *Molecular Microbiology,* 55**,** 1413-1422.

CLAING, A., LAPORTE, S. A., CARON, M. G. & LEFKOWITZ, R. J. (2002) Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins. *Progress in Neurobiology,* 66**,** 61-79.

CLARKLEWIS, I., DEWALD, B., GEISER, T., MOSER, B. & BAGGIOLINI, M. (1993) PLATELET FACTOR-IV BINDS TO INTERLEUKIN-8 RECEPTORS AND ACTIVATES NEUTROPHILS WHEN ITS N-TERMINUS IS MODIFIED WITH GLU-LEU-ARG. *Proceedings of the National Academy of Sciences of the United States of America,* 90**,** 3574-3577.

CLARKLEWIS, I., KIM, K. S., RAJARATHNAM, K., GONG, J. H., DEWALD, B., MOSER, B., BAGGIOLINI, M. & SYKES, B. D. (1995) STRUCTURE-ACTIVITY-RELATIONSHIPS OF CHEMOKINES. *Journal of Leukocyte Biology,* 57**,** 703-711.

COCCHI, F., DEVICO, A. L., GARZINODEMO, A., ARYA, S. K., GALLO, R. C. & LUSSO, P. (1995) IDENTIFICATION OF RANTES, MIP-1-ALPHA, AND MIP-1-BETA AS THE MAJOR HIV-SUPPRESSIVE FACTORS PRODUCED BY CD8(+) T-CELLS. *Science,* 270**,** 1811-1815.

COCHAIN, C., AUVYNET, C., POUPEL, L., VILAR, J., DUMEAU, E., RICHART, A., RECALDE, A., ZOUGGARI, Y., YIN, K., BRUNEVAL, P., RENAULT, G., MARCHIOL, C., BONNIN, P., LEVY, B., BONECCHI, R., LOCATI, M., COMBADIERE, C. & SILVESTRE, J. S. (2012) The Chemokine Decoy Receptor D6 Prevents Excessive Inflammation and Adverse Ventricular Remodeling After Myocardial Infarction. *Arteriosclerosis Thrombosis and Vascular Biology,* 32**,** 2206-+.

CODULLO, V., BALDWIN, H. M., SINGH, M. D., FRASER, A. R., WILSON, C., GILMOUR, A., HUEBER, A. J., BONINO, C., MCINNES, I. B., MONTECUCCO, C. & GRAHAM, G. J. (2011) An investigation of the inflammatory cytokine and chemokine network in systemic sclerosis. *Annals of the Rheumatic Diseases,* 70**,** 1115-1121.

COLLINS, C. B., MCNAMEE, E. N., WERMERS, J. D., LEBSACK, M. D. P. & RIVERA-NIEVES, J. (2010) Chemokine decoy receptor D6 in inflammatory bowel disease (IBD) and IBD-associated colon cancer. *Gut,* 59**,** 151-152.

COLVIN, R. A., CAMPANELLA, G. S. V., MANICE, L. A. & LUSTER, A. D. (2006) CXCR3 requires tyrosine sulfation for ligand binding and a second extracellular loop arginine residue for ligand-induced chemotaxis. *Molecular and Cellular Biology,* 26**,** 5838-5849.

COMERFORD, I., HARATA-LEE, Y., BUNTING, M. D., GREGOR, C., KARA, E. E. & MCCOLL, S. R. (2013) A myriad of functions and complex regulation of the CCR7/CCL19/CCL21 chemokine axis in the adaptive immune system. *Cytokine & Growth Factor Reviews,* 24**,** 269-283.

COMERFORD, I., MILASTA, S., MORROW, V., MILLIGAN, G. & NIBBS, R. (2006) The chemokine receptor CCX-CKR mediates effective scavenging of CCL19 in vitro. *European Journal of Immunology,* 36**,** 1904-1916.

COMERFORD, I. & NIBBS, R. J. B. (2005) Post-translational control of chemokines: a role for,decoy receptors? *Immunology Letters,* 96**,** 163-174.

COMERFORD, I., NIBBS, R. J. B., LITCHFIELD, W., BUNTING, M., HARATA-LEE, Y., HAYLOCK-JACOBS, S., FORROW, S., KORNER, H. & MCCOLL, S. R. (2010) The atypical chemokine receptor CCX-CKR scavenges homeostatic chemokines in circulation and tissues and suppresses Th17 responses. *Blood,* 116**,** 4130-4140.

CONNOR, R. I., SHERIDAN, K. E., CERADINI, D., CHOE, S. & LANDAU, N. R. (1997) Change in coreceptor use correlates with disease progression in HIV-1-infected individuals. *Journal of Experimental Medicine,* 185**,** 621-628.

CORMIER, E. G., PERSUH, M., THOMPSON, D. A. D., LIN, S. W., SAKMAR, T. P., OLSON, W. C. & DRAGIC, T. (2000) Specific interaction of CCR5 amino-terminal domain peptides containing sulfotyrosines with HIV-1 envelope glycoprotein gp120. *Proceedings of the National Academy of Sciences of the United States of America,* 97**,** 5762-5767.

COSTIN, J. M. (2007) Cytopathic mechanisms of HIV-I. *Virology Journal,* 4**,** 22.

COTTON, M. & CLAING, A. (2009) G protein-coupled receptors stimulation and the control of cell migration. *Cellular Signalling,* 21**,** 1045-1053.

COUCHMAN, J. R. & PATAKI, C. A. (2012) An Introduction to Proteoglycans and Their Localization. *Journal of Histochemistry & Cytochemistry,* 60**,** 885-897.

COUSSENS, L. M. & WERB, Z. (2002) Inflammation and cancer. *Nature,* 420**,** 860-867.

DAIRAGHI, D. J., ZHANG, P., WANG, Y., SEITZ, L. C., JOHNSON, D. A., MIAO, S., ERTL, L. S., ZENG, Y., POWERS, J. P., PENNELL, A. M., BEKKER, P., SCHALL, T. J. & JAEN, J. C. (2011) Pharmacokinetic and Pharmacodynamic Evaluation of the Novel CCR1 Antagonist CCX354 in Healthy Human Subjects: Implications for Selection of Clinical Dose. *Clinical Pharmacology & Therapeutics,* 89**,** 726-734.

DANAN, L. M., YU, Z. H., HOFFHINES, A. J., MOORE, K. L. & LEARY, J. A. (2008) Mass Spectrometric Kinetic Analysis of Human Tyrosylprotein Sulfotransferase-1 and-2. *Journal of the American Society for Mass Spectrometry,* 19**,** 1459-1466.

DANIELS, G. D., ZOU, J., CHARLEMAGNE, J., PARTULA, S., CUNNINGHAM, C. & SECOMBES, C. J. (1999) Cloning of two chemokine receptor homologs (CXC-R4 and CC-R7) in rainbow trout Oncorhynchus mykiss. *Journal of Leukocyte Biology,* 65**,** 684-690.

DAWSON, T. C., LENTSCH, A. B., WANG, Z. X., COWHIG, J. E., ROT, A., MAEDA, N. & PEIPER, S. C. (2000) Exaggerated response to endotoxin in mice lacking the Duffy antigen/receptor for chemokines (DARC). *Blood,* 96**,** 1681-1684.

DE RODA HUSMAN, A.-M., KOOT, M., CORNELISSEN, M., KEET, I. P. M., BROUWER, M., BROERSEN, S. M., BAKKER, M., ROOS, M. T. L., PRINS, M., DE WOLF, F., COUTINHO, R. A., MIEDEMA, F., GOUDSMIT, J. & SCHUITEMAKER, H. (1997) Association between CCR5 genotype and the clinical course of HIV-1 infection. *Annals of Internal Medicine,* 127**,** 882-890.

DERUAZ, M., FRAUENSCHUH, A., ALESSANDRI, A. L., DIAS, J. M., COELHO, F. M., RUSSO, R. C., FERREIRA, B. R., GRAHAM, G. J., SHAW, J. P., WELLS, T. N. C., TEIXEIRA, M. M., POWER, C. A. & PROUDFOOT, A. E. I. (2008) Ticks produce highly selective chemokine binding proteins with antiinflammatory activity. *Journal of Experimental Medicine,* 205**,** 2019-2031.

DEVRIES, M. E., KELVIN, A. A., XU, L. L., RAN, L. S., ROBINSON, J. & KELVIN, D. J. (2006) Defining the origins and evolution of the chemokine/chemokine receptor system. *Journal of Immunology,* 176**,** 401-415.

DI LIBERTO, D., LOCATI, M., CACCAMO, N., VECCHI, A., MERAVIGLIA, S., SALERNO, A., SIRECI, G., NEBULONI, M., CACERES, N., CARDONA, P. J., DIELI, F. & MANTOVANI, A. (2008) Role of the chemokine decoy receptor D6 in balancing inflammation, immune activation, and antimicrobial resistance in Mycobacterium tuberculosis infection. *Journal of Experimental Medicine,* 205**,** 2075-2084.

DIMITROV, D. S. (1997) How do viruses enter cells? The HIV coreceptors teach us a lesson of complexity. *Cell,* 91**,** 721-730.

DING, Y. Z., SHIMADA, Y., MAEDA, M., KAWABE, A., KAGANOI, J., KOMOTO, I., HASHIMOTO, Y., MIYAKE, M., HASHIDA, H. & IMAMURA, M. (2003) Association of CC chemokine receptor 7 with lymph node metastasis of esophageal squamous cell carcinoma. *Clinical Cancer Research,* 9**,** 3406-3412.

DOITSIDOU, M., REICHMAN-FRIED, M., STEBLER, J., KOPRUNNER, M., DORRIES, J., MEYER, D., ESGUERRA, C. V., LEUNG, T. & RAZ, E. (2002) Guidance of primordial germ cell migration by the chemokine SDF-1. *Cell,* 111**,** 647-659.

DORR, P., WESTBY, M., DOBBS, S., GRIFFIN, P., IRVINE, B., MACARTNEY, M., MORI, J., RICKETT, G., SMITH-BURCHNELL, C., NAPIER, C., WEBSTER, R., ARMOUR, D., PRICE, D., STAMMEN, B., WOOD, A. & PERROS, M. (2005) Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. *Antimicrobial Agents and Chemotherapy,* 49**,** 4721-4732.

DRURY, L. J., ZIAREK, J. J., GRAVEL, S., VELDKAMP, C. T., TAKEKOSHI, T., HWANG, S. T., HEVEKER, N., VOLKMAN, B. F. & DWINELL, M. B. (2011) Monomeric and dimeric CXCL12 inhibit metastasis through distinct CXCR4 interactions and signaling pathways. *Proceedings of the National Academy of Sciences of the United States of America,* 108**,** 17655-17660.

DUMA, L., HAUSSINGER, D., ROGOWSKI, M., LUSSO, P. & GRZESIEK, S. (2007) Recognition of RANTES by extracellular parts of the CCR5 receptor. *Journal of Molecular Biology,* 365**,** 1063-1075.

EL-ASMAR, L., SPRINGAEL, J. Y., BALLET, S., ANDRIEU, E. U., VASSART, G. & PARMENTIER, M. (2005) Evidence for negative binding cooperativity within CCR5-CCR2b heterodimers. *Molecular Pharmacology,* 67**,** 460-469.

EPPERSON, M. L., LEE, C. A. & FREMONT, D. H. (2012) Subversion of cytokine networks by virally encoded decoy receptors. *Immunological Reviews,* 250**,** 199-215.

ESKO, J. D., STEWART, T. E. & TAYLOR, W. H. (1985) ANIMAL-CELL MUTANTS DEFECTIVE IN GLYCOSAMINOGLYCAN BIOSYNTHESIS. *Proceedings of the National Academy of Sciences of the United States of America,* 82**,** 3197-3201.

FARZAN, M., MIRZABEKOV, T., KOLCHINSKY, P., WYATT, R., CAYABYAB, M., GERARD, N. P., GERARD, C., SODROSKI, J. & CHOE, H. (1999) Tyrosine sulfation of the amino terminus of CCR5 facilitates HIV-1 entry. *Cell,* 96**,** 667-676.

FARZAN, M., VASILIEVA, N., SCHNITZLER, C. E., CHUNG, S., ROBINSON, J., GERARD, N. P., GERARD, C., CHOE, H. & SODROSKI, J. (2000) A tyrosine-

sulfated peptide based on the N terminus of CCR5 interacts with a CD4-enhanced epitope of the HIV-1 gp120 envelope glycoprotein and inhibits HIV-1 entry. *Journal of Biological Chemistry,* 275**,** 33516-33521.

FATKENHEUER, G., POZNIAK, A. L., JOHNSON, M. A., PLETTENBERG, A., STASZEWSKI, S., HOEPELMAN, A. I. M., 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. & VAN DER RYST, E. (2005) Efficacy of short-term monotherapy with maraviroc, a new CCR5 antagonist, in patients infected with HIV-1. *Nature Medicine,* 11**,** 1170-1172.

FENG, L. Y., OU, Z. L., WU, F. Y., SHEN, Z. Z. & SHAO, Z. M. (2009) Involvement of a Novel Chemokine Decoy Receptor CCX-CKR in Breast Cancer Growth, Metastasis and Patient Survival. *Clinical Cancer Research,* 15**,** 2962-2970.

FERNANDO, H., CHIN, C., ROSGEN, J. & RAJARATHNAM, K. (2004) Dimer dissociation is essential for interleukin-8 (IL-8) binding to CXCR1 receptor. *Journal of Biological Chemistry,* 279**,** 36175-36178.

FIRESTEIN, G. S. (2003) Evolving concepts of rheumatoid arthritis. *Nature,* 423**,** 356-361.

FONG, A. M., ALAM, S. M., IMAI, T., HARIBABU, B. & PATEL, D. D. (2002) CX(3)CR1 tyrosine sulfation enhances fractalkine-induced cell adhesion. *Journal of Biological Chemistry,* 277**,** 19418-19423.

FONG, A. M., ROBINSON, L. A., STEEBER, D. A., TEDDER, T. F., YOSHIE, O., IMAI, T. & PATEL, D. D. (1998) Fractalkine and CX(3)CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. *Journal of Experimental Medicine,* 188**,** 1413-1419.

FORSTER, R., DAVALOS-MISSLITZ, A. C. & ROT, A. (2008) CCR7 and its ligands: balancing immunity and tolerance. *Nature Reviews Immunology,* 8**,** 362-371.

FRA, A. M., LOCATI, M., OTERO, K., SIRONI, M., SIGNORELLI, P., MASSARDI, M. L., GOBBI, M., VECCHI, A., SOZZANI, S. & MANTOVANI, A. (2003) Cutting edge: Scavenging of inflammatory CC chemokines by the promiscuous putatively silent chemokine receptor D6(1). *Journal of Immunology,* 170**,** 2279-2282.

FUKUMA, N., AKIMITSU, N., HAMAMOTO, H., KUSUHARA, H., SUGIYAMA, Y. & SEKIMIZU, K. (2003) A role of the Duffy antigen for the maintenance of plasma chemokine concentrations. *Biochemical and Biophysical Research Communications,* 303**,** 137-139.

GALLIERA, E., JALA, V. R., TRENT, J. O., BONECCHI, R., SIGNORELLI, P., LEFKOWITZ, R. J., MANTOVANI, A., LOCATI, M. & HARIBABU, B. (2004) beta-arrestin-dependent constitutive internalization of the human chemokine decoy receptor D6. *Journal of Biological Chemistry,* 279**,** 25590-25597.

GALLO, R. C. & MONTAGNIER, L. (2003) Retrospective: The discovery of HIV as the cause of AIDS. *New England Journal of Medicine,* 349**,** 2283-2285.

GALZI, J. L., HACHET-HAAS, M., BONNET, D., DAUBEUF, F., LECAT, S., HIBERT, M., HAIECH, J. & FROSSARD, N. (2010) Neutralizing endogenous chemokines with small molecules Principles and potential therapeutic applications. *Pharmacology & Therapeutics,* 126**,** 39-55.

GAO, P., ZHOU, X. Y., YASHIRO-OHTANI, Y., YANG, Y. F., SUGIMOTO, N., ONO, S., NAKANISHI, T., OBIKA, S., IMANISHI, T., EGAWA, T., NAGASAWA, T., FUJIWARA, H. & HAMAOKA, T. (2003) The unique target specificity of a nonpeptide chemokine receptor antagonist: selective blockade of two Th1 chemokine receptors CCR5 and CXCR3. *Journal of Leukocyte Biology,* 73**,** 273-280.

GARDNER, L., PATTERSON, A. M., ASHTON, B. A., STONE, M. A. & MIDDLETON, J. (2004) The human Duffy antigen binds selected inflammatory but not

homeostatic chemokines. *Biochemical and Biophysical Research Communications,* 321**,** 306-312.

GARLANDA, C., MAINA, V., MARTINEZ DE LA TORRE, Y., NEBULONI, M. & LOCATI, M. (2008) Inflammatory reaction and implantation: the new entries PTX3 and D6. *Placenta,* 29 Suppl B**,** 129-34.

GARRED, P., MADSEN, H. O., PETERSEN, J., MARQUART, H., HANSEN, T. M., SORENSEN, S. F., VOLCK, B., SVEJGAARD, A. & ANDERSEN, V. (1998) CC chemokine receptor 5 polymorphism in rheumatoid arthritis. *Journal of Rheumatology,* 25**,** 1462-1465.

GERARD, C. & ROLLINS, B. J. (2001) Chemokines and disease. *Nature Immunology,* 2**,** 108-115.

GERRITS, H., SCHENAU, D., BAKKER, N. E. C., VAN DISSELDORP, A. J. M., STRIK, A., HERMENS, L. S., KOENEN, T. B., KRAJNC-FRANKEN, M. A. M. & GOSSEN, J. A. (2008) Early postnatal lethality and cardiovascular defects in CXCR7-deficient mice. *Genesis,* 46**,** 235-245.

GILLITZER, R., WOLFF, K., TONG, D., MULLER, C., YOSHIMURA, T., HARTMANN, A. A., STINGL, G. & BERGER, R. (1993) MCP-1 MESSENGER-RNA EXPRESSION IN BASAL KERATINOCYTES OF PSORIATIC LESIONS. *Journal of Investigative Dermatology,* 101**,** 127-131.

GLUSHAKOVA, S., GRIVEL, J. C., FITZGERALD, W., SYLWESTER, A., ZIMMERBERG, J. & MARGOLIS, L. B. (1998) Evidence for the HIV-1 phenotype switch as a causal factor in acquired immunodeficiency. *Nature Medicine,* 4**,** 346-349.

GOMEZ-REINO, J. J., PABLOS, J. L., CARREIRA, P. E., SANTIAGO, B., SERRANO, L., VICARIO, J. L., BALSA, A., FIGUEROA, M. & DE JUAN, M. D. (1999) Association of rheumatoid arthritis with a functional chemokine receptor, CCR5. *Arthritis and Rheumatism,* 42**,** 989-992.

GONG, J. H. & CLARKLEWIS, I. (1995) ANTAGONISTS OF MONOCYTE CHEMOATTRACTANT PROTEIN-1 IDENTIFIED BY MODIFICATION OF FUNCTIONALLY CRITICAL NH2-TERMINAL RESIDUES. *Journal of Experimental Medicine,* 181**,** 631-640.

GONG, J. H., RATKAY, L. G., WATERFIELD, J. D. & CLARKLEWIS, I. (1997) An antagonist of monocyte chemoattractant protein 1 (MCP-1) inhibits arthritis in the MRL-lpr mouse model. *Journal of Experimental Medicine,* 186**,** 131-137.

GONG, J. H., UGUCCIONI, M., DEWALD, B., BAGGIOLINI, M. & CLARKLEWIS, I. (1996) RANTES and MCP-3 antagonists bind multiple chemokine receptors. *Journal of Biological Chemistry,* 271**,** 10521-10527.

GONZALEZ, E., KULKARNI, H., BOLIVAR, H., MANGANO, A., SANCHEZ, R., CATANO, G., NIBBS, R. J., FREEDMAN, B. I., QUINONES, M. P., BAMSHAD, M. J., MURTHY, K. K., ROVIN, B. H., BRADLEY, W., CLARK, R. A., ANDERSON, S. A., O'CONNELL, R. J., AGAN, B. K., AHUJA, S. S., BOLOGNA, R., SEN, L., DOLAN, M. J. & AHUJA, S. K. (2005) The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. *Science,* 307**,** 1434-1440.

GORRY, P. R., DUNFEE, R. L., MEFFORD, M. E., KUNSTMAN, K., MORGAN, T., MOORE, J. P., MASCOLA, J. R., AGOPIAN, K., HOLM, G. H., MEHLE, A., TAYLOR, J., FARZAN, M., WANG, H., ELLERY, P., WILLEY, S. J., CLAPHAM, P. R., WOLINSKY, S. M., CROWE, S. M. & GABUZDA, D. (2007) Changes in the V3 region of gp 120 contribute to unusually broad coreceptor usage of an HIV-1 isolate from a CCR5 Delta 32 heterozygote. *Virology,* 362**,** 163-178.

GOZANSKY, E. K., LOUIS, J. M., CAFFREY, M. C. & CLORE, G. M. (2005) Mapping the binding of the tail of the CXCR4 receptor n-terminal extracellular to stromal cell-derived factor-1 alpha. *Journal of Molecular Biology,* 345**,** 651-658.

GRAHAM, G. J. (2009) D6 and the atypical chemokine receptor family: Novel regulators of immune and inflammatory processes. *European Journal of Immunology,* 39**,** 342-351.

GRAHAM, G. J. & LOCATI, M. (2013) Regulation of the immune and inflammatory responses by the 'atypical' chemokine receptor D6. *Journal of Pathology,* 229**,** 168-175.

GRAHAM, G. J., LOCATI, M., MANTOVANI, A., ROT, A. & THELEN, M. (2012) The biochemistry and biology of the atypical chemokine receptors. *Immunology Letters,* 145**,** 30-38.

GRAHAM, G. J., MACKENZIE, J., LOWE, S., TSANG, M. L. S., WEATHERBEE, J. A., ISSACSON, A., MEDICHERLA, J., FANG, F., WILKINSON, P. C. & PRAGNELL, I. B. (1994) AGGREGATION OF THE CHEMOKINE MIP-1-ALPHA IS A DYNAMIC AND REVERSIBLE PHENOMENON - BIOCHEMICAL AND BIOLOGICAL ANALYSES. *Journal of Biological Chemistry,* 269**,** 4974-4978.

GRAHAM, G. J. & MCKIMMIE, C. S. (2006) Chemokine scavenging by D6: a movable feast? *Trends in Immunology,* 27**,** 381-386.

GRAHAM, G. J., WILKINSON, P. C., NIBBS, R. J. B., LOWE, S., KOLSET, S. O., PARKER, A., FRESHNEY, M. G., TSANG, M. L. S. & PRAGNELL, I. B. (1996) Uncoupling of stem cell inhibition from monocyte chemoattraction in MIP-1 alpha by mutagenesis of the proteoglycan binding site. *Embo Journal,* 15**,** 6506-6515.

GRAHAM, G. J., WRIGHT, E. G., HEWICK, R., WOLPE, S. D., WILKIE, N. M., DONALDSON, D., LORIMORE, S. & PRAGNELL, I. B. (1990) IDENTIFICATION AND CHARACTERIZATION OF AN INHIBITOR OF HEMATOPOIETIC STEM-CELL PROLIFERATION. *Nature,* 344**,** 442-444.

GRAY, L., STERJOVSKI, J., CHURCHILL, M., ELLERY, P., NASR, N., LEWIN, S. R., CROWE, S. M., WESSELINGH, S. L., CUNNINGHAM, A. L. & GORRY, P. R. (2005) Uncoupling coreceptor usage of human immunodeficiency virus type 1 (HIV-1) from macrophage tropism reveals biological properties of CCR5-restricted HIV-1 isolates from patients with acquired immunodeficiency syndrome. *Virology,* 337**,** 384-398.

GREENE, C. M. & MCELVANEY, N. G. (2009) Proteases and antiproteases in chronic neutrophilic lung disease - relevance to drug discovery. *British Journal of Pharmacology,* 158**,** 1048-1058.

GUAN, E., WANG, J. H., RODERIQUEZ, G. & NORCROSS, M. A. (2002) Natural truncation of the chemokine MIP-1 beta/CCL4 affects receptor specificity but not Anti-HIV-1 activity. *Journal of Biological Chemistry,* 277**,** 32348-32352.

GUNTHER, K., LEIER, J., HENNING, G., DIMMLER, A., WEISSBACH, R., HOHENBERGER, W. & FORSTER, R. (2005) Prediction of lymph node metastasis in colorectal carcinoma by expression of chemokine receptor CCR7. *International Journal of Cancer,* 116**,** 726-733.

GUPTA, S. K., PILLARISETTI, K., GRAY, S. L. & STADEL, J. M. (1998) Molecular cloning of a novel chemokine receptor-like gene from early stage chick embryos. *Biochemistry and Molecular Biology International,* 44**,** 673-681.

GUTIERREZ, J., KREMER, L., ZABALLOS, A., GOYA, I., MARTINEZ, C. & MARQUEZ, G. (2004) Analysis of post-translational CCR8 modifications and their influence on receptor activity. *Journal of Biological Chemistry,* 279**,** 14726-14733.

HAAS, C. S., MARTINEZ, R. J., ATTIA, N., HAINES, G. K., CAMPBELL, P. L. & KOCH, A. E. (2005) Chemokine receptor expression in rat adjuvant-induced arthritis. *Arthritis and Rheumatism,* 52**,** 3718-3730.

HAESSLER, U., PISANO, M., WU, M. M. & SWARTZ, M. A. (2011) Dendritic cell chemotaxis in 3D under defined chemokine gradients reveals differential response

to ligands CCL21 and CCL19. *Proceedings of the National Academy of Sciences of the United States of America,* 108**,** 5614-5619.

HAN, K. H., GREEN, S. R., TANGIRALA, R. K., TANAKA, S. & QUEHENBERGER, O. (1999) Role of the first extracellular loop in the functional activation of CCR2 - The first extracellular loop contains distinct domains necessary for both agonist binding and transmembrane signaling. *Journal of Biological Chemistry,* 274**,** 32055-32062.

HANAHAN, D. & WEINBERG, R. A. (2000) The hallmarks of cancer. *Cell,* 100**,** 57-70.

HANDEL, T. M., JOHNSON, Z., CROWN, S. E., LAU, E. K., SWEENEY, M. & PROUDFOOT, A. E. (2005) Regulation of protein function by glycosaminoglycans - as exemplified by chemokines. *Annual Review of Biochemistry,* 74**,** 385-410.

HANDEL, T. M., JOHNSON, Z., RODRIGUES, D. H., DOS SANTOS, A. C., CIRILLO, R., MUZIO, V., RIVA, S., MACK, M., DERUAZ, M., BORLAT, F., VITTE, P. A., WELLS, T. N. C., TEIXEIRA, M. M. & PROUDFOOT, A. E. I. (2008) An engineered monomer of CCL2 has anti-inflammatory properties emphasizing the importance of oligomerization for chemokine activity in vivo. *Journal of Leukocyte Biology,* 84**,** 1101-1108.

HANSELL, C. A. H., SCHIERING, C., KINSTRIE, R., FORD, L., BORDON, Y., MCINNES, I. B., GOODYEAR, C. S. & NIBBS, R. J. B. (2011) Universal expression and dual function of the atypical chemokine receptor D6 on innate-like B cells in mice. *Blood,* 117**,** 5413-5424.

HARTL, D., LATZIN, P., HORDIJK, P., MARCOS, V., RUDOLPH, C., WOISCHNIK, M., KRAUSS-ETSCHMANN, S., KOLLER, B., REINHARDT, D., ROSCHER, A. A., ROOS, D. & GRIESE, M. (2007) Cleavage of CXCR1 on neutrophils disables bacterial killing in cystic fibrosis lung disease. *Nature Medicine,* 13**,** 1423-1430.

HARTLEY, O., KLASSE, P. J., SATTENTAU, Q. J. & MOORE, J. P. (2005) V3: HIV's switch-hitter. *Aids Research and Human Retroviruses,* 21**,** 171-189.

HASKELL, C. A., CLEARY, M. D. & CHARO, I. F. (1999) Molecular uncoupling of fractalkine-mediated cell adhesion and signal transduction - Rapid flow arrest of CX(3)CR1-expressing cells is independent of G-protein activation. *Journal of Biological Chemistry,* 274**,** 10053-10058.

HELBIG, G., CHRISTOPHERSON, K. W., BHAT-NAKSHATRI, P., KUMAR, S., KISHIMOTO, H., MILLER, K. D., BROXMEYER, H. E. & NAKSHATRI, H. (2003) NF-kappa B promotes breast cancer cell migration and metastasis by inducing the expression of the chemokine receptor CXCR4. *Journal of Biological Chemistry,* 278**,** 21631-21638.

HEMMERICH, S., PAAVOLA, C., BLOOM, A., BHAKTA, S., FREEDMAN, R., GRUNBERGER, D., KRSTENANSKY, J., LEE, S., MCCARLEY, D., MULKINS, M., WONG, B., PEASE, J., MIZOUE, L., MIRZADEGAN, T., POLSKY, I., THOMPSON, K., HANDEL, T. M. & JARNAGIN, K. (1999) Identification of residues in the monocyte chemotactic protein-1 that contact the MCP-1 receptor, CCR2. *Biochemistry,* 38**,** 13013-13025.

HENDRIX, C. W., COLLIER, A. C., LEDERMAN, M. M., SCHOLS, D., POLLARD, R. B., BROWN, S., JACKSON, J. B., COOMBS, R. W., GLESHY, M. J., FLEXNER, C. W., BRIDGER, G. J., BADEL, K., MACFARLAND, R. T., HENSON, G. W., CALANDRA, G. & GRP, A. H. S. (2004) Safety, pharmacokinetics, and antiviral activity of AMD3100, a selective CXCR4 receptor inhibitor, in HIV-1 infection. *Jaids-Journal of Acquired Immune Deficiency Syndromes,* 37**,** 1253-1262.

HEWIT, K. D., FRASER, A., NIBBS, R. J. & GRAHAM, G. J. (2014) The N-terminal region of the atypical chemokine receptor ACKR2 is a key determinant of ligand binding. *J Biol Chem.*

HIROTA, K., YOSHITOMI, H., HASHIMOTO, M., MAEDA, S., TERADAIRA, S., SUGIMOTO, N., YAMAGUCHI, T., NOMURA, T., ITO, H., NAKAMURA, T., SAKAGUCHI, N. & SAKAGUCHI, S. (2007) Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. *Journal of Experimental Medicine,* 204**,** 2803-2812.

HOFFHINES, A. J., DAMOC, E., BRIDGES, K. G., LEARY, J. A. & MOORE, K. L. (2006) Detection and purification of tyrosine-sulfated proteins using a novel anti-sulfotyrosine monoclonal antibody. *Journal of Biological Chemistry,* 281**,** 37877-37887.

HOLST, P. J., LUTTICHAU, H. R., SCHWARTZ, T. W. & ROSENKILDE, M. M. (2003) Virally encoded chemokines and chemokine receptors in the role of viral infections. *Contributions to microbiology,* 10**,** 232-52.

HOMEY, B., ALENIUS, H., MULLER, A., SOTO, H., BOWMAN, E. P., YUAN, W., MCEVOY, L., LAUERMA, A. I., ASSMANN, T., BUNEMANN, E., LEHTO, M., WOLFF, H., YEN, D., MARXHAUSEN, H., TO, W., SEDGWICK, J., RUZICKA, T., LEHMANN, P. & ZLOTNIK, A. (2002) CCL27-CCR10 interactions regulate T cell-mediated skin inflammation. *Nature Medicine,* 8**,** 157-165.

HOMEY, B., DIEU-NOSJEAN, M. C., WIESENBORN, A., MASSACRIER, C., PIN, J. J., OLDHAM, E., CATRON, D., BUCHANAN, M. E., MULLER, A., MALEFYT, R. D., DENG, G., OROZCO, R., RUZICKA, T., LEHMANN, P., LEBECQUE, S., CAUX, C. & ZLOTNIK, A. (2000) Up-regulation of macrophage inflammatory protein-3 alpha/CCL20 and CC chemokine receptor 6 in psoriasis. *Journal of Immunology,* 164**,** 6621-6632.

HOPKINS, A. L. & GROOM, C. R. (2002) The druggable genome. *Nature Reviews Drug Discovery,* 1**,** 727-730.

HORUK, R. (2009) OPINION Chemokine receptor antagonists: overcoming developmental hurdles. *Nature Reviews Drug Discovery,* 8**,** 23-33.

HORUK, R., CHITNIS, C. E., DARBONNE, W. C., COLBY, T. J., RYBICKI, A., HADLEY, T. J. & MILLER, L. H. (1993) A RECEPTOR FOR THE MALARIAL PARASITE PLASMODIUM-VIVAX - THE ERYTHROCYTE CHEMOKINE RECEPTOR. *Science,* 261**,** 1182-1184.

HOU, T., LIANG, D., XU, L., HUANG, X., HUANG, Y. & ZHANG, Y. (2013) Atypical chemokine receptors predict lymph node metastasis and prognosis in patients with cervical squamous cell cancer. *Gynecologic oncology,* 130**,** 181-7.

HOWARD, O. M. Z., DONG, H. F., SU, S. B., CASPI, R. R., CHEN, X., PLOTZ, P. & OPPENHEIM, J. J. (2005) Autoantigens signal through chemokine receptors: uveitis antigens induce CXCR3- and CXCR5-expressing lymphocytes and immature dendritic cells to migrate. *Blood,* 105**,** 4207-4214.

HUISING, M. O., STET, R. J. M., KRUISWIJK, C. P., SAVELKOUL, H. F. J. & VERBURG-VAN KEMENADE, B. M. L. (2003) Molecular evolution of CXC chemokines: extant CXC chemokines originate from the CNS. *Trends in Immunology,* 24**,** 307-313.

ISSAFRAS, H., ANGERS, S., BULENGER, S., BLANPAIN, C., PARMENTIER, M., LABBE-JULLIE, C., BOUVIER, M. & MARULLO, S. (2002) Constitutive agonist-independent CCR5 oligomerization and antibody-mediated clustering occurring at physiological levels of receptors. *Journal of Biological Chemistry,* 277**,** 34666-34673.

JAMIESON, T., COOK, D. N., NIBBS, R. J. B., ROT, A., NIXON, C., MCLEAN, P., ALCAMI, A., LIRA, S. A., WIEKOWSKI, M. & GRAHAM, G. J. (2005) The chemokine receptor D6 limits the inflammatory response in vivo. *Nature Immunology,* 6**,** 403-411.

JILMA-STOHLAWETZ, P., HOMONCIK, M., DRUCKER, C., MARSIK, C., ROT, A., MAYR, W. R., SEIBOLD, B. & JILMA, B. (2001) Fy phenotype and gender

determine plasma levels of monocyte chemotactic protein. *Transfusion,* 41**,** 378-381.

JOHN, S., SMITH, S., MORRISON, J. F., SYMMONS, D., WORTHINGTON, J., SILMAN, A. & BARTON, A. (2003) Genetic variation in CCR5 does not predict clinical outcome in inflammatory arthritis. *Arthritis and Rheumatism,* 48**,** 3615-3616.

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. C. & PROUDFOOT, A. E. I. (2004) Interference with heparin binding and oligomerization creates a novel anti-inflammatory strategy targeting the chemokine system. *Journal of Immunology,* 173**,** 5776-5785.

JOHNSON, Z., PROUDFOOT, A. E. & 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 Reviews,* 16**,** 625-636.

JOYCE, J. A. & POLLARD, J. W. (2009) Microenvironmental regulation of metastasis. *Nature Reviews Cancer,* 9**,** 239-252.

KATADA, T. (2012) The Inhibitory G Protein G(i) Identified as Pertussis Toxin-Catalyzed ADP-Ribosylation. *Biological & Pharmaceutical Bulletin,* 35**,** 2103-2111.

KAWAMURA, T., STEPHENS, B., QIN, L., YIN, X., DORES, M. R., SMITH, T. H., GRIMSEY, N., ABAGYAN, R., TREJO, J., KUFAREVA, I., FUSTER, M. M., SALANGA, C. L. & HANDEL, T. M. (2014) A General Method for Site Specific Fluorescent Labeling of Recombinant Chemokines. *Plos One,* 9**,** 10.

KEHOE, J. W. & BERTOZZI, C. R. (2000) Tyrosine sulfation: a modulator of extracellular protein-protein interactions. *Chemistry & Biology,* 7**,** R57-R61.

KNAUT, H., WERZ, C., GEISLER, R., NUSSLEIN-VOLHARD, C. & TUBINGEN SCREEN, C. (2003) A zebrafish homologue of the chemokine receptor Cxcr4 is a germ-cell guidance receptor. *Nature,* 421**,** 279-282.

KOCH, A. E., KUNKEL, S. L., HARLOW, L. A., JOHNSON, B., EVANOFF, H. L., HAINES, G. K., BURDICK, M. D., POPE, R. M. & STRIETER, R. M. (1992) ENHANCED PRODUCTION OF MONOCYTE CHEMOATTRACTANT PROTEIN-1 IN RHEUMATOID-ARTHRITIS. *Journal of Clinical Investigation,* 90**,** 772-779.

KOCHI, Y., OKADA, Y., SUZUKI, A., IKARI, K., TERAO, C., TAKAHASHI, A., YAMAZAKI, K., HOSONO, N., MYOUZEN, K., TSUNODA, T., KAMATANI, N., FURUICHI, T., IKEGAWA, S., OHMURA, K., MIMORI, T., MATSUDA, F., IWAMOTO, T., MOMOHARA, S., YAMANAKA, H., YAMADA, R., KUBO, M., NAKAMURA, Y. & YAMAMOTO, K. (2010) A regulatory variant in CCR6 is associated with rheumatoid arthritis susceptibility. *Nature Genetics,* 42**,** 515-U63.

KOENEN, R. R., VON HUNDELSHAUSEN, P., NESMELOVA, I. V., ZERNECKE, A., LIEHN, E. A., SARABI, A., KRAMP, B. K., PICCININI, A. M., PALUDAN, S. R., KOWALSKA, M. A., KUNGL, A. J., HACKENG, T. M., MAYO, K. H. & WEBER, C. (2009) Disrupting functional interactions between platelet chemokines inhibits atherosclerosis in hyperlipidemic mice. *Nature Medicine,* 15**,** 97-103.

KOLTSOVA, E. & LEY, K. (2009) Tyrosine Sulfation of Leukocyte Adhesion Molecules and Chemokine Receptors Promotes Atherosclerosis. *Arteriosclerosis Thrombosis and Vascular Biology,* 29**,** 1709-1711.

KOOPMANN, W. & KRANGEL, M. S. (1997) Identification of a glycosaminoglycan-binding site in chemokine macrophage inflammatory protein-1 alpha. *Journal of Biological Chemistry,* 272**,** 10103-10109.

KOZIEL, J., MACIAG-GUDOWSKA, A., MIKOLAJCZYK, T., BZOWSKA, M., STURDEVANT, D. E., WHITNEY, A. R., SHAW, L. N., DELEO, F. R. & POTEMPA, J. (2009) Phagocytosis of Staphylococcus aureus by Macrophages Exerts Cytoprotective Effects Manifested by the Upregulation of Antiapoptotic Factors. *Plos One,* 4**,** 14.

KRIEGOVA, E., TSYRULNYK, A., ARAKELYAN, A., MRAZEK, F., ORDELTOVA, M., PETZMANN, S., ZATLOUKAL, J., KOLEK, V., DU BOIS, R. M., POPPER, H. & PETREK, M. (2006) Expression of CCX CKR in pulmonary sarcoidosis. *Inflammation Research,* 55**,** 441-445.

KRUEGER, J. G. (2002) The immunologic basis for the treatment of psoriasis with new biologic agents. *Journal of the American Academy of Dermatology,* 46**,** 1-23.

KUBICA, M., GUZIK, K., KOZIEL, J., ZAREBSKI, M., RICHTER, W., GAJKOWSKA, B., GOLDA, A., MACIAG-GUDOWSKA, A., BRIX, K., SHAW, L., FOSTER, T. & POTEMPA, J. (2008) A Potential New Pathway for Staphylococcus aureus Dissemination: The Silent Survival of S. aureus Phagocytosed by Human Monocyte-Derived Macrophages. *Plos One,* 3**,** 16.

KUCIA, M., RECA, R., MIEKUS, K., WANZECK, J., WOJAKOWSKI, W., JANOWSKA-WIECZOREK, A., RATAJCZAK, J. & RATAJCZAK, M. Z. (2005) Trafficking of normal stem cells and metastasis of cancer stem cells involve similar mechanisms: Pivotal role of the SDF-1-CXCR4 axis. *Stem Cells,* 23**,** 879-894.

KUSCHERT, G. S. V., COULIN, F., POWER, C. A., PROUDFOOT, A. E. I., HUBBARD, R. E., HOOGEWERF, A. J. & WELLS, T. N. C. (1999) Glycosaminoglycans interact selectively with chemokines and modulate receptor binding and cellular responses. *Biochemistry,* 38**,** 12959-12968.

LAARMAN, A. J., MIJNHEER, G., MOOTZ, J. M., VAN ROOIJEN, W. J. M., RUYKEN, M., MALONE, C. L., HEEZIUS, E. C., WARD, R., MILLIGAN, G., VAN STRIJP, J. A. G., DE HAAS, C. J. C., HORSWILL, A. R., VAN KESSEL, K. P. M. & ROOIJAKKERS, S. H. M. (2012) Staphylococcus aureus Staphopain A inhibits CXCR2-dependent neutrophil activation and chemotaxis. *Embo Journal,* 31**,** 3607-3619.

LAINE, R. A. (1994) A CALCULATION OF ALL POSSIBLE OLIGOSACCHARIDE ISOMERS BOTH BRANCHED AND LINEAR YIELDS 1.05X10(12) STRUCTURES FOR A REDUCING HEXASACCHARIDE - THE ISOMER-BARRIER TO DEVELOPMENT OF SINGLE-METHOD SACCHARIDE SEQUENCING OR SYNTHESIS SYSTEMS. *Glycobiology,* 4**,** 759-767.

LAPIDOT, T. & PETIT, I. (2002) Current understanding of stem cell mobilization: The roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. *Experimental Hematology,* 30**,** 973-981.

LAU, E. K., PAAVOLA, C. D., JOHNSON, Z., GAUDRY, J. P., GERETTI, E., BORLAT, F., KUNGL, A. J., PROUDFOOT, A. E. & HANDEL, T. M. (2004) Identification of the glycosaminoglycan binding site of the CC chemokine, MCP-1 - Implications for structure and function in vivo. *Journal of Biological Chemistry,* 279**,** 22294-22305.

LAX, P., LIMATOLA, C., FUCILE, S., TRETTEL, F., DI BARTOLOMEO, S., RENZI, M., RAGOZZINO, D. & EUSEBI, F. (2002) Chemokine receptor CXCR2 regulates the functional properties of AMPA-type glutamate receptor GluR 1 in HEK cells. *Journal of Neuroimmunology,* 129**,** 66-73.

LEBRE, M. C., VERGUNST, C. E., CHOI, I. Y. K., AARRASS, S., OLIVEIRA, A. S. F., WYANT, T., HORUK, R., REEDQUIST, K. A. & TAK, P. P. (2011) Why CCR2 and CCR5 Blockade Failed and Why CCR1 Blockade Might Still Be Effective in the Treatment of Rheumatoid Arthritis. *Plos One,* 6**,** 7.

LEE, B., SHARRON, M., BLANPAIN, C., DORANZ, B. J., VAKILI, J., SETOH, P., BERG, E., LIU, G., GUY, H. R., DURELL, S. R., PARMENTIER, M., CHANG,

C. N., PRICE, K., TSANG, M. & DOMS, R. W. (1999) Epitope mapping of CCR5 reveals multiple conformational states and distinct but overlapping structures involved in chemokine and coreceptor function. *Journal of Biological Chemistry,* 274**,** 9617-9626.

LEE, K. M., MCKIMMIE, C. S., GILCHRIST, D. S., PALLAS, K. J., NIBBS, R. J., GARSIDE, P., MCDONALD, V., JENKINS, C., RANSOHOFF, R., LIU, L. P., MILLING, S., CEROVIC, V. & GRAHAM, G. J. (2011) D6 facilitates cellular migration and fluid flow to lymph nodes by suppressing lymphatic congestion. *Blood,* 118**,** 6220-6229.

LEE, K. M., NIBBS, R. J. B. & GRAHAM, G. J. (2013a) D6: the 'crowd controller' at the immune gateway. *Trends in Immunology,* 34**,** 7-12.

LEE, Y. H., BAE, S. C. & SONG, G. G. (2013b) Association between the chemokine receptor 5 delta32 polymorphism and rheumatoid arthritis: a meta-analysis. *Modern Rheumatology,* 23**,** 304-310.

LEI, Y. & TAKAHAMA, Y. (2012) XCL1 and XCR1 in the immune system. *Microbes and Infection,* 14**,** 262-267.

LEVER, R. & PAGE, C. R. (2002) Novel drug development opportunities for heparin. *Nature Reviews Drug Discovery,* 1**,** 140-148.

LEVESQUE, J. P., HENDY, J., TAKAMATSU, Y., SIMMONS, P. J. & BENDALL, L. J. (2003) Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by GCSF or cyclophosphamide. *Journal of Clinical Investigation,* 111**,** 187-196.

LEVESQUE, J. P., LIU, F., SIMMONS, P. J., BETSUYAKU, T., SENIOR, R. M., PHAM, C. & LINK, D. C. (2004) Characterization of hematopoietic progenitor mobilization in protease-deficient mice. *Blood,* 104**,** 65-72.

LEVOYE, A., BALABANIAN, K., BALEUX, F., BACHELERIE, F. & LAGANE, B. (2009) CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling. *Blood,* 113**,** 6085-6093.

LIDER, O., BAHARAV, E., MEKORI, Y. A., MILLER, T., NAPARSTEK, Y., VLODAVSKY, I. & COHEN, I. R. (1989) SUPPRESSION OF EXPERIMENTAL AUTOIMMUNE-DISEASES AND PROLONGATION OF ALLOGRAFT SURVIVAL BY TREATMENT OF ANIMALS WITH LOW-DOSES OF HEPARINS. *Journal of Clinical Investigation,* 83**,** 752-756.

LINDNER, E., NORDANG, G. B. N., MELUM, E., FLATO, B., SELVAAG, A. M., THORSBY, E., KVIEN, T. K., FORRE, O. T. & LIE, B. A. (2007) Lack of association between the chemokine receptor 5 polymorphism CCR5delta32 in rheumatoid arthritis and juvenile idiopathic arthritis. *Bmc Medical Genetics,* 8**,** 5.

LIU, J., LOUIE, S., HSU, W., YU, K. M., NICHOLAS, H. B. & ROSENQUIST, G. L. (2008) Tyrosine sulfation is prevalent in human chemokine receptors important in lung disease. *American Journal of Respiratory Cell and Molecular Biology,* 38**,** 738-743.

LIU, R., PAXTON, W. A., CHOE, S., CERADINI, D., MARTIN, S. R., HORUK, R., MACDONALD, M. E., STUHLMANN, H., KOUP, R. A. & LANDAU, N. R. (1996) Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell,* 86**,** 367-377.

LOCATI, M., TORRE, Y. M. D. L., GALLIERA, E., BONECCHI, R., BODDULURI, H., VAGO, G., VECCHI, A. & MANTOVANI, A. (2005) Silent chemoattractant receptors: D6 as a decoy and scavenger receptor for inflammatory CC chemokines. *Cytokine & Growth Factor Reviews,* 16**,** 679-686.

LOOS, T., MORTIER, A., GOUWY, M., RONSSE, I., PUT, W., LENAERTS, J. P., VAN DAMME, J. & 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**,** 2648-2656.

LOVE, M., SANDBERG, J. L., ZIAREK, J. J., GERARDEN, K. P., RODE, R. R., JENSEN, D. R., MCCASLIN, D. R., PETERSON, F. C. & VELDKAMP, C. T. (2012) Solution Structure of CCL21 and Identification of a Putative CCR7 Binding Site. *Biochemistry,* 51**,** 733-735.

LUDWIG, A., EHLERT, J. E., FLAD, H. D. & BRANDT, E. (2000) Identification of distinct surface-expressed and intracellular CXC-chemokine receptor 2 glycoforms in neutrophils: N-glycosylation is essential for maintenance of receptor surface expression. *Journal of Immunology,* 165**,** 1044-1052.

LUKACS, N. W. & HARRISON, J. K. (2007) *The birth and maturation of chemokines and their receptors,* Humana Press Inc.

LUKER, K. E., STEELE, J. M., MIHALKO, L. A., RAY, P. & LUKER, G. D. (2010) Constitutive and chemokine-dependent internalization and recycling of CXCR7 in breast cancer cells to degrade chemokine ligands. *Oncogene,* 29**,** 4599-4610.

LUROSS, J. A. & WILLIAMS, N. A. (2001) The genetic and immunopathological processes underlying collagen-induced arthritis. *Immunology,* 103**,** 407-416.

LUSTER, A. D. (1998) Chemokines - Chemotactic cytokines that mediate inflammation. *New England Journal of Medicine,* 338**,** 436-445.

LUTHER, S. A., BIDGOL, A., HARGREAVES, D. C., SCHMIDT, A., XU, Y., PANIYADI, J., MATLOUBIAN, M. & CYSTER, J. G. (2002) Differing activities of homeostatic chemokines CCL19, CCL21, and CXCL12 in lymphocyte and dendritic cell recruitment and lymphoid neogenesis. *Journal of Immunology,* 169**,** 424-433.

LUTTICHAU, H. R., STINE, J., BOESEN, T. P., JOHNSEN, A. H., CHANTRY, D., GERSTOFT, J. & SCHWARTZ, T. W. (2000) A highly selective CC chemokine receptor (CCR)8 antagonist encoded by the poxvirus molluscum contagiosum. *Journal of Experimental Medicine,* 191**,** 171-179.

MA, Q., JONES, D., BORGHESANI, P. R., SEGAL, R. A., NAGASAWA, T., KISHIMOTO, T., BRONSON, R. T. & SPRINGER, T. A. (1998) Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America,* 95**,** 9448-9453.

MADIGAN, J., FREEMAN, D. J., MENZIES, F., FORROW, S., NELSON, S. M., YOUNG, A., SHARKEY, A., MOFFET, A., GRAHAM, G. J., GREER, I. A., ROT, A. & NIBBS, R. J. B. (2010) Chemokine Scavenger D6 Is Expressed by Trophoblasts and Aids the Survival of Mouse Embryos Transferred into Allogeneic Recipients. *Journal of Immunology,* 184**,** 3202-3212.

MANTOVANI, A. (1999) The chemokine system: redundancy for robust outputs. *Immunology Today,* 20**,** 254-257.

MANTOVANI, A., BONECCHI, R. & LOCATI, M. (2006) Tuning inflammation and immunity by chemokine sequestration: decoys and more. *Nature Reviews Immunology,* 6**,** 907-918.

MARTINEZ DE LA TORRE, Y., LOCATI, M., BURACCHI, C., DUPOR, J., COOK, D. N., BONECCHI, R., NEBULONI, M., RUKAVINA, D., VAGO, L., VECCHI, A., LIRA, S. A. & MANTOVANI, A. (2005) Increased inflammation in mice deficient for the chemokine decoy receptor D6. *European Journal of Immunology,* 35**,** 1342-6.

MASHINO, K., SADANAGA, N., YAMAGUCHI, H., TANAKA, F., OHTA, M., SHIBUTA, K., INOUE, H. & MORI, M. (2002) Expression of chemokine receptor CCR7 is associated with lymph node metastasis of gastric carcinoma. *Cancer Research,* 62**,** 2937-2941.

MATLOUBIAN, M., DAVID, A., ENGEL, S., RYAN, J. E. & CYSTER, J. G. (2000) A transmembrane CXC chemokine is a ligand for HIV-coreceptor Bonzo. *Nature Immunology,* 1**,** 298-304.

MCCULLOCH, C. V., MORROW, V., MILASTA, S., COMERFORD, I., MILLIGAN, G., GRAHAM, G. J., ISAACS, N. W. & NIBBS, R. J. B. (2008) Multiple roles for the C-terminal tail of the chemokine scavenger D6. *Journal of Biological Chemistry,* 283**,** 7972-7982.

MCKIMMIE, C. S., FRASER, A. R., HANSELL, C., GUTIERREZ, L., PHILIPSEN, S., CONNELL, L., ROT, A., KUROWSKA-STOLARSKA, M., CARRENO, P., PRUENSTER, M., CHU, C. C., LOMBARDI, G., HALSEY, C., MCINNES, L. B., LIEW, F. Y., NIBBS, R. J. & GRAHAM, G. J. (2008) Hemopoietic cell expression of the chemokine decoy receptor D6 is dynamic and regulated by GATA1. *Journal of Immunology,* 181**,** 3353-3363.

MCKIMMIE, C. S. & GRAHAM, G. J. (2006) Leucocyte expression of the chemokine scavenger D6. *Biochemical Society Transactions,* 34**,** 1002-1004.

MCKIMMIE, C. S., SINGH, M. D., HEWIT, K., LOPEZ-FRANCO, O., LE BROCQ, M., ROSE-JOHN, S., LEE, K. M., BAKER, A. H., WHEAT, R., BLACKBOURN, D. J., NIBBS, R. J. B. & GRAHAM, G. J. (2013) An analysis of the function and expression of D6 on lymphatic endothelial cells. *Blood,* 121**,** 3768-77.

MCLEAN, K. A., HOLST, P. J., MARTINI, L., SCHWARTZ, T. W. & ROSENKILDE, M. M. (2004) Similar activation of signal transduction pathways by the herpesvirus-encoded chemokine receptors US28 and ORF74. *Virology,* 325**,** 241-251.

MCQUIBBAN, G. A., GONG, J. H., TAM, E. M., MCCULLOCH, C. A. G., CLARK-LEWIS, I. & OVERALL, C. M. (2000) Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. *Science,* 289**,** 1202-1206.

MENARD, D., BARNADAS, C., BOUCHIER, C., HENRY-HALLDIN, C., GRAY, L. R., RATSIMBASOA, A., THONIER, V., CAROD, J. F., DOMARLE, O., COLIN, Y., BERTRAND, O., PICOT, J., KING, C. L., GRIMBERG, B. T., MERCEREAU-PUIJALON, O. & ZIMMERMAN, P. A. (2010) Plasmodium vivax clinical malaria is commonly observed in Duffy-negative Malagasy people. *Proceedings of the National Academy of Sciences of the United States of America,* 107**,** 5967-5971.

MIAO, Z., LUKER, K. E., SUMMERS, B. C., BERAHOVICH, R., BHOJANI, M. S., REHEMTULLA, A., KLEER, C. G., ESSNER, J. J., NASEVICIUS, A., LUKER, G. D., HOWARD, M. C. & SCHALL, T. J. (2007) CXCR7 (RDC1) promotes breast and lung tumor growth in vivo and is expressed on tumor-associated vasculature. *Proceedings of the National Academy of Sciences of the United States of America,* 104**,** 15735-15740.

MILLER, L. H., MASON, S. J., DVORAK, J. A., MCGINNISS, M. H. & ROTHMAN, I. K. (1975) ERYTHROCYTE RECEPTORS FOR (PLASMODIUM-KNOWLESI) MALARIA - DUFFY BLOOD-GROUP DETERMINANTS. *Science,* 189**,** 561-563.

MISHIRO, E., SAKAKIBARA, Y., LIU, M. C. & SUIKO, M. (2006) Differential enzymatic characteristics and tissue-specific expression of human TPST-1 and TPST-2. *Journal of Biochemistry,* 140**,** 731-737.

MITHAL, D. S., BANISADR, G. & MILLER, R. J. (2012) CXCL12 Signaling in the Development of the Nervous System. *Journal of Neuroimmune Pharmacology,* 7**,** 820-834.

MIZOUE, L. S., BAZAN, J. F., JOHNSON, E. C. & HANDEL, T. M. (1999) Solution structure and dynamics of the CX3C chemokine domain of fractalkine and its interaction with an N-terminal fragment of CX(3)CR1. *Biochemistry,* 38**,** 1402-1414.

MOLYNEAUX, K. A., ZINSZNER, H., KUNWAR, P. S., SCHAIBLE, K., STEBLER, J., SUNSHINE, M. J., O'BRIEN, W., RAZ, E., LITTMAN, D., WYLIE, C. & LEHMANN, R. (2003) The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival. *Development,* 130**,** 4279-4286.

MONIGATTI, F., HEKKING, B. & STEEN, H. (2006) Protein sulfation analysis - A primer. *Biochimica Et Biophysica Acta-Proteins and Proteomics,* 1764**,** 1904-1913.

MONTECLARO, F. S. & CHARO, I. F. (1996) The amino-terminal extracellular domain of the MCP-1 receptor, but not the RANTES/MIP-1 alpha receptor, confers chemokine selectivity - Evidence for a two-step mechanism for MCP-1 receptor activation. *Journal of Biological Chemistry,* 271**,** 19084-19092.

MONTECLARO, F. S. & CHARO, I. F. (1997) The amino-terminal domain of CCR2 is both necessary and sufficient for high affinity binding of monocyte chemoattractant protein 1 - Receptor activation by a pseudo-tethered ligand. *Journal of Biological Chemistry,* 272**,** 23186-23190.

MOORE, J. P., KITCHEN, S. G., PUGACH, P. & ZACK, J. A. (2004) The CCR5 and CXCR4 coreceptors - Central to understanding the transmission and pathogenesis of human immunodeficiency virus type 1 infection. *Aids Research and Human Retroviruses,* 20**,** 111-126.

MORTIER, A., GOUWY, M., VAN DAMME, J. & PROOST, P. (2011) Effect of posttranslational processing on the in vitro and in vivo activity of chemokines. *Experimental Cell Research,* 317**,** 642-654.

MORTIER, A., LOOS, T., GOUWY, M., RONSSE, I., VAN DAMME, J. & PROOST, P. (2010) Posttranslational Modification of the NH2-terminal Region of CXCL5 by Proteases or Peptidylarginine Deiminases (PAD) Differently Affects Its Biological Activity. *Journal of Biological Chemistry,* 285**,** 29750-29759.

MOSER, B., WOLF, M., WALZ, A. & LOETSCHER, P. (2004) Chemokines: multiple levels of leukocyte migration control. *Trends in Immunology,* 25**,** 75-84.

MULLER, A., HOMEY, B., SOTO, H., GE, N. F., CATRON, D., BUCHANAN, M. E., MCCLANAHAN, T., MURPHY, E., YUAN, W., WAGNER, S. N., BARRERA, J. L., MOHAR, A., VERASTEGUI, E. & ZLOTNIK, A. (2001) Involvement of chemokine receptors in breast cancer metastasis. *Nature,* 410**,** 50-56.

MURAKAMI, T., MAKI, W., CARDONES, A. R., FANG, H., KYI, A. T., NESTLE, F. O. & HWANG, S. T. (2002) Expression of CXC chemokine receptor-4 enhances the pulmonary metastatic potential of murine B16 melanoma cells. *Cancer Research,* 62**,** 7328-7334.

MURPHY, P. M., BAGGIOLINI, M., CHARO, I. F., HEBERT, C. A., HORUK, R., MATSUSHIMA, K., MILLER, L. H., OPPENHEIM, J. J. & POWER, C. A. (2000) International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacological Reviews,* 52**,** 145-176.

NAGASAWA, T., HIROTA, S., TACHIBANA, K., TAKAKURA, N., NISHIKAWA, S., KITAMURA, Y., YOSHIDA, N., KIKUTANI, H. & KISHIMOTO, T. (1996) Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature,* 382**,** 635-638.

NAGASAWA, T., TACHIBANA, K. & KISHIMOTO, T. (1998) A novel CXC chemokine PBSF/SDF-1 and its receptor CXCR4: their functions in development, hematopoiesis and HIV infection. *Seminars in Immunology,* 10**,** 179-185.

NAKAMURA, K., WILLIAMS, I. R. & KUPPER, T. S. (1995) KERATINOCYTE-DERIVED MONOCYTE CHEMOATTRACTANT PROTEIN-1 (MCP-1) - ANALYSIS IN A TRANSGENIC MODEL DEMONSTRATES MCP-1 CAN RECRUIT DENDRITIC AND LANGERHANS CELLS TO SKIN. *Journal of Investigative Dermatology,* 105**,** 635-643.

NAUMANN, U., CAMERONI, E., PRUENSTER, M., MAHABALESHWAR, H., RAZ, E., ZERWES, H. G., ROT, A. & THELEN, M. (2010) CXCR7 Functions as a Scavenger for CXCL12 and CXCL11. *Plos One,* 5**,** 11.

NEEL, N. F., SCHUTYSER, E., SAI, J., FAN, G. H. & RICHMOND, A. (2005) Chemokine receptor internalization and intracellular trafficking. *Cytokine & Growth Factor Reviews,* 16**,** 637-658.

NEGUS, R. P. M., STAMP, G. W. H., HADLEY, J. & BALKWILL, F. R. (1997) Quantitative assessment of the leukocyte infiltrate in ovarian cancer and its relationship to the expression of C-C chemokines. *American Journal of Pathology,* 150**,** 1723-1734.

NEIL, S. J. D., AASA-CHAPMAN, M. M. I., CLAPHAM, P. R., NIBBS, R. J., MCKNIGHT, A. & WEISS, R. A. (2005) The promiscuous CC chemokine receptor D6 is a functional coreceptor for primary isolates of human immunodeficiency virus type 1 (HIV-1) and HIV-2 on astrocytes. *Journal of Virology,* 79**,** 9618-9624.

NIBBS, R., GRAHAM, G. & ROT, A. (2003) Chemokines on the move: control by the chemokine "interceptors" Duffy blood group antigen and D6. *Seminars in Immunology,* 15**,** 287-294.

NIBBS, R. J. B., GILCHRIST, D. S., KING, V., FERRA, A., FORROW, S., HUNTER, K. D. & GRAHAM, G. J. (2007) The atypical chemokine receptor D6 suppresses the development of chemically induced skin tumors. *Journal of Clinical Investigation,* 117**,** 1884-1892.

NIBBS, R. J. B. & GRAHAM, G. J. (2013) Immune regulation by atypical chemokine receptors. *Nature Reviews Immunology,* 13**,** 815-829.

NIBBS, R. J. B., KRIEHUBER, E., PONATH, P. D., PARENT, D., QIN, S. X., CAMPBELL, J. D. M., HENDERSON, A., KERJASCHKI, D., MAURER, D., GRAHAM, G. J. & ROT, A. (2001) The beta-chemokine receptor D6 is expressed by lymphatic endothelium and a subset of vascular tumors. *American Journal of Pathology,* 158**,** 867-877.

NIBBS, R. J. B., WYLIE, S. M., YANG, J. Y., LANDAU, N. R. & GRAHAM, G. J. (1997) Cloning and characterization of a novel promiscuous human beta-chemokine receptor D6. *Journal of Biological Chemistry,* 272**,** 32078-32083.

NIBBS, R. J. B., YANG, J. Y., LANDAU, N. R., MAO, J. H. & GRAHAM, G. J. (1999) LD78 beta, a non-allelic variant of human MIP-1 alpha (LD78 alpha), has enhanced receptor interactions and potent HIV suppressive activity. *Journal of Biological Chemistry,* 274**,** 17478-17483.

NICKOLOFF, B. J., XIN, H., NESTLE, F. O. & QIN, J. Z. (2007) The cytokine and chemokine network in psoriasis. *Clinics in Dermatology,* 25**,** 568-573.

NOMIYAMA, H., OSADA, N. & YOSHIE, O. (2013) Systematic classification of vertebrate chemokines based on conserved synteny and evolutionary history. *Genes to Cells,* 18**,** 1-16.

O'HAYRE, M., SALANGA, C. L., HANDEL, T. M. & HAMEL, D. J. (2010) Emerging concepts and approaches for chemokine-receptor drug discovery. *Expert Opinion on Drug Discovery,* 5**,** 1109-1122.

OGATA, H., TAKEYA, M., YOSHIMURA, T., TAKAGI, K. & TAKAHASHI, K. (1997) The role of monocyte chemoattractant protein-1 (MCP-1) in the pathogenesis of collagen-induced arthritis in rats. *Journal of Pathology,* 182**,** 106-114.

OLDHAM, W. M. & HAMM, H. E. (2008) Heterotrimeric G protein activation by G-protein-coupled receptors. *Nature Reviews Molecular Cell Biology,* 9**,** 60-71.

ORAVECZ, T., PALL, M., RODERIQUEZ, G., GORRELL, M. D., DITTO, M., NGUYEN, N. Y., BOYKINS, R., UNSWORTH, E. & NORCROSS, M. A. (1997) Regulation of the receptor specificity and function of the chemokine RANTES (regulated on activation, normal T cell expressed and secreted) by dipeptidyl peptidase IV (CD26)-mediated cleavage. *Journal of Experimental Medicine,* 186**,** 1865-1872.

OUYANG, Y. B., CRAWLEY, J. T. B., ASTON, C. E. & MOORE, K. L. (2002) Reduced body weight and increased postimplantation fetal death in tyrosylprotein sulfotransferase-1-deficient mice. *Journal of Biological Chemistry,* 277**,** 23781-23787.

PAAVOLA, C. D., HEMMERICH, S., GRUNBERGER, D., POLSKY, I., BLOOM, A., FREEDMAN, R., MULKINS, M., BHAKTA, S., MCCARLEY, D., WIESENT, L., WONG, B., JARNAGIN, K. & HANDEL, T. M. (1998) Monomeric monocyte chemoattractant protein-1 (MCP-1) binds and activates the MCP-1 receptor CCR2B. *Journal of Biological Chemistry,* 273**,** 33157-33165.

PARK, S. H., DAS, B. B., CASAGRANDE, F., TIAN, Y., NOTHNAGEL, H. J., CHU, M. N., KIEFER, H., MAIER, K., DE ANGELIS, A. A., MARASSI, F. M. & OPELLA, S. J. (2012) Structure of the chemokine receptor CXCR1 in phospholipid bilayers. *Nature,* 491**,** 779-+.

PARKS, W. C., WILSON, C. L. & LOPEZ-BOADO, Y. S. (2004) Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nature Reviews Immunology,* 4**,** 617-629.

PARRY, C. M., SIMAS, J. P., SMITH, V. P., STEWART, C. A., MINSON, A. C., EFSTATHIOU, S. & ALCAMI, A. (2000) A broad spectrum secreted chemokine binding protein encoded by a herpesvirus. *Journal of Experimental Medicine,* 191**,** 573-578.

PARSONAGE, G., FILER, A. D., HAWORTH, O., NASH, G. B., RAINGER, G. E., SALMON, M. & BUCKLEY, C. D. (2005) A stromal address code defined by fibroblasts. *Trends in Immunology,* 26**,** 150-156.

PEASE, J. E. & HORUK, R. (2014) Recent progress in the development of antagonists to the chemokine receptors CCR3 and CCR4. *Expert Opinion on Drug Discovery,* 9**,** 467-483.

PEETERS, M. C., VAN WESTEN, G. J. P., LI, Q. & IJZERMAN, A. P. (2011) Importance of the extracellular loops in G protein-coupled receptors for ligand recognition and receptor activation. *Trends in Pharmacological Sciences,* 32**,** 35-42.

PERCHERANCIER, Y., BERCHICHE, Y. A., SLIGHT, I., VOLKMER-ENGERT, R., TAMAMURA, H., FUJII, N., BOUVIER, M. & HEVEKER, N. (2005) Bioluminescence resonance energy transfer reveals ligand-induced conformational changes in CXCR4 homo- and heterodimers. *Journal of Biological Chemistry,* 280**,** 9895-9903.

PERCHERANCIER, Y., PLANCHENAULT, T., VALENZUELA-FERNANDEZ, A., VIRELIZIER, J. L., ARENZANA-SEISDEDOS, F. & BACHELERIE, F. (2001) Palmitoylation-dependent control of degradation, life span, and membrane expression of the CCR5 receptor. *Journal of Biological Chemistry,* 276**,** 31936-31944.

PERERA, G. K., DI MEGLIO, P. & NESTLE, F. O. (2012) Psoriasis. IN ABBAS, A. K., GALLI, S. J. & HOWLEY, P. M. (Eds.) *Annual Review of Pathology: Mechanisms of Disease, Vol 7.* Palo Alto, Annual Reviews.

PLATERZYBERK, C., HOOGEWERF, A. J., PROUDFOOT, A. E. I., POWER, C. A. & WELLS, T. N. C. (1997) Effect of a CC chemokine receptor antagonist on collagen induced arthritis in DBA/1 mice. *Immunology Letters,* 57**,** 117-120.

PRAHALAD, S. (2006) Negative association between the chemokine receptor CCR5-Delta 32 polymorphism and rheumatoid arthritis: a meta-analysis. *Genes and Immunity,* 7**,** 264-268.

PREOBRAZHENSKY, A. A., DRAGAN, S., KAWANO, T., GAVRILIN, M. A., GULINA, I. V., CHAKRAVARTY, L. & KOLATTUKUDY, P. E. (2000) Monocyte chemotactic protein-1 receptor CCR2B is a glycoprotein that has tyrosine sulfation in a conserved extracellular N-terminal region. *Journal of Immunology,* 165**,** 5295-5303.

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. & VAN DAMME, J. (2008) Citrullination of CXCL8 by

peptidylarginine deiminase alters receptor usage, prevents proteolysis, and dampens tissue inflammation. *Journal of Experimental Medicine,* 205**,** 2085-2097.

PROUDFOOT, A. E. I. (2002) Chemokine receptors: Multifaceted therapeutic targets. *Nature Reviews Immunology,* 2**,** 106-115.

PROUDFOOT, A. E. I. (2006) The biological relevance of chemokine-proteoglycan interactions. *Biochemical Society Transactions,* 34**,** 422-426.

PROUDFOOT, A. E. I., FRITCHLEY, S., BORLAT, F., SHAW, J. P., VILBOIS, F., ZWAHLEN, C., TRKOLA, A., MARCHANT, D., CLAPHAM, P. R. & WELLS, T. N. C. (2001) The BBXB motif of RANTES is the principal site for heparin binding and controls receptor selectivity. *Journal of Biological Chemistry,* 276**,** 10620-10626.

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. (2003) Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proceedings of the National Academy of Sciences of the United States of America,* 100**,** 1885-1890.

PRUENSTER, M., MUDDE, L., BOMBOSI, P., DIMITROVA, S., ZSAK, M., MIDDLETON, J., RICHMOND, A., GRAHAM, G. J., SEGERER, S., NIBBS, R. J. B. & ROT, A. (2009) The Duffy antigen receptor for chemokines transports chemokines and supports their promigratory activity. *Nature Immunology,* 10**,** 101-108.

QANBAR, R. & BOUVIER, M. (2003) Role of palmitoylation/depalmitoylation reactions in G-protein-coupled receptor function. *Pharmacology & Therapeutics,* 97**,** 1-33.

QIN, S. X., ROTTMAN, J. B., MYERS, P., KASSAM, N., WEINBLATT, M., LOETSCHER, M., KOCH, A. E., MOSER, B. & MACKAY, C. R. (1998) The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *Journal of Clinical Investigation,* 101**,** 746-754.

QUINONES, M. P., AHUJA, S. K., JIMENEZ, F., SCHAEFER, J., GARAVITO, E., RAO, A., CHENAUX, G., REDDICK, R. L., KUZIEL, W. A. & AHUJA, S. S. (2004) Experimental arthritis in CC chemokine receptor 2-null mice closely mimics severe human rheumatoid arthritis. *Journal of Clinical Investigation,* 113**,** 856-866.

RAJAGOPAL, S., KIM, J., AHN, S., CRAIG, S., LAM, C. M., GERARD, N. P., GERARD, C. & LEFKOWITZ, R. J. (2010) beta-arrestin- but not G protein-mediated signaling by the "decoy" receptor CXCR7. *Proceedings of the National Academy of Sciences of the United States of America,* 107**,** 628-632.

RAJAGOPALAN, L. & RAJARATHNAM, K. (2006) Structural basis of chemokine receptor function - A model for binding affinity and ligand selectivity. *Bioscience Reports,* 26**,** 325-339.

RATAJCZAK, M. Z., ZUBA-SURMA, E., KUCIA, M., RECA, R., WOJAKOWSKI, W. & RATAJCZAK, J. (2006) The pleiotropic effects of the SDF-1-CXCR4 axis in organogenesis, regeneration and tumorigenesis. *Leukemia,* 20**,** 1915-1924.

RAVINDRAN, A., SAWANT, K. V., SARMIENTO, J., NAVARRO, J. & RAJARATHNAM, K. (2013) Chemokine CXCL1 Dimer Is a Potent Agonist for the CXCR2 Receptor. *Journal of Biological Chemistry,* 288**,** 12244-12252.

REGOES, R. R. & BONHOEFFER, S. (2005) The HIV coreceptor switch: a population dynamical perspective. *Trends in Microbiology,* 13**,** 269-277.

RIBEIRO, S. & HORUK, R. (2007) *Pharmaceutical targeting of chemokine receptors,* Humana Press Inc.

RODRIGUEZ-FRADE, J. M., DEL REAL, G., SERRANO, A., HERNANZ-FALCON, P., SORIANO, S. F., VILA-CORO, A. J., DE ANA, A. M., LUCAS, P., PRIETO, I., MARTINEZ-A, C. & MELLADO, M. (2004) Blocking HIV-1 infection via CCR5 and CXCR4 receptors by acting in trans on the CCR2 chemokine receptor. *Embo Journal,* 23**,** 66-76.

RODRIGUEZ-FRADE, J. M., VILA-CORO, A. J., DE ANA, A. M., ALBAR, J. P., MARTINEZ-A, C. & MELLADO, M. (1999) The chemokine monocyte chemoattractant protein-1 induces functional responses through dimerization of its receptor CCR2. *Proceedings of the National Academy of Sciences of the United States of America,* 96**,** 3628-3633.

ROLLINS, B. J. (1997) Chemokines. *Blood,* 90**,** 909-928.

ROSE, J. J., FOLEY, J. F., MURPHY, P. M. & VENKATESAN, S. (2004) On the mechanism and significance of ligand-induced internalization of human neutrophil chemokine receptors CXCR1 and CXCR2. *Journal of Biological Chemistry,* 279**,** 24372-24386.

ROSENKILDE, M. M. (2005) Virus-encoded chemokine receptors - putative novel antiviral drug targets. *Neuropharmacology,* 48**,** 1-13.

ROSENKILDE, M. M., KLEDAL, T. N., BRAUNER-OSBORNE, H. & SCHWARTZ, T. W. (1999) Agonists and inverse agonists for the herpesvirus 8-encoded constitutively active seven-transmembrane oncogene product, ORF-74. *Journal of Biological Chemistry,* 274**,** 956-961.

ROSSI, D. & ZLOTNIK, A. (2000) The biology of chemokines and their receptors. *Annual Review of Immunology,* 18**,** 217-243.

ROT, A. (1993) NEUTROPHIL ATTRACTANT ACTIVATION PROTEIN-1 (INTERLEUKIN-8) INDUCES INVITRO NEUTROPHIL MIGRATION BY HAPTOTACTIC MECHANISM. *European Journal of Immunology,* 23**,** 303-306.

ROT, A. (2005) Contribution of Duffy antigen to chemokine function. *Cytokine & Growth Factor Reviews,* 16**,** 687-694.

ROT, A., MCKIMMIE, C., BURT, C. L., PALLAS, K. J., JAMIESON, T., PRUENSTER, M., HORUK, R., NIBBS, R. J. B. & GRAHAM, G. J. (2013) Cell-autonomous regulation of neutrophil migration by the d6 chemokine decoy receptor. *Journal of immunology (Baltimore, Md. : 1950),* 190**,** 6450-6.

ROT, A. & VON ANDRIAN, U. H. (2004) Chemokines in innate and adaptive host defense: Basic chemokinese grammar for immune cells. *Annual Review of Immunology,* 22**,** 891-928.

RUTKOWSKI, J. M., BOARDMAN, K. C. & SWARTZ, M. A. (2006) Characterization of lymphangiogenesis in a model of adult skin regeneration. *American Journal of Physiology-Heart and Circulatory Physiology,* 291**,** H1402-H1410.

SADIR, R., IMBERTY, A., BALEUX, F. & LORTAT-JACOB, H. (2004) Heparan sulfate/heparin oligosaccharides protect stromal cell-derived factor-1 (SDF-1)/CXCL12 against proteolysis induced by CD26/dipeptidyl peptidase IV. *Journal of Biological Chemistry,* 279**,** 43854-43860.

SAFAIYAN, F., KOLSET, S. O., PRYDZ, K., GOTTFRIDSSON, E., LINDAHL, U. & SALMIVIRTA, M. (1999) Selective effects of sodium chlorate treatment on the sulfation of heparan sulfate. *Journal of Biological Chemistry,* 274**,** 36267-36273.

SAVINO, B., BORRONI, E. M., TORRES, N. M., PROOST, P., STRUYF, S., MORTIER, A., MANTOVANI, A., LOCATI, M. & BONECCHI, R. (2009) Recognition Versus Adaptive Up-regulation and Degradation of CC Chemokines by the Chemokine Decoy Receptor D6 Are Determined by Their N-terminal Sequence. *Journal of Biological Chemistry,* 284**,** 26207-26215.

SCHALL, T. J. & PROUDFOOT, A. E. I. (2011) Overcoming hurdles in developing successful drugs targeting chemokine receptors. *Nature Reviews Immunology,* 11**,** 355-363.

SCHIOPPA, T., URANCHIMEG, B., SACCANI, A., BISWAS, S. K., DONI, A., RAPISARDA, A., BERNASCONI, S., SACCANI, S., NEBULONI, M., VAGO, L., MANTOVANI, A., MELILLO, G. & SICA, A. (2003) Regulation of the chemokine receptor CXCR4 by hypoxia. *Journal of Experimental Medicine,* 198**,** 1391-1402.

SCHNABEL, R. B., BAUMERT, J., BARBALIC, M., DUPUIS, J., ELLINOR, P. T., DURDA, P., DEHGHAN, A., BIS, J. C., ILLIG, T., MORRISON, A. C., JENNY, N. S., KEANEY, J. F., GIEGER, C., TILLEY, C., YAMAMOTO, J. F., KHUSEYINOVA, N., HEISS, G., DOYLE, M., BLANKENBERG, S., HERDER, C., WALSTON, J. D., ZHU, Y. Y., VASAN, R. S., KLOPP, N., BOERWINKLE, E., LARSON, M. G., PSATY, B. M., PETERS, A., BALLANTYNE, C. M., WITTEMAN, J. C. M., HOOGEVEEN, R. C., BENJAMIN, E. J., KOENIG, W. & TRACY, R. P. (2010) Duffy antigen receptor for chemokines (Darc) polymorphism regulates circulating concentrations of monocyte chemoattractant protein-1 and other inflammatory mediators. *Blood,* 115**,** 5289-5299.

SCHURGERS, E., BILLIAU, A. & MATTHYS, P. (2011) Collagen-Induced Arthritis as an Animal Model for Rheumatoid Arthritis: Focus on Interferon-gamma. *Journal of Interferon and Cytokine Research,* 31**,** 917-926.

SCOTTON, C., MILLIKEN, D., WILSON, J., RAJU, S. & BALKWILL, F. (2001a) Analysis of CC chemokine and chemokine receptor expression in solid ovarian tumours. *British Journal of Cancer,* 85**,** 891-897.

SCOTTON, C. J., WILSON, J. L., MILLIKEN, D., STAMP, G. & BALKWILLL, F. R. (2001b) Epithelial cancer cell migration: A role for chemokine receptors? *Cancer Research,* 61**,** 4961-4965.

SEIBERT, C., CADENE, M., SANFIZ, A., CHAIT, B. T. & SAKMAR, T. P. (2002) Tyrosine sulfation of CCR5 N-terminal peptide by tyrosylprotein sulfotransferases 1 and 2 follows a discrete pattern and temporal sequence. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 11031-11036.

SEIBERT, C. & SAKMAR, T. P. (2008) Toward a framework for sulfoproteomics: Synthesis and characterization of sulfotyrosine-containing peptides. *Biopolymers,* 90**,** 459-477.

SHANKARAPPA, R., MARGOLICK, J. B., GANGE, S. J., RODRIGO, A. G., UPCHURCH, D., FARZADEGAN, H., GUPTA, P., RINALDO, C. R., LEARN, G. H., HE, X., HUANG, X. L. & MULLINS, J. I. (1999) Consistent viral evolutionary changes associated with the progression of human immunodeficiency virus type 1 infection. *Journal of Virology,* 73**,** 10489-10502.

SHEN, H., SCHUSTER, R., STRINGER, K. F., WALTZ, S. E. & LENTSCH, A. B. (2006) The Duffy antigen/receptor for chemokines (DARC) regulates prostate tumor growth. *Faseb Journal,* 20**,** 59-64.

SHERRY, B., TEKAMPOLSON, P., GALLEGOS, C., BAUER, D., DAVATELIS, G., WOLPE, S. D., MASIARZ, F., COIT, D. & CERAMI, A. (1988) RESOLUTION OF THE 2 COMPONENTS OF MACROPHAGE INFLAMMATORY PROTEIN-1, AND CLONING AND CHARACTERIZATION OF ONE OF THOSE COMPONENTS, MACROPHAGE INFLAMMATORY PROTEIN-1-BETA. *Journal of Experimental Medicine,* 168**,** 2251-2259.

SHERRY, D. M., MURRAY, A. R., KANAN, Y., ARBOGAST, K. L., HAMILTON, R. A., FLIESLER, S. J., BURNS, M. E., MOORE, K. L. & AL-UBAIDI, M. R. (2010) Lack of protein-tyrosine sulfation disrupts photoreceptor outer segment morphogenesis, retinal function and retinal anatomy. *European Journal of Neuroscience,* 32**,** 1461-1472.

SHULMAN, N. R. (1952) STUDIES ON THE INHIBITION OF PROTEOLYTIC ENZYMES BY SERUM .1. THE MECHANISM OF THE INHIBITION OF TRYPSIN, PLASMIN, AND CHYMOTRYPSIN BY SERUM USING FIBRIN TAGGED WITH I-131 AS A SUBSTRATE. *Journal of Experimental Medicine,* 95**,** 571-591.

SIERRO, F., BIBEN, C., MARTINEZ-MUNOZ, L., MELLADO, M., RANSOHOFF, R. M., LI, M., WOEHL, B., LEUNG, H., GROOM, J., BATTEN, M., HARVEY, R. P., MARTINEZ-A, C., MACKAY, C. R. & MACKAY, F. (2007) Disrupted

cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. *Proceedings of the National Academy of Sciences of the United States of America,* 104**,** 14759-14764.

SIGNORET, N., HEWLETT, L., WAVRE, S., PELCHEN-MATTHEWS, A., OPPERMANN, M. & MARSH, M. (2005) Agonist-induced endocytosis of CC chemokine receptor 5 is clathrin dependent. *Molecular Biology of the Cell,* 16**,** 902-917.

SIMPSON, L. S., ZHU, J. Z., WIDLANSKI, T. S. & STONE, M. J. (2009) Regulation of Chemokine Recognition by Site-Specific Tyrosine Sulfation of Receptor Peptides. *Chemistry & Biology,* 16**,** 153-161.

SINGH, M. D., KING, V., BALDWIN, H., BURDEN, D., THORRAT, A., HOLMES, S., MCINNES, I. B., NICOLL, R., SHAMS, K., PALLAS, K., JAMIESON, T., LEE, K. M., CARBALLIDO, J. M., ROT, A. & GRAHAM, G. J. (2012) Elevated Expression of the Chemokine-Scavenging Receptor D6 Is Associated with Impaired Lesion Development in Psoriasis. *American Journal of Pathology,* 181**,** 1158-1164.

SORIANO, S. F., SERRANO, A., HERNANZ-FALCON, P., DE ANA, A. M., MONTERRUBIO, M., MARTINEZ, C., RODRIGUEZ-FRADE, J. M. & MELLADO, M. (2003) Chemokines integrate JAK/STAT and G-protein pathways during chemotaxis and calcium flux responses. *European Journal of Immunology,* 33**,** 1328-1333.

SOULIE, C., CALVEZ, V. & MARCELIN, A. G. (2012) Coreceptor usage in different reservoirs. *Current Opinion in Hiv and Aids,* 7**,** 450-455.

SPAAN, A. N., SUREWAARD, B. G. J., NIJLAND, R. & VAN STRIJP, J. A. G. (2013) Neutrophils Versus Staphylococcus aureus: A Biological Tug of War. IN GOTTESMAN, S. (Ed.) *Annual Review of Microbiology, Vol 67.* Palo Alto, Annual Reviews.

SPARMANN, A. & BAR-SAGI, D. (2004) Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. *Cancer Cell,* 6**,** 447-458.

SPRINGAEL, J. Y., URIZAR, E., COSTAGLIOLA, S., VASSART, G. & PARMENTIER, M. (2007) Allosteric properties of G protein-coupled receptor oligomers. *Pharmacology & Therapeutics,* 115**,** 410-418.

SPRINGER, T. A. (1995) TRAFFIC SIGNALS ON ENDOTHELIUM FOR LYMPHOCYTE RECIRCULATION AND LEUKOCYTE EMIGRATION. *Annual Review of Physiology,* 57**,** 827-872.

STIEVANO, L., PIOVAN, E. & AMADORI, A. (2004) C and CX3C chemokines: Cell sources and physiopathological implications. *Critical Reviews in Immunology,* 24**,** 205-228.

STONE, M. J., CHUANG, S., HOU, X., SHOHAM, M. & ZHU, J. Z. (2009) Tyrosine sulfation: an increasingly recognised post-translational modification of secreted proteins. *New Biotechnology,* 25**,** 299-317.

STRIETER, R. M., BURDICK, M. D., GOMPERTS, B. N., BELPERIO, J. A. & KEANE, M. P. (2005) CXC chemokines in angiogenesis. *Cytokine & Growth Factor Reviews,* 16**,** 593-609.

STRIETER, R. M., POLVERINI, P. J., KUNKEL, S. L., ARENBERG, D. A., BURDICK, M. D., KASPER, J., DZUIBA, J., VANDAMME, J., WALZ, A., MARRIOTT, D., CHAN, S. Y., ROCZNIAK, S. & SHANAFELT, A. B. (1995) THE FUNCTIONAL-ROLE OF THE ELR MOTIF IN CXC CHEMOKINE-MEDIATED ANGIOGENESIS. *Journal of Biological Chemistry,* 270**,** 27348-27357.

STRUYF, S., PROOST, P. & VAN DAMME, J. (2003) Regulation of the immune response by the interaction of chemokines and proteases. *Advances in Immunology, Vol 81.* San Diego, Elsevier Academic Press Inc.

SUZUKI, A., YAMADA, R., CHANG, X. T., TOKUHIRO, S., SAWADA, T., SUZUKI, M., NAGASAKI, M., NAKAYAMA-HAMADA, M., KAWAIDA, R., ONO, M., OHTSUKI, M., FURUKAWA, H., YOSHINO, S., YUKIOKA, M., TOHMA, S., MATSUBARA, T., WAKITANI, S., TESHIMA, R., NISHIOKA, Y., SEKINE, A., IIDA, A., TAKAHASHI, A., TSUNODA, T., NAKAMURA, Y. & YAMAMOTO, K. (2003) Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. *Nature Genetics,* 34**,** 395-402.

SZEKANECZ, Z., KOCH, A. E. & TAK, P. P. (2011) Chemokine and chemokine receptor blockade in arthritis, a prototype of immune-mediated inflammatory diseases. *Netherlands Journal of Medicine,* 69**,** 356-366.

SZEKANECZ, Z., VEGVARI, A., SZABO, Z. & KOCH, A. E. (2010) Chemokines and chemokine receptors in arthritis. *Frontiers in bioscience (Scholar edition),* 2**,** 153-67.

SZPAKOWSKA, M., FIEVEZ, V., ARUMUGAN, K., VAN NULAND, N., SCHMIT, J. C. & CHEVIGNE, A. (2012) Function, diversity and therapeutic potential of the N-terminal domain of human chemokine receptors. *Biochemical Pharmacology,* 84**,** 1366-1380.

TAKASHIMA, A. & FALLER, D. V. (2013) Targeting the RAS oncogene. *Expert Opinion on Therapeutic Targets,* 17**,** 507-531.

TAKEKOSHI, T., ZIAREK, J. J., VOLKMAN, B. F. & HWANG, S. T. (2012) A Locked, Dimeric CXCL12 Variant Effectively Inhibits Pulmonary Metastasis of CXCR4-Expressing Melanoma Cells Due to Enhanced Serum Stability. *Molecular Cancer Therapeutics,* 11**,** 2516-2525.

TALMADGE, J. E. (2011) Immune cell infiltration of primary and metastatic lesions: Mechanisms and clinical impact. *Seminars in Cancer Biology,* 21**,** 131-138.

TAN, J. H. Y., CANALS, M., LUDEMAN, J. P., WEDDERBURN, J., BOSTON, C., BUTLER, S. J., CARRICK, A. M., PARODY, T. R., TALESKI, D., CHRISTOPOULOS, A., PAYNE, R. J. & STONE, M. J. (2012) Design and Receptor Interactions of Obligate Dimeric Mutant of Chemokine Monocyte Chemoattractant Protein-1 (MCP-1). *Journal of Biological Chemistry,* 287**,** 14692-14702.

TAN, J. H. Y., LUDEMAN, J. P., WEDDERBURN, J., CANALS, M., HALL, P., BUTLER, S. J., TALESKI, D., CHRISTOPOULOS, A., HICKEY, M. J., PAYNE, R. J. & STONE, M. J. (2013a) Tyrosine Sulfation of Chemokine Receptor CCR2 Enhances Interactions with Both Monomeric and Dimeric Forms of the Chemokine Monocyte Chemoattractant Protein-1 (MCP-1). *Journal of Biological Chemistry,* 288**,** 10024-10034.

TAN, Q. X., ZHU, Y., LI, J., CHEN, Z. X., HAN, G. W., KUFAREVA, I., LI, T. T., MA, L. M., FENALTI, G., ZHANG, W. R., XIE, X., YANG, H. Y., JIANG, H. L., CHEREZOV, V., LIU, H., STEVENS, R. C., ZHAO, Q. & WU, B. L. (2013b) Structure of the CCR5 Chemokine Receptor-HIV Entry Inhibitor Maraviroc Complex. *Science,* 341**,** 1387-1390.

THELEN, M. (2001) Dancing to the tune of chemokines. *Nature Immunology,* 2**,** 129-134.

THELEN, M., MUNOZ, L. M., RODRIGUEZ-FRADE, J. M. & MELLADO, M. (2010) Chemokine receptor oligomerization: functional considerations. *Current Opinion in Pharmacology,* 10**,** 38-43.

THORNTON, S., DUWEL, L. E., BOIVIN, G. P., MA, Y. H. & HIRSCH, R. (1999) Association of the course of collagen-induced arthritis with distinct patterns of cytokine and chemokine messenger RNA expression. *Arthritis and Rheumatism,* 42**,** 1109-1118.

THWAITES, G. E. & GANT, V. (2011) Are bloodstream leukocytes Trojan Horses for the metastasis of Staphylococcus aureus? *Nature Reviews Microbiology,* 9**,** 215-222.

TSOU, C. L., HASKELL, C. A. & CHARO, I. F. (2001) Tumor necrosis factor-alpha-converting enzyme mediates the inducible cleavage of fractalkine. *Journal of Biological Chemistry,* 276**,** 44622-44626.

TURNBULL, J., POWELL, A. & GUIMOND, S. (2001) Heparan sulfate: decoding a dynamic multifunctional cell regulator. *Trends in Cell Biology,* 11**,** 75-82.

ULLUM, H., LEPRI, A. C., VICTOR, J., ALADDIN, H., PHILLIPS, A. N., GERSTOFT, J., SKINHOJ, P. & PEDERSEN, B. K. (1998) Production of beta-chemokines in human immunodeficiency virus (HIV) infection: Evidence that high levels of macrophage in inflammatory protein-1 beta are associated with a decreased risk of HIV disease progression. *Journal of Infectious Diseases,* 177**,** 331-336.

ULVMAR, M. H., HUB, E. & ROT, A. (2011) Atypical chemokine receptors. *Experimental Cell Research,* 317**,** 556-568.

UMEHARA, H., BLOOM, E. T., OKAZAKI, T., NAGANO, Y., YOSHIE, O. & IMAI, T. (2004) Fractalkine in vascular biology - From basic research to clinical disease. *Arteriosclerosis Thrombosis and Vascular Biology,* 24**,** 34-40.

UNUTMAZ, D. & LITTMAN, D. R. (1997) Expression pattern of HIV-1 coreceptors on T cells: Implications for viral transmission and lymphocyte homing. *Proceedings of the National Academy of Sciences of the United States of America,* 94**,** 1615-1618.

VALENTIN, G., HAAS, P. & GILMOUR, D. (2007) The chemokine SDF1a coordinates tissue migration through the spatially restricted activation of Cxcr7 and Cxcr4b. *Current Biology,* 17**,** 1026-1031.

VALENZUELA-FERNANDEZ, A., PLANCHENAULT, T., BALEUX, F., STAROPOLI, I., LE-BARILLEC, K., LEDUC, D., DELAUNAY, T., LAZARINI, F., VIRELIZIER, J. L., CHIGNARD, M., PIDARD, D. & ARENZANA-SEISDEDOS, F. (2002) Leukocyte elastase negatively regulates stromal cell-derived factor-1 (SDF-1)/CXCR4 binding and functions by amino-terminal processing of SDF-1 and CXCR4. *Journal of Biological Chemistry,* 277**,** 15677-15689.

VAN DEN STEEN, P. E., PROOST, P., WUYTS, A., VAN DAMME, J. & OPDENAKKER, G. (2000) Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. *Blood,* 96**,** 2673-2681.

VAN SINDEREN, M., CUMAN, C., WINSHIP, A., MENKHORST, E. & DIMITRIADIS, E. (2013) The chrondroitin sulfate proteoglycan (CSPG4) regulates human trophoblast function. *Placenta,* 34**,** 907-912.

VELDKAMP, C. T., SEIBERT, C., PETERSON, F. C., SAKMAR, T. P. & VOLKMAN, B. F. (2006) Recognition of a CXCR4 sulfotyrosine by the chemokine stromal cell-derived factor-1 alpha (SDF-1 alpha/CXCL12). *Journal of Molecular Biology,* 359**,** 1400-1409.

VESTERGAARD, C., JUST, H., NIELSEN, J. B., THESTRUP-PEDERSEN, K. & DELEURAN, M. (2004) Expression of CCR2 on monocytes and macrophages in chronically inflamed skin in atopic dermatitis and psoriasis. *Acta Dermato-Venereologica,* 84**,** 353-358.

VETRANO, S., BORRONI, E. M., SARUKHAN, A., SAVINO, B., BONECCHI, R., CORREALE, C., ARENA, V., FANTINI, M., RONCALLI, M., MALESCI, A., MANTOVANI, A., LOCATI, M. & DANESE, S. (2010) The lymphatic system controls intestinal inflammation and inflammation-associated colon cancer through the chemokine decoy receptor D6. *Gut,* 59**,** 197-206.

VIERBOOM, M. P. M., ZAVODNY, P. J., CHOU, C. C., TAGAT, J. R., PUGLIESE-SIVO, C., STRIZKI, J., STEENSMA, R. W., MCCOMBIE, S. W., CELEBI-PAUL, L., REMARQUE, E., JONKER, M., NARULA, S. K. & T HART, B. (2005) Inhibition of the development of collagen-induced arthritis in rhesus

monkeys by a small molecular weight antagonist of CCR5. *Arthritis and Rheumatism,* 52**,** 627-636.

VINET, J., VAN ZWAM, M., DIJKSTRA, I. M., BROUWER, N., VAN WEERING, H. R. J., WATTS, A., MEIJER, M., FOKKENS, M. R., KANNAN, V., VERZIJL, D., VISCHER, H. F., SMIT, M. J., LEURS, R., BIBER, K. & BODDEKE, H. (2013) Inhibition of CXCR3-mediated chemotaxis by the human chemokine receptor-like protein CCX-CKR. *British Journal of Pharmacology,* 168**,** 1375-1387.

VINEY, J. M., ANDREW, D. P., PHILLIPS, R. M., MEISER, A., PATEL, P., LENNARTZ-WALKER, M., COUSINS, D. J., BARTON, N. P., HALL, D. A. & PEASE, J. E. (2014) Distinct Conformations of the Chemokine Receptor CCR4 with Implications for Its Targeting in Allergy. *Journal of Immunology,* 192**,** 3419-3427.

VOLEJNIKOVA, S., LASKARI, M., MARKS, S. C. & GRAVES, D. T. (1997) Monocyte recruitment and expression of monocyte chemoattractant protein-1 are developmentally regulated in remodeling bone in the mouse. *American Journal of Pathology,* 150**,** 1711-1721.

VON STEBUT, E., METZ, M., MILON, G., KNOP, J. & MAURER, M. (2003) Early macrophage influx to sites of cutaneous granuloma formation is dependent on MIP-1 alpha/beta released from neutrophils recruited by mast cell-derived TNF alpha. *Blood,* 101**,** 210-215.

WAGNER, L., YANG, O. O., GARCIA-ZEPEDA, E. A., GE, Y. M., KALAMS, S. A., WALKER, B. D., PASTERNACK, M. S. & LUSTER, A. D. (1998) beta-chemokines are released from HIV-1-specific cytolytic T-cell granules complexed to proteoglycans. *Nature,* 391**,** 908-911.

WANG, J., OU, Z. L., HOU, Y. F., LUO, J. M., SHEN, Z. Z., DING, J. & SHAO, Z. M. (2006) Enhanced expression of Duffy antigen receptor for chemokines by breast cancer cells attenuates growth and metastasis potential. *Oncogene,* 25**,** 7201-7211.

WANG, L. C., FUSTER, M., SRIRAMARAO, P. & ESKO, J. D. (2005) Endothelial heparan sulfate deficiency impairs L-selectin- and chemokine-mediated neutrophil trafficking during inflammatory responses. *Nature Immunology,* 6**,** 902-910.

WATTS, A. O., VERKAAR, F., VAN DER LEE, M. M. C., TIMMERMAN, C. A. W., KUIJER, M., VAN OFFENBEEK, J., VAN LITH, L., SMIT, M. J., LEURS, R., ZAMAN, G. J. R. & VISCHER, H. F. (2013) beta-Arrestin Recruitment and G Protein Signaling by the Atypical Human Chemokine Decoy Receptor CCX-CKR. *Journal of Biological Chemistry,* 288**,** 7169-7181.

WEBB, L. M. C. & ALCAMI, A. (2005) Virally encoded chemokine binding proteins. *Mini-Reviews in Medicinal Chemistry,* 5**,** 833-848.

WEBER, M., BLAIR, E., SIMPSON, C. V., O'HARA, M., BLACKBURN, P. E., ROT, A., GRAHAM, G. J. & NIBBS, R. J. B. (2004) The chemokine receptor D6 constitutively traffics to and from the cell surface to internalize and degrade chemokine. *Molecular Biology of the Cell,* 15**,** 2492-2508.

WEBER, M., HAUSCHILD, R., SCHWARZ, J., MOUSSION, C., DE VRIES, I., LEGLER, D. F., LUTHER, S. A., BOLLENBACH, T. & SIXT, M. (2013) Interstitial Dendritic Cell Guidance by Haptotactic Chemokine Gradients. *Science,* 339**,** 328-332.

WESTMUCKETT, A. D., HOFFHINES, A. J., BORGHEI, A. & MOORE, K. L. (2008) Early postnatal pulmonary failure and primary hypothyroidism in mice with combined TPST-1 and TPST-2 deficiency. *General and Comparative Endocrinology,* 156**,** 145-153.

WESTMUCKETT, A. D. & MOORE, K. L. (2009) Lack of Tyrosylprotein Sulfotransferase Activity in Hematopoietic Cells Drastically Attenuates Atherosclerosis in Ldlr(-/-) Mice. *Arteriosclerosis Thrombosis and Vascular Biology,* 29**,** 1730-1736.

WHITEHEAD, G. S., WANG, T., DEGRAFF, L. M., CARD, J. W., LIRA, S. A., GRAHAM, G. J. & COOK, D. N. (2007) The chemokine receptor D6 has opposing effects on allergic inflammation and airway reactivity. *American Journal of Respiratory and Critical Care Medicine,* 175**,** 243-249.

WILEN, C. B., TILTON, J. C. & DOMS, R. W. (2012) Molecular Mechanisms of HIV Entry. IN ROSSMANN, M. G. & RAO, V. B. (Eds.) *Viral Molecular Machines.* Berlin, Springer-Verlag Berlin.

WOLF, M., ALBRECHT, S. & MARKI, C. (2008) Proteolytic processing of chemokines: Implications in physiological and pathological conditions. *International Journal of Biochemistry & Cell Biology,* 40**,** 1185-1198.

WONG, M. M. & FISH, E. N. (2003) Chemokines: attractive mediators of the immune response. *Seminars in Immunology,* 15**,** 5-14.

WU, B. L., CHIEN, E. Y. T., MOL, C. D., FENALTI, G., LIU, W., KATRITCH, V., ABAGYAN, R., BROOUN, A., WELLS, P., BI, F. C., HAMEL, D. J., KUHN, P., HANDEL, T. M., CHEREZOV, V. & STEVENS, R. C. (2010) Structures of the CXCR4 Chemokine GPCR with Small-Molecule and Cyclic Peptide Antagonists. *Science,* 330**,** 1066-1071.

WU, F. Y., OU, Z. L., FENG, L. Y., LUO, J. M., WANG, L. P., SHEN, Z. Z. & SHAO, Z. M. (2008) Chemokine decoy receptor D6 plays a negative role in human breast cancer. *Molecular Cancer Research,* 6**,** 1276-1288.

YANG, Y. F., MUKAI, T., GAO, P., YAMAGUCHI, N., ONO, S., IWAKI, H., OBIKA, S., IMANISHI, T., TSUJIMURA, T., HAMAOKA, T. & FUJIWARA, H. (2002) A non-peptide CCR5 antagonist inhibits collagen-induced arthritis by modulating T cell migration without affecting anti-collagen T cell responses. *European Journal of Immunology,* 32**,** 2124-2132.

YE, J. P., KOHLI, L. L. & STONE, M. J. (2000) Characterization of binding between the chemokine eotaxin and peptides derived from the chemokine receptor CCR3. *Journal of Biological Chemistry,* 275**,** 27250-27257.

YIN, X., TRUTY, J., LAWRENCE, R., JOHNS, S. C., SRINIVASAN, R. S., HANDEL, T. M. & FUSTER, M. M. (2010) A critical role for lymphatic endothelial heparan sulfate in lymph node metastasis. *Molecular Cancer,* 9**,** 20.

YOSHIDA, T., IMAI, T., TAKAGI, S., NISHIMURA, M., ISHIKAWA, I., YAOI, T. & YOSHIE, O. (1996) Structure and expression of two highly related genes encoding SCM-1 human lymphotactin. *Febs Letters,* 395**,** 82-88.

ZENG, X. H., OU, Z. L., YU, K. D., FENG, L. Y., YIN, W. J., LI, J., SHEN, Z. Z. & SHAO, Z. M. (2011) Coexpression of atypical chemokine binders (ACBs) in breast cancer predicts better outcomes. *Breast Cancer Research and Treatment,* 125**,** 715-727.

ZHU, J. Z., MILLARD, C. J., LUDEMAN, J. P., SIMPSON, L. S., CLAYTON, D. J., PAYNE, R. J., WIDLANSKI, T. S. & STONE, M. J. (2011) Tyrosine Sulfation Influences the Chemokine Binding Selectivity of Peptides Derived from Chemokine Receptor CCR3. *Biochemistry,* 50**,** 1524-1534.

ZHU, Z., SUN, Z., WANG, Z., GUO, P., ZHENG, X. & XU, H. (2013) Prognostic impact of atypical chemokine receptor expression in patients with gastric cancer. *The Journal of surgical research,* 183**,** 177-83.

ZIMMERMAN, P. A., FERREIRA, M. U., HOWES, R. E. & MERCEREAU-PUIJALON, O. (2013) Red Blood Cell Polymorphism and Susceptibility to Plasmodium vivax. IN HAY, S. I., PRICE, R. & BAIRD, J. K. (Eds.) *Advances in Parasitology, Vol 81: Epidemiology of Plasmodium Vivax: History, Hiatus and Hubris, Pt B.* San Diego, Elsevier Academic Press Inc.

ZLOTNIK, A. & YOSHIE, O. (2000) Chemokines: A new classification system and their role in immunity. *Immunity,* 12**,** 121-127.

ZLOTNIK, A., YOSHIE, O. & NOMIYAMA, H. (2006) The chemokine and chemokine receptor superfamilies and their molecular evolution. *Genome Biology,* 7**,** 11.

ZOU, P., ISEGAWA, Y., NAKANO, K., HAQUE, M., HORIGUCHI, Y. & YAMANISHI, K. (1999) Human herpesvirus 6 open reading frame U83 encodes a functional chemokine. *Journal of Virology,* 73**,** 5926-5933.