Trafficking regulation of the chemokine receptor CCR2B compared to CCR5

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Abstract

The closely-related CC chemokine receptors 2B and 5 are seven-transmembrane domain receptors coupled to heterotrimeric G proteins. The two receptors bind inflammatory chemokines and play important complementary roles in the recruitment of specific leukocyte sub-populations to sites of infection. To enable fine-tuning of cellular responses to chemokines, CCR2B and CCR5, like other GPCRs, can be desensitised in response to agonist stimulation or cross-talk with other receptors. This involves down-modulation of cell surface active receptor through two essential transportation events, endocytosis and recycling. The CCR5 endocytic and recycling pathways are well established and several mechanisms involved have been clearly defined. Conversely, less is known about the route followed by CCR2B upon stimulation.

This study investigated the regulation, trafficking and fate of CCR2B in the context of THP-1 cells endogenously expressing the receptor and HEK293 transfectants. Comparison with CCR5 highlighted marked differences in the behaviours of the two receptors. However, my initial findings indicate that certain aspects of the regulation of CCR5 as well as CCR2B may be cell type-dependent.

Flow cytometry, immunofluorescence and biochemical analyses showed that unlike CCR5, internalised CCR2B can be both degraded and recycled following agonist stimulation. In HEK293, CCR2B follows an EGF receptor-like pathway, transiting through early endosomes containing EEA1, transferrin and Rab4, reaching CD63 and Lamp1 positive late endosomes/lysosomes before being degraded.

Importantly, I showed that CCR2B cell surface molecules are N- and O-glycosylated, and only this glycosylated form of the receptor is targeted for agonist-induced degradation.

This thesis also presents findings from proteomics approaches developed in an attempt to identify interacting proteins implicated in the trafficking of each receptor.

This study brings new insights to the endocytic regulation of agonist-treated CC chemokine receptors, revealing receptor- and cell type-specific behaviours, which add complexity to a relatively conserved process.
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Author’s declaration

All data presented in this thesis is original. With the exception of Figures 3.3 and 3.5, which were compiled from data obtained by Laura Fell in the Laboratory of Dr Signoret, all the work here was performed by Laura Bennett. Figures 1.2 and 1.4, and Tables 1.1 and 1.2 are modified from Bennett et al., (2011), a copy of which is included as an appendix to this thesis.
1 Introduction

1.1 Chemokine receptors

1.1.1 Definition and nomenclature

Chemokine receptors belong to the G protein coupled receptor (GPCR) superfamily and are divided into classes based upon the type of chemokine with which they interact (IUIS/WHO 2003). The four classes of chemokines are named according to the motif displayed by their first two or single conserved N-terminal cysteines (CC, CXC and CX3C or XC respectively) that form disulfide bridges with other downstream conserved cysteine residues to stabilise the tertiary structure (Rossi and Zlotnik, 2000). Since the cloning of the interleukin-8 (CXCL8) receptor (Murphy and Tiffany, 1991), a total of ten CC, seven CXC, one CX3C and one XC classical chemokine receptors have been identified (IUIS/WHO 2003; Schall and Proudfoot, 2011). In addition there are at least four atypical scavenger chemokine receptors (Ulvmar et al., 2011). CCR2B and CCR5 belong to the CC chemokine receptor subfamily.

1.1.2 Roles

Chemokine receptors are predominantly expressed in the immune system on a wide range of leukocytes, with some expression on other cell types including epithelial and stromal cells (Le et al., 2004). Individual cell types display a specific chemokine receptor expression profile that can be further modified during development and according to the local microenvironment experienced by the cell (Rossi and Zlotnik, 2000). Chemokine receptors have a wide range of biological functions and can be grouped as constitutive or inflammatory receptors depending on the nature of the functional response induced by their chemokine ligands (Johnson et al., 2005). Homeostatic chemokines and their receptors regulate the basal trafficking of leukocytes, which influences lymphocyte development and immune surveillance as well as the development and organization of secondary lymphoid organs. In contrast, binding of inflammatory chemokines to their receptors is involved in the host response to inflammation and infection via the recruitment of specific leukocyte sub-populations to sites of injury. CCR2B and CCR5 are both inflammatory chemokine receptors and their main function is the recruitment of monocytes/macrophages, to sites of inflammation.
(Boring et al., 1997; Weber et al., 2001). In addition, they have both been shown to be upregulated on murine neutrophils under certain inflammatory conditions, where they play an important role in the adherence and transmigration processes involved in neutrophil recruitment (Johnston et al., 1999; Maus et al., 2002; Reichel et al., 2006; Souto et al., 2011). Chemokine receptor functions are dependent on the ability of the receptor to induce chemotaxis in response to chemokine binding enabling directed migration of the cell towards the source of chemokine gradient. An additional group of atypical chemokine receptors can bind, internalise and either degrade or transcytose chemokines, whilst displaying an apparent inability to independently activate the classical signalling pathways that lead to chemotaxis (Ulvmar et al., 2011).

### 1.1.3 Structure

Chemokine receptors are seven-transmembrane receptors with an extracellular N-terminus and an intracellular C-terminus (Figure 1.1). Until recently, no crystal structures for any chemokine receptors were available. However, despite low sequence homology, the high structural similarity observed between GPCRs for which crystal structures have been solved, has enabled homology modelling to be carried out for several chemokine receptors including CCR2 (Berkhout et al., 2003; Carter and Tebben, 2009; Kim et al., 2011; Kimura et al., 2008; Mirzadegan et al., 2000; Shi et al., 2002) and CCR5 (Carter and Tebben, 2009; Castonguay et al., 2003; Fano et al., 2006; Garcia-Perez et al., 2011; Kondru et al., 2008; Li et al., 2009; Maeda et al., 2006; Manikandan and Malik, 2008; Seibert et al., 2006; Shahlai et al., 2011; Xu et al., 2004). Traditionally the high resolution bovine rhodopsin crystal structure (Palczewski et al., 2000) or more recently the human β2 adrenergic receptor crystal structure (Cherezov et al., 2007), was used as a template. Then biochemical data, such as the effects of mutations on ligand binding, were typically used to improve the models (Carter and Tebben, 2009). Several crystal structures have now been solved for CXCR4 and they show important differences in the localisation and shape of the ligand binding sites compared to those observed for other typical GPCRs (Wu et al., 2010). The existence of an actual chemokine receptor crystal structure should provide a better base for homology mapping of other chemokine receptors. Indeed it was recently used for homology modelling of CCR2 and CCR5 to enable investigation of antagonist binding interactions (Kothandan et al., 2012).
Chemokine receptors have seven transmembrane regions (TM) linked by three intracellular (ICL) and three extracellular (ECL) loops. The N-terminus is located extracellularly and the C-terminus is intracellular. The four conserved extracellular cysteines that form disulfide bonds in CC, CXC and CX₃C chemokine receptors are highlighted in black and the bonds are represented by dashed lines. Green asterisks (*) mark the locations of glycosylation and sulphation sites: CCR2 N-glycosylation site (N₁₄), CCR2 sulphation site (Y₂₆), major CCR5 O-glycosylation site (S₉/S₁₇) and CCR5 sulphation sites (Y₁₁, Y₁₀, Y₁₄ and Y₁₅). The DRY sequence, which is conserved as part of a larger motif in almost all chemokine receptors, is highlighted in the second intracellular loop. The cytoplasmic tail amino acid sequences of CCR2B and CCR5 are shown for comparison. The three cysteines that are palmitoylated in CCR5 are highlighted in blue. Phosphorylation sites are highlighted in red. The CCR5 PDZ domain and dileucine motif are underlined in purple and green respectively.

The chemokine receptor tertiary structure provides different binding sites for orthosteric chemokine ligands, small molecule ligands, and in the case of CCR5 and CXCR4, the human immunodeficiency virus (HIV). The chemokine binding site is located on the extracellular side of the receptor and involves the N-terminus plus certain extracellular...
loops (ECLs) and transmembrane domains. On receptors that bind multiple chemokines, the precise binding sites are usually distinct but overlapping (Blanpain et al., 2003; Jensen et al., 2008; Xanthou et al., 2003). It has been suggested that chemokine binding is a two-step process where the first step is important for ligand binding and the second step is more important for activation of the receptor and signalling (Allen et al., 2007). Firstly, the N-loop and the core domain of the chemokine bind to the chemokine recognition site 1, which consists of the N-terminus and extracellular loops of the receptor. Secondly, the chemokine N-terminus is inserted into chemokine recognition site 2, which is located within the transmembrane helical bundle/transmembrane domain of the receptor and may also involve some extracellular loops. In contrast, small molecule antagonists do not typically use the receptor N-terminus for binding. Instead they often have binding sites located deeper within the TM helix bundle, involving either TM1, 2, 3, 7 (minor binding pocket) or TM3, 4, 5, 6 (major binding pocket) or both (Scholten et al., 2012). Therefore, they can modulate their effect on chemokine binding not by competitive binding but by allosteric modulation of the receptor conformation. HIV gp120 binds sequentially to its primary receptor CD4 and then to a CCR5 or CXCR4 co-receptor using binding sites involving the N-terminus and second extracellular loop of the co-receptor (Brelot et al., 1997; Doranz et al., 1999; Wu et al., 1997).

Chemokine receptors can be subject to different types of post-translational modification that can impact both on the overall structure and on the various binding sites. CCR5, like many other GPCRs (Qanbar and Bouvier, 2003), has been shown to be palmitoylated on C-terminal tail cysteines, residues 321, 323 and 324, which is thought to enable interactions with plasma membrane lipids and lead to the formation of an extra fourth intracellular loop, thus reducing the flexibility of the tail (Figure 1.1; Blanpain et al., 2001; Kraft et al., 2001; Percherancier et al., 2001). Despite the presence of C-terminal cysteines as potential palmitoylation sites in most but not all chemokine receptors, so far no other receptors have actually been demonstrated to be palmitoylated (Neel et al., 2005).

Chemokine receptors can be N-glycosylated on asparagine residues or O-glycosylated on hydroxyl groups of serine/threonine residues located on their N-terminus or extracellular loops. The extent and type of glycosylation varies between receptors.
CCR2 (Figure 1.1, Preobrazhensky et al., 2000), CXCR2 (Ludwig et al., 2000), CXCR4 (Berson et al., 1996; Chabot et al., 2000), the Duffy antigen receptor for chemokines (DARC) (Tournamille et al., 2003) and D6 (Blackburn et al., 2004) have been shown to undergo N-linked glycosylation. In contrast, despite the presence of potential sites in its third ECL, CCR5 is not N-glycosylated but does undergo O-linked glycosylation at serines 6 or 7 (Figure 1.1; Bannert et al., 2001; Farzan et al., 1999).

The majority of chemokine receptors contain predicted N-terminal tyrosine sulphation sites (Liu et al., 2008) and sulphation has been demonstrated experimentally for human CCR2 (Figure 1.1; Preobrazhensky et al., 2000), CCR5 (Figure 1.1; Bannert et al., 2001; Farzan et al., 1999), CXCR3 (Colvin et al., 2006; Gao et al., 2009), CXCR4 (Farzan et al., 2002; Farzan et al., 1999), CX3CR1 (Fong et al., 2002), D6 (Blackburn et al., 2004) and DARC (Choe et al., 2005), and murine CCR8 (Gutierrez et al., 2004). In addition, chemokine receptors can also be modified by the attachment of sugar chains containing sulphate groups to the hydroxyl group of serine residues as has been shown for CXCR4 (Farzan et al., 2002).

Glycosylation has been reported to play an important role in cell surface expression of certain GPCRs (Dong et al., 2007; Duvernay et al., 2005). In contrast, despite being a common post-translational modification of secreted and transmembrane proteins (Moore, 2003), tyrosine sulphation appears to play no major role in the cell surface expression of most GPCRs, including chemokine receptors, studied to date (Choe et al., 2005; Colvin et al., 2006; Costagliola et al., 2002; Farzan et al., 1999; Farzan et al., 2001; Fieger et al., 2005; Fong et al., 2002; Preobrazhensky et al., 2000). However, tyrosine sulphation and glycosylation have both been shown to be important for chemokine binding to many chemokine receptors including CXCR4 and CCR5 (Neel et al., 2005). Additionally, tyrosine sulphation appears to have a positive impact on HIV gp120 binding to CCR5 (Farzan et al., 1999) and to a lesser extent to CXCR4 (Farzan et al., 2002). In contrast the impact of receptor glycosylation on HIV gp120 binding is less clear. No significant influence on HIV infection has been described for O-linked glycosylation of CCR5 (Bannert et al., 2001). However, N-linked glycosylation of CXCR4 has been differentially reported to have either no (Brelot et al., 2000; Picard et al., 1997) or a negative (Wang et al., 2004b) impact on HIV binding, or alternatively to be important for HIV X4 strain specificity (Chabot et al., 2000; Thordsen et al., 2002).
To be functionally active, in addition to being expressed at the cell surface with the correct post-translational modifications, chemokine receptors have to be presented in a conformation that is compatible with agonist binding and be coupled to a heterotrimeric G protein, so that they are ready to transmit intracellular signals (reviewed in Bennett et al., 2011, see Appendix). Other GPCRs are thought to reside in the plasma membrane in equilibrium between multiple active and inactive states (Vauquelin and Van Liefde, 2005). This equilibrium is thought to depend on complex allosteric interactions and conformational changes affected by ligands, as well as cell-specific parameters such as receptor expression level, G protein and accessory protein availability, and local membrane environment (Gilchrist, 2007; Nelson and Challiss, 2007; Vauquelin and Van Liefde, 2005; Wess et al., 2008). This is still relatively uncharted territory for chemokine receptors but experimental findings suggest that they may be subject to similar regulation. Indeed, there is evidence for conformational heterogeneity in cell surface CCR2 (Berchiche et al., 2011), CCR5 (Berro et al., 2011; Lee et al., 1999a) and CXCR4 (Baribaud et al., 2001; Sloane et al., 2005) receptor populations under either ligand-stimulated or non-stimulated conditions.

It is now accepted that GPCRs not only operate as single entities (monomers), but can also function as multimers regulated by allosteric mechanisms (Fuxe et al., 2010; Smith and Milligan, 2010). Chemokine receptors have been shown to form homomers as well as heteromers with other chemokine receptors, GPCRs or distinct types of cell surface receptors (Tables 1.1 and 1.2). Early work indicated that chemokine receptor dimerisation was ligand-induced, as described for CCR2 (Mellado et al., 2001; Rodriguez-Frade et al., 1999a), CCR5 (Rodriguez-Frade et al., 1999b; Vila-Coro et al., 2000) and CXCR4 (Toth et al., 2004; Vila-Coro et al., 1999b) homodimers, and CCR2/CCR5 heterodimers (Mellado et al., 2001). However, the current view is that chemokine receptor dimers are constitutively formed (Tables 1.1 and 1.2), and that ligand binding stabilizes or reorganizes pre-existing complexes (Salanga et al., 2009; Thelen et al., 2010; Wang and Norcross, 2008). CXCR1 and CXCR2 exemplify this: a recent study revealed that CXCL8 binding stabilizes homodimers but alters heterodimers (Martinez Munoz et al., 2009). In fact, dimers are thought to assemble during biosynthesis prior to arriving at the cell surface, as shown for CCR5 homomers (Issafras et al., 2002) or CXCR1/CXCR2 heterodimers (Wilson et al., 2005). For oligomerisation with non-chemokine receptors, other factors, such as the type of
molecules complexed with the chemokine receptor or the cellular background, could affect where and how dimers form. For CCR5, there are reports of constitutive intracellular interactions with CD4 in a monocytic cell line (Achour et al., 2009) and stable cell surface CCR5/CD4 heteromers complexed with or without CXCR4 on transfected cells (Baker et al., 2007; Wang et al., 2004a) or blood-derived dendritic cells (Xiao et al., 2000). Other studies described CCR5 and CD4 as being co-localised but independent monomeric molecules (Steffens and Hope, 2003) and interacting upon binding of HIV-gp120 at the surface of transfected cells (Yi et al., 2006).

Table 1.1 Identified chemokine receptor homomers.
Modified from Bennett et al. (2011).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Formation</th>
<th>Methods</th>
<th>Cells</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR2</td>
<td>Constitutive</td>
<td>BRET</td>
<td>HEK293</td>
<td>(El-Asmar et al., 2005; Percherancier et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>Inducible</td>
<td>IP</td>
<td>HEK293 MM-1</td>
<td>(Mellado et al., 2001; Rodriguez-Frade et al., 1999a)</td>
</tr>
<tr>
<td>CCR5</td>
<td>Constitutive</td>
<td>IP, Y2H, FRET, FLIM</td>
<td>HeLa, RBLs</td>
<td>(Benkirane et al., 1997; El-Asmar et al., 2005; Hernanz-Falcon et al., 2004; Huttenrauch et al., 2005; Issafras et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>Inducible</td>
<td>IP</td>
<td>HEK293 L1.2</td>
<td>(Hernanz-Falcon et al., 2004; Rodriguez-Frade et al., 1999b; Vila-Coro et al., 2000)</td>
</tr>
<tr>
<td>CXCR1</td>
<td>Constitutive</td>
<td>CO-IP, FRET, BRET</td>
<td>HEK293</td>
<td>(Wilson et al., 2005)</td>
</tr>
<tr>
<td>CXCR2</td>
<td>Constitutive</td>
<td>IP, FRET, BRET</td>
<td>HEK293</td>
<td>(Wilson et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>Constitutive</td>
<td>IP, WB</td>
<td>HEK293 Neurons</td>
<td>(Trettel et al., 2003)</td>
</tr>
<tr>
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<td>HEK293, tsA201</td>
<td>(Babcock et al., 2003; Percherancier et al., 2005; Toth et al., 2004; Wang et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>Inducible</td>
<td>IP</td>
<td>MOLT4</td>
<td>(Vila-Coro et al., 1999b)</td>
</tr>
<tr>
<td>DARC</td>
<td>Constitutive</td>
<td>BRET</td>
<td>HEK293</td>
<td>(Chakera et al., 2008)</td>
</tr>
</tbody>
</table>

BRET, bioluminescence resonance energy transfer; CO-IP, co-immunoprecipitation; DARC, duffy antigen receptor for chemokines; FLIM, fluorescence lifetime imaging; FRET, fluorescence resonance energy transfer; IP, immunoprecipitation; Y2H, yeast-2-hybrid.
Table 1.2 Identified chemokine receptor heteromers and their functional outcomes. Modified from Bennett et al. (2011).

### CHEMOKINE RECEPTORS

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Formation</th>
<th>Methods</th>
<th>Cells</th>
<th>Cooperativity (Assays)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Constitutive</td>
<td>CO-IP, FRET, BRET</td>
<td>HEK293</td>
<td>No</td>
<td>(Martinez Munoz et al., 2009; Wilson et al., 2005)</td>
</tr>
<tr>
<td>CXCR3/CCR5</td>
<td>Constitutive</td>
<td>FRET</td>
<td>Activated T cells</td>
<td>Negative (chemotaxis)</td>
<td>(O’Boyle et al., 2012)</td>
</tr>
<tr>
<td>CXCR4/ CXCR7</td>
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<td>CO-IP, FRET, BRET</td>
<td>HEK293, IM-9</td>
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<td>(Sierro et al., 2007)</td>
</tr>
<tr>
<td>CXCR4/ CCR2</td>
<td>Constitutive</td>
<td>BRET</td>
<td>CHO-K1, HEK293</td>
<td>Negative (binding, chemotaxis)</td>
<td>(Sohy et al., 2007)</td>
</tr>
<tr>
<td>CXCR4/ CCR5</td>
<td>Constitutive</td>
<td>CO-IP, BRET</td>
<td>NIH 3T3</td>
<td>Positive (chemotaxis)</td>
<td>(Gouwy et al., 2011; Wang et al., 2004a)</td>
</tr>
<tr>
<td>CXCR4/CCR2/ CCR5</td>
<td>Constitutive</td>
<td>BRET</td>
<td>HEK293</td>
<td>Negative (binding)</td>
<td>(Sohy et al., 2009)</td>
</tr>
<tr>
<td>CCR2/CCR5</td>
<td>Inducible</td>
<td>CO-IP</td>
<td>HEK293, PBMCs</td>
<td>Positive (Ca2+ flux)</td>
<td>(Mellado et al., 2001)</td>
</tr>
<tr>
<td>DARC/ CCR5</td>
<td>Constitutive</td>
<td>CO-IP, BRET, CO-IP, BRET</td>
<td>CHO-K1, CD4&lt;sup&gt;+&lt;/sup&gt; T cells, HEK293</td>
<td>Negative (binding)</td>
<td>(El-Asmar et al., 2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Chakera et al., 2008)</td>
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</tbody>
</table>

### GPCRs

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Formation</th>
<th>Methods</th>
<th>Cells</th>
<th>Cooperativity (Assays)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR5/ C5aR</td>
<td>Constitutive</td>
<td>CO-IP, BRET</td>
<td>RBLs, HEK293</td>
<td>Negative (co-internalisation)</td>
<td>(Huttenrauch et al., 2005)</td>
</tr>
<tr>
<td>CXCR2/ DOP</td>
<td>Constitutive</td>
<td>CO-IP, FRET, BRET</td>
<td>HEK293</td>
<td>Positive (G protein activation)</td>
<td>(Parenty et al., 2008)</td>
</tr>
<tr>
<td>CXCR4/DOP</td>
<td>Constitutive</td>
<td>CO-IP, FRET, BRET</td>
<td>HEK293, MM-1 Monocytes</td>
<td>Negative (chemotaxis, adhesion, Ca2+ flux)</td>
<td>(Pello et al., 2008)</td>
</tr>
<tr>
<td>CCR5/ opioid receptors</td>
<td>Constitutive</td>
<td>CO-IP</td>
<td>CHO, CEMx174</td>
<td>Negative (chemotaxis)</td>
<td>(Chen et al., 2004; Suzuki et al., 2002)</td>
</tr>
</tbody>
</table>
Table 1.2 Identified chemokine receptor heteromers and their functional outcomes.

<table>
<thead>
<tr>
<th>OTHERS</th>
<th>Receptors</th>
<th>Formation</th>
<th>Methods</th>
<th>Cells</th>
<th>Cooperativity (Assays)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Overexp.   Endog.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CXXCR2/ AMPA GluR1</td>
<td>Constitutive</td>
<td>CO-IP</td>
<td>HEK293</td>
<td>Neurons</td>
<td>Positive (chemotaxis)</td>
</tr>
<tr>
<td></td>
<td>CXXCR4/ CD4</td>
<td>Inducible (HIV)</td>
<td>CO-IP</td>
<td>PBMCs</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CXXCR4/ TCR</td>
<td>Inducible</td>
<td>CO-IP, FRET</td>
<td>Jurkat T</td>
<td>PBMCs T cells</td>
<td>Positive (Ca2+ flux)</td>
</tr>
<tr>
<td></td>
<td>CXXCR4/IGF-R1</td>
<td>Constitutive</td>
<td>CO-IP</td>
<td>MCF-7</td>
<td>T cells</td>
<td>Positive (chemotaxis)</td>
</tr>
<tr>
<td></td>
<td>CXXCR4/CD63</td>
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<td>CO-IP</td>
<td>HEK293</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCR5/ CD4</td>
<td>Constitutive</td>
<td>FRET BRET,</td>
<td>HEK293</td>
<td>CHO K1</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CO-IP</td>
<td></td>
<td>DCs</td>
<td></td>
</tr>
</tbody>
</table>

AMPA GluR1, a-amino-3-hydroxy-5-methyl-4-isoxazolepropionate-type glutamate receptor 1; BRET, bioluminescence resonance energy transfer; C5aR, complement component 5a receptor; CO-IP, co-immunoprecipitation; DARC, duffy antigen receptor for chemokines; DCs, Dendritic cells; DOP, μ-opioid receptor; FRET, fluorescence resonance energy transfer; IGF-R1, insulin-like growth factor-1 receptor; PBMCs, Peripheral blood mononuclear cells.
1.1.4 Signalling

Like other GPCRs, chemokine receptors present at the plasma membrane signal intracellularly in response to extracellular agonist binding. The most well-studied and characterized form of GPCR signalling is the canonical G protein-dependent signalling pathway where receptors signal via their associated heterotrimeric G proteins, which consist of a Gβ subunit, a Gγ subunit and one of several different Gα subunits. Traditionally it was thought that each receptor can only signal via a single specific G protein, however it has since been discovered that although receptors have a preferred G protein, different agonist binding can lead to signalling via different G proteins (Gilchrist, 2007; Oldham and Hamm, 2008). Agonist binding to the receptor results in conformational changes within the receptor that lead to dissociation of the G protein into guanosine triphosphate (GTP)-bound Gα and the Gβ/γ complex. These subunits can then activate or inhibit enzymes, such as phospholipase C-β (PLCβ) and adenylate cyclase (AC), and thus regulate downstream kinases, including protein kinase A (PKA) and protein kinase C (PKC), as well as other second messengers such as inositol-trisphosphate (IP3), diacylglycerol (DAG) and Ca²⁺ (New and Wong, 2003). This G protein-mediated signalling can regulate transcription events in the nucleus via the activation of transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), cAMP response element-binding (CREB), c-jun, c-fos and c-myc.

It is now established that GPCRs can also elicit G protein-independent signals through interaction with β-arrestins, which act as scaffolds for various signalling pathway proteins (DeFea, 2011). β-arrestin signalling can activate and/or inhibit various pathways including those involving mitogen-activated protein kinases (MAPKs), LIM domain kinase 1 (LIMK)/chronophin/cofilin, PKA, and PI3K/Akt and can act independently of, in synergy with or in opposition to G protein-dependent signalling (DeFea, 2011). Whereas G protein-mediated signalling is thought to be mainly restricted to plasma membrane GPCRs, signalling utilising β-arrestin scaffolds can occur from receptors in the endocytic pathway (von Zastrow and Sorkin, 2007). Evidence of β-arrestin-dependent signalling from chemokine receptors has been reported for CCR5 (Cheung et al., 2009), CCR7 (Kohout et al., 2004), CXCR4 (Sun et al., 2002) and CXCR7 (Rajagopal et al., 2010). For CCR5, β-arrestin has been suggested to act as a
scaffold for extracellular signal-regulated kinase (ERK) and a multimeric complex consisting of Pyk2, PI3K and Lyn involved in CCL4 stimulated chemotaxis (Cheung et al., 2009).

In addition to transcriptional regulation mediated by intracellular signalling cascades resulting from extracellular agonist binding to plasma membrane GPCRs, another more controversial form of direct nuclear GPCR signalling has been recently proposed for some receptors. This was suggested to involve GPCRs relocating to the nucleus where the receptors themselves or their ligands can regulate nuclear events, often with different or opposite outcomes to plasma membrane based signalling (Goetzl, 2007; Planque, 2006). Some nuclear expression of CXCR4 in tumour cells (Na et al., 2008; Speetjens et al., 2009; Wang et al., 2009; Wang et al., 2005; Wang et al., 2010b; Woo et al., 2008; Xiang et al., 2009; Yao et al., 2011) and CCR2 in transfected HEK293 and HeLa cells (Favre et al., 2008) has been reported, although it remains to be established if this is true expression or an artefact due to the experimental conditions used. CXCR4 nuclear localisation has been linked to poor prognosis for cancer patients (Na et al., 2008; Speetjens et al., 2009; Wang et al., 2009; Wang et al., 2010b; Woo et al., 2008; Xiang et al., 2009; Yao et al., 2011) but if or how precisely CXCR4 and CCR2 might signal at the nucleus remains to be investigated. For both receptors, there is some limited evidence that nuclear localisation may be mediated by agonist stimulation (Favre et al., 2008; Wang et al., 2009). However, nuclear localisation of GPCRs remains a debatable topic that challenges the traditional ideas of GPCR signalling, and no evidence has yet been provided for the mechanisms that would facilitate the necessary intracellular trafficking of the receptors.

An additional group of atypical chemokine receptors often called scavenger or decoy receptors, consisting of DARC, D6, CCRL1 and CCRL2, bind a wide range of chemokines (Table 1.3) but are unable to activate classical G protein-dependent signalling pathways (Ulvmar et al., 2011). Scavenger receptors are believed to regulate the signalling activity of other typical chemokine receptors via modulation of the local chemokine concentrations, gradients and expression profiles, or through heterodimerisation with chemokine receptors (Ulvmar et al., 2011). Due to its inability to mediate chemotaxis or calcium mobilisation in response to CXCL12 binding, CXCR7 was originally thought to belong to this group of atypical chemokine receptors.
(Burns et al., 2006; Thelen and Thelen, 2008). However, it has since been shown that in some circumstances it can signal via β-arrestin-mediated pathways in response to binding of its ligand CXCL11 (Rajagopal et al., 2010). Additionally, recent developments have suggested that it may also undergo Gi/o-dependent signalling in response to another ligand CXCL12 in astrocytes (Odemis et al., 2012). Thus it can act as a scavenger receptor, a β-arrestin signalling receptor and a classical chemokine receptor, and it has been proposed that the choice of role played by CXCR7 may be in part cell type dependent (Odemis et al., 2012).

It has long been thought that there is much functional redundancy in the chemokine ligand/receptor system, as many chemokines bind multiple receptors of one class and more than one receptor can interact with each chemokine (Table 1.3). However, recently some groups have found different receptor signalling and trafficking responses to individual chemokines, suggesting that this redundancy may not be as widespread as previously thought (Borroni et al., 2010; Zidar, 2011). Functional selectivity or biased agonism, where binding of different agonists leads to differential activation of downstream signalling pathways, has been reported for several chemokine receptors including both CCR2 and CCR5 (Berchiche et al., 2011; Leach et al., 2007; Mueller et al., 2002b; O'Boyle et al., 2007; Odemis et al., 2012; Oppermann et al., 1999; Wain et al., 2002). For CCR2, the bias towards β-arrestin 1 or 2 binding following ligand stimulation and the stability of this interaction, which influences the endocytic fate of the receptor, have been reported to be dependent on the identity of the chemokine involved (Berchiche et al., 2011). In addition, the specific G protein utilisation of CCR2 for chemotaxis and the kinetics of ERK and Akt activation vary dependent on the chemokine ligand engaged (O'Boyle et al., 2007; Wain et al., 2002). A range of CCR5 chemokine ligands exhibit different abilities to induce Gα- and Gβγ-dependent signalling cascades and to induce Gai/o-independent signalling, leading to different cellular responses (Leach et al., 2007; Mueller et al., 2002b; Oppermann et al., 1999). In addition, for several GPCRs (Violin and Lefkowitz, 2007) and recently the chemokine receptor CXCR7 (Odemis et al., 2012), different ligands have been reported to result in a bias towards either G protein-dependent or β-arrestin-dependent signalling. Finally, in vivo the expression of active chemokine receptors is tightly controlled in a spatial and temporal manner, thus limiting the actual signalling ability of a cell dependent on cell
type and the local microenvironment (Allen et al., 2007; Locati et al., 2005; Norment and Bevan, 2000; van der Veen et al., 2009).

Table 1.3 Chemokine receptors and their chemokine ligands.
This table is compiled from data from the IUPHAR database (Sharman et al., 2011), with the incorporation of additional data from other sources (Graham, 2009; Schall and Proudfoot, 2011; Scholten et al., 2012; Ulvmar et al., 2011; personal communication from James Fox, University of York, UK).

<table>
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<tr>
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<th>Antagonist</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
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<td>CCL4</td>
<td></td>
</tr>
<tr>
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<td>CCL11/24/26</td>
<td></td>
</tr>
<tr>
<td>CCR3</td>
<td>CCL2/5/7/8/13/15/24/26/28</td>
<td>CCL11</td>
<td></td>
</tr>
<tr>
<td>CCR4</td>
<td>CCL17/22</td>
<td></td>
<td></td>
</tr>
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</tr>
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<td>CX3CL1</td>
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</table>

1.2 Chemokine receptors and disease

Chemokine receptors play roles in many infectious and non-infectious diseases that have a major impact on world-wide human health. Firstly, several receptors are exploited by viruses and parasites to gain entry into human cells. In the mid 1990s, CCR5 and CXCR4 were identified as major co-receptors for HIV entry (Alkhatib et al., 1996; Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996). The transmission and asymptomatic stages of early infection predominantly involve CCR5 targeting by R5 HIV-1 strains. In many individuals, more promiscuous R5X4 HIV-1 strains, targeting CCR5, CXCR4 and possibly other minor coreceptors, typically emerge later in infection, and in the terminal stages of disease X4 HIV-1 strains targeting only CXCR4
can develop (Schuitemaker et al., 2010). Additionally, DARC is an atypical chemokine receptor expressed on erythrocytes, which is targeted by the human malaria parasite, *Plasmodium vivax* (Horuk et al., 1993).

Secondly, the chemotactic property of chemokine receptors is thought to contribute to an array of non-infectious diseases where inappropriate recruitment of specific chemokine receptor-expressing subsets of leukocytes is observed. These pathologies include auto-immune and allergic inflammatory disorders such as rheumatoid arthritis (RA), multiple sclerosis (MS), atherosclerosis, psoriasis, asthma and irritable bowel diseases (reviewed in Koelink et al., 2012), in addition to allograft transplant rejection (reviewed in Tan and Zhou, 2005) and cancer (reviewed in Balkwill, 2012). The inappropriate or chronic inflammatory responses central to these diseases can be due to mis-regulation of specific chemokine receptors themselves or changes in availability of their chemokine ligands.

1.2.1 Examples of diseases involving CCR2 and CCR5

Most inflammatory disease pathologies involve multiple chemokine receptors and their relative contributions can change during disease progression. The importance of a specific chemokine receptor for any given disease generally correlates with the level of involvement of cell types expressing that receptor. Due to their key role in the recruitment of monocytes and T cells, CCR2 and CCR5 have been implicated as playing minor roles in many inflammatory diseases. This includes several allergic disorders where these cells are not the major players but do contribute in some capacity (Fuchimoto et al., 2011; Hogaboam et al., 2005; Medoff et al., 2005; Pease, 2011). In addition, they are thought to play major roles in inflammatory diseases that have a high dependence on monocyte and/or T cell recruitment. Of these diseases, RA, has received much attention over the last two decades due to the associated high expression levels of CCR2 (Ellingsen et al., 2007), CCR5 (Haringman et al., 2006b) and their main ligands (Haringman et al., 2006b; Koch et al., 1992). Although targeting these receptors individually showed promise in animal models (Brodmerkel et al., 2005; Gong et al., 1997; Matsukawa et al., 1998; Ogata et al., 1997; Rafei et al., 2009; Schimmer et al., 1998; Schrier et al., 1998; Shahrara et al., 2008; Shahrara et al., 2005; Vierboom et al., 2005; Xia et al., 2007; Yang et al., 2002), years of failed clinical trials have given rise to
much debate over whether they are actually the best therapeutic targets for RA and if a multiple chemokine receptor blockade would be a better approach (Koelink et al., 2012; Lebre et al., 2011; Pease and Horuk, 2010; Proudfoot, 2008; Schall and Proudfoot, 2011; Zhao, 2010). However, robust roles have been shown for CCR2 and CCR5 in other diseases including MS and atherosclerosis, which are discussed here (Koelink et al., 2012).

CCR2, and to a lesser extent CCR5, is thought to play a major role in MS (Szczucinski and Losy, 2007), a chronic inflammatory disease of the central nervous system (CNS). The CNS is normally protected by the blood brain barrier, however in MS this barrier is broken down and CCR2 is thought to play a significant role in the recruitment of T cells and monocytes into the CNS (Koelink et al., 2012). T cells recognise myelin debris from apoptosed oligodendrocytes as being foreign and produce cytokines that stimulate macrophages and resident microglial cells and induce an inflammatory response causing further neuron demyelination and eventually development of MS lesions (Koelink et al., 2012). The resultant axonal damage negatively affects the signalling ability of neurons leading to both physical and cognitive disabilities in the patient. The involvement of CCR2 and CCR5 in MS has been demonstrated both in the mouse model of MS, Experimental Autoimmune Encephalomyelitis (EAE), and in the human disease itself. Elevated expression levels of CCR2 and CCR5 within active human MS lesions on macrophages and microglia have been reported (Simpson et al., 2000). In addition, the presence of the main CCR2 agonist, CCL2 (McManus et al., 1998; Simpson et al., 1998) and three CCR5 agonists, CCL3, CCL4 and CCL5 (Simpson et al., 1998) has also been observed. CCR2-/- (Izikson et al., 2000) or CCL2-/- (Huang et al., 2001) mice were shown to be resistant to induction of EAE, whereas upregulation of the receptor or ligand was associated with relapse of the disease (Jee et al., 2002).

Another type of chronic inflammatory disease, atherosclerosis, involves narrowing and hardening of the arteries that results in reduced blood flow and can lead to multiple different types of cardiovascular disease including myocardial infarction, ischemic coronary artery disease and ultimately congestive heart failure. Atherosclerosis is typified by the development of arterial plaques that consist of lipid deposits, connective tissue elements and inflammatory leukocytes (Stary et al., 1995). Multiple studies have confirmed that CCR2 and CCR5 play important complementary roles in the initiation
and progression of atherosclerosis. Agonists for both receptors (CCL2, CCL3, CCL4 and CCL5) are present in atherosclerotic lesions in animal disease models (Veillard et al., 2004; von Hundelshausen et al., 2001; Yu et al., 1992) and human atherosclerosis patients (Nelken et al., 1991; Schechter et al., 2000; Wilcox et al., 1994; Yla-Herttuala et al., 1991), with CCL2 levels in particular being upregulated. Indeed, over expression of CCL2 in the ApoE−/− mouse atherosclerosis model increased disease progression (Aiello et al., 1999). Knocking out CCR2 in ApoE−/− mice (Boring et al., 1998; Dawson et al., 1999) or CCL2 in LDLR−/− mice fed a high lipid diet (Gu et al., 1998), another atherosclerosis mouse model, resulted in reduced disease progression. Knocking out CCR5 in both ApoE−/− and LDLR−/− mice was also shown to protect against atherosclerosis disease progression (Braunersreuther et al., 2007; Potteaux et al., 2006; Quinones et al., 2007; Zernecke et al., 2006). However, the results were not as clear cut as for CCR2, as some groups showed that CCR5 only protected against later stages of the disease (Quinones et al., 2007) or had only a minor effect on lesion size (Potteaux et al., 2006). Furthermore, it has been suggested that the naturally occurring CCR5Δ32 mutant may provide a level of protection against related cardiovascular diseases, including myocardial infarction (Gonzalez et al., 2001; Incalcaterra et al., 2010) and severe coronary artery disease (Szalai et al., 2001), in some human populations. In addition, FROUNT, a binding partner for CCR2B and CCR5 that positively regulates directional chemotaxis (Terashima et al., 2005; Toda et al., 2009), has been implicated as playing a role in inflammatory cardiovascular disease (Satoh et al., 2007).

### 1.2.2 Chemokine receptors as therapeutic targets

Despite the existence of licensed drugs to treat many of the diseases in which chemokine receptors have been implicated, the development of novel therapeutics is still required to treat non-responders and to tackle the increasing problem of drug resistance. Drugs acting on members of the GPCR super family make up more than 45 % of all drugs currently in use (Pease and Horuk, 2009b), thus setting a good precedent for targeting chemokine receptors. The involvement in many inflammatory diseases of CCR2 and CCR5 in particular, makes them attractive targets for the pharmaceutical industry.

So far, at least 10 of the 19 classical chemokine receptors have been targeted for a variety of diseases by the development of either small molecule antagonists of the
receptor or therapeutic antibodies against the receptor or its chemokine ligands (Pease and Horuk, 2009a). However, to date only two anti-chemokine receptor drugs have been licensed. The CCR5 antagonist Maraviroc is used in HIV therapy (Westby and van der Ryst, 2010). Plerixafor (AMD3100), a CXCR4 antagonist, is used in combination with granulocyte-colony stimulating factor (G-CSF) to mobilize hematopoietic stem cells from the bone marrow to the peripheral blood for collection and subsequent autologous transplantation in patients with multiple myeloma and non-Hodgkin’s lymphoma (Wagstaff, 2009). Importantly, despite encouraging pre-clinical results in animal disease models and many phase I and II clinical trials in humans, there are currently no licensed drugs designed to modulate the negative roles of chemokine receptors in inflammatory diseases.

Clinical trials have been carried out to test various CCR2 and CCR5 antagonists, and antibodies targeting CCR2 or CCL2 in RA, MS and atherosclerotic cardiovascular disease, with mostly negative results (Gerlag et al., 2010; Haringman et al., 2006a; Horuk, 2009; Pease and Horuk, 2009a; Scholten et al., 2012; van Kuijk et al., 2010; Vergunst et al., 2008). Recent reviews have discussed several different theories to try to explain why so many drugs targeting individual chemokine receptors, and in particular CCR2 and CCR5, have failed to show efficacy in clinical trials (Horuk, 2009; Proudfoot, 2008; Zhao, 2010).

A common argument is the concept of redundancy within the chemokine system where a single chemokine may bind to multiple receptors and some receptors bind more than one chemokine. Whilst the traditional idea of complete redundancy is losing favour, the possibility that multiple chemokine/receptor combinations are involved in a particular disease and may potentially play the same or similar roles in different subtypes or stages of the disease, is accepted. Indeed in complex diseases such as MS and RA, several other chemokine receptors, in addition to CCR2 and CCR5, have been shown to help drive the pathophysiology of the diseases, and for MS the chemokine receptor expression profile can vary according to the disease subtype (Jalonen et al., 2002; Sorensen and Sellebjerg, 2001). Thus, mis-targeting or partial compensation of therapeutically targeted chemokines/receptors may lead to an apparent lack of effect. Therefore, it has been suggested that promiscuous antagonists, targeting groups of receptors with significant homology, may be a better approach, and work is currently
underway developing dual-specific antagonists that target CCR2 and CCR5 (Norman, 2011; Zheng et al., 2011)

A major problem facing the development of antagonists targeting chemokines and their receptors is the fact that pre-clinical trial validation work is carried out using animal disease models that do not perfectly mimic human pathologies. The species differences of the immune system include variation in chemokine receptor expression. While the expression profile of CCR2 on murine leukocytes closely resembles that observed in humans, some variability is observed for CCR5 with a much greater population of murine NK cells expressing this receptor (Mack et al., 2001). In addition, rodent and human CCR2/CCL2 and CCR5 orthologues show relatively low sequence homology, suggesting that some antibodies and antagonists developed against human receptors or chemokines may exhibit lower affinities for the rodent equivalents, leading to species selectivity of drugs (Saita et al., 2007).

Incomplete knowledge of chemokine receptor regulation has hindered the development of drugs targeting this system in several ways. Although in vitro studies have provided information on the drug concentration required to inhibit functional receptor responses on isolated cells, the level of receptor occupancy required to inhibit the role of the receptor in the disease in vivo has not been accurately defined (Horuk, 2009). In order to effectively block recruitment, it has been hypothesized that more than 90% receptor coverage is needed (Schall and Proudfoot, 2011), however some clinical trials achieving this level have still been unsuccessful (Vergunst et al., 2008; Zipp et al., 2006). Current therapeutics are commonly targeted at a single chemokine receptor in isolation. However, as many chemokine receptors are subject to cross-regulation from other cell surface proteins, the full extent of which is still being uncovered, targeting a specific receptor can have knock-on effects.

Despite the negative results so far, the future does look positive. There are currently many chemokine receptor drugs in clinical trials tackling some of these problems. The first crystal structures for a chemokine receptor, CXCR4, were recently published and will help improve future modelling of antagonist-receptor interactions. It has become apparent that better characterisation of the specific regulation of individual CRs is required in order to more accurately anticipate how the receptor will respond following
drug binding. Coupled with an improved understanding of the impact of chemokine receptor cross-talk, this knowledge will be vital to the future development of safe and effective therapeutics that target disease via manipulation of chemokine receptor activation states.

1.3 **Chemokine receptor regulation**

Tight regulation of the chemokine system is essential to enable individual cells to fine-tune their responses according to the specific composition of the local environment (Weber and Koenen, 2006). This control can be exerted by mechanisms affecting the receptor and/or its chemokine ligands, and can have both positive and negative effects on the cell’s ability to respond to a given chemokine (Bennett et al., 2011).

Regulation of chemokine receptor expression can be targeted at the level of gene regulation, mRNA and protein synthesis. However, these processes, taking many minutes to hours, are too slow to be solely responsible for the rapid changes in cellular responses that are typically observed (Thelen, 2001). Therefore, tight modulation of the presentation of functional chemokine receptors at the cell surface is essential, and can be achieved by affecting the activation state, signalling ability and/or cellular localization of the receptor. This rapid control can be mediated in response to ligand binding but also as a consequence of cross-talk from other receptors (Bennett et al., 2011). Some of the molecular mechanisms involved in the regulation are common to multiple chemokine receptors while others appear to be receptor-specific (Kelly et al., 2008).

1.3.1 **Long-term chemokine receptor regulation**

Long-term up- or down-regulation of chemokine receptors is achieved by controlling the cellular levels of receptor molecules through changes in gene expression, mRNA stability and protein degradation. With regards to leukocytes, the expression of chemokine receptors is tightly regulated on the different subtypes, and changes through the processes of cell differentiation, activation and polarization (Fantuzzi et al., 1999; Mantovani et al., 2004; Patel et al., 2001; Sallusto et al., 1999; Sallusto et al., 1998; Sebastiani et al., 2001). Chemokine receptors, including CCR2 and CCR5, are believed to be subject to epigenetic regulation in the form of histone modifications. CCR2 gene expression can be modulated by histone methylation in response to monocyte
differentiation (Tserel et al., 2010) or to stimulation with drugs such as methamphetamine (Ikegami et al., 2010). CCR5 transcription has been reported to be modulated by both histone methylation and acetylation in response to hypoxic conditions in macrophages (Tausendschon et al., 2011) or dependent on leukocyte cell type (Wierda et al., 2012). Host–pathogen interactions can also regulate chemokine receptor expression. For example, it was shown that bacterial lipopolysaccharide (LPS) interfered with CCL2-mediated recruitment of monocytes in vivo by down-regulating CCR2 expression (Zhou et al., 1999). LPS was found to act in vitro by affecting CCR2 mRNA stability (Sica et al., 1997; Xu et al., 1997), as did the inflammatory cytokines interleukin-1 (IL-1), tumour necrosis factor (TNF-a) and interferon-gamma (IFN-γ) (Penton-Rol et al., 1998; Sica et al., 1997), but with no major effect on CCR5 transcripts. In contrast, reactive oxygen intermediates produced by phagocytes for killing pathogens increased CCR2, CCR5 and CXCR4 mRNA expression and opposed the down-regulation induced by LPS (Saccani et al., 2000). Interestingly, chemokine receptor switch and modulation of mRNA expression has also been reported with Mycobacterium tuberculosis antigens and proposed to be part of a normal programme of cell co-ordination needed to contain infection (Arias et al., 2006).

Desensitisation is a term that is widely and variably used to describe a range of different processes negatively regulating chemokine receptors. It can encompass both the long-term negative regulation of receptor expression mentioned in the previous section and the more rapid process of down-modulation. Down-modulation is a feedback mechanism protecting cells from over-stimulation by transiently controlling the level of active receptors at the cell surface. The process of down-modulation can be either ligand-induced (homologous) or a result of cross-talk (heterologous), and various different mechanisms have been suggested (Kelly et al., 2008; Salanga et al., 2009).

1.3.2 Agonist-induced (homologous)

Receptors are typically desensitised in response to prolonged agonist stimulation via the process of homologous down-modulation (Figure 1.2A) (Kelly et al., 2008). Following agonist binding, signalling receptors are rapidly phosphorylated on their cytoplasmic tail, usually by a member of the G protein receptor kinase (GRK) family, which uncouples the G protein from the receptor and prevents further activation. Phosphorylated
receptors typically then interact with one of the β-arrestins that act as a scaffold enabling interaction with adapter proteins and targeting receptors for internalisation. This can lead to a permanent or transient loss of cell surface receptors due to degradation or subsequent recycling back to the cell surface of internalised molecules, respectively (Borroni et al., 2010).

(Figure 1.2) Homologous (A) and heterologous (B) chemokine receptor down-modulation. (A) Following agonist binding, the signalling chemokine receptor is rapidly phosphorylated on its cytoplasmic tail, usually by a G protein receptor kinase (GRK); this uncouples the G protein, which dissociates into guanosine triphosphate (GTP)-bound Ga and the Gβγ complex. Phosphorylated receptors then interact with a β-arrestin, which acts as a scaffold targeting the receptor for internalisation. Once internalised, the receptor follows recycling or degradation pathways. (B) Receptor X mediates cross-phosphorylation of the chemokine receptor, which may involve protein kinase C (PKC), leading to inhibition of chemokine-induced signalling and in some cases internalisation of the receptor. This figure was modified from Bennett et al., (2011).
1.3.3 Negative Cross-talk (heterologous desensitisation)

A chemokine receptor can also be negatively regulated via indirect cross-talk from other cell surface proteins and receptors, which often leads to down-modulation (Salanga et al., 2009), or in some cases to down-regulation (McKimmie et al., 2009; Zhou et al., 1999), of the chemokine receptor. The cross-talk can be targeted at the receptor itself, the heterotrimeric G protein it is coupled to or downstream signalling components, resulting in trans-inhibition of chemokine receptor activity. Heterologous down-modulation (Figure 1.2B) often involves rapid signalling inactivation of surface chemokine receptors, inhibiting chemokine-induced intracellular calcium mobilization. It happens in both human cells and immortalized cell lines whether the cross-talk comes from another chemokine receptor such as for CXCR4 with CCR5 (Hecht et al., 2003; Honczarenko et al., 2002), another GPCR as for CCR5 with formyl peptide receptors (Le et al., 2001), or an unrelated surface receptor such as the T cell receptor (TCR) with CXCR4 (Schneider et al., 2009). In many reports, the inactivation has been linked to rapid cross-phosphorylation of the chemokine receptor, with some studies identifying protein kinase C (PKC) as the point of convergence between the different receptor pathways (Le et al., 2001; Nasser et al., 2005; Richardson et al., 1998; Richardson et al., 2000). Alternatively, receptor inactivation can result from indirect effects as reported for CXCR4 either in pre-B cells, where CD24 altered its distribution in membrane lipid rafts by changing cholesterol levels (Schabath et al., 2006), or in leukaemia cells, where an oncoprotein has been shown to highjack kinases of the CXCR4-dependent calcium pathway (Ptasznik et al., 2002). The process of signalling inactivation involved in heterologous down-modulation can be, but is not always, followed by a reduction in cell surface expression of chemokine receptors believed to be due to internalisation (Finley et al., 2008; Le et al., 2001; Richardson et al., 2003). Conversely, cross-talk induced receptor internalisation can occur without prior desensitisation of chemokine-mediated signalling, as recently shown with the cross-regulation of CC chemokine receptors 1, 2 and 5 by TLR2 on human blood monocytes (Fox et al., 2011). In this instance, activation of TLR2 via lipoteichoic acid (LTA) triggered relatively slow phosphorylation and removal of cell surface CCR5 molecules by activating the machinery used to support chemokine-dependent endocytosis (Fox et al., 2011). Different pathways of chemokine receptor desensitisation following TLR2 cross-talk have been suggested by other groups, supporting the view that desensitisation is a
ligand, receptor and cell type specific process (Alves-Filho et al., 2009; McKimmie et al., 2009).

1.3.4 Transactivation and Synergy

Cross-talk can also positively regulate chemokine receptors via transactivation or synergistic mechanisms. Chemokine receptors can transactivate or be transactivated by several different types of receptors, but one of the most well-studied is the case of receptor tyrosine kinases (RTKs) including various growth factor receptors (Salanga et al., 2009). Stimulated RTKs transactivate chemokine receptors leading to functional signalling responses, either by modulating the availability and activity of their chemokine ligands via transcriptional or post-transcriptional regulation, or by as yet uncharacterised ligand-independent mechanisms potentially involving cytosolic tyrosine kinases and receptor phosphorylation. Reciprocally, chemokine receptors can transactivate RTKs.

Some combinations of chemokines and cytokines can act synergistically to amplify inflammatory responses, probably due to integration of multiple different signalling pathways. The chemokinetic effect of cytokines is believed to prime cells to increase their migratory response to chemokines, as found with IL-5-enhancing eosinophil chemotaxis in response to CCL11 (Gouwy et al., 2005). In addition, synergy between different chemokine receptors has been involved in the migration of primary cells. For example, CXCL8 has been shown to increase monocyte migration towards suboptimal concentrations of CCL2 and CCL7 (Gouwy et al., 2008), while CCL2 and CCL7 can enhance neutrophil chemotaxis towards a suboptimal concentration of CXCL8 (Gouwy et al., 2004). Furthermore, cross-talk initiated from non-chemokine or cytokine receptors is also emerging as an important but complex phenomenon that modulates innate immune responses to pathogens. Synergy between CCR2 and N-formyl peptide receptor (FPR) agonists has recently been shown to co-operate with TLR4 for production of the inflammatory chemokine CXCL8 upon LPS stimulation, which in turn synergizes with CCL2 as described previously to mediate monocyte chemotaxis (Gouwy et al., 2009). Finally, synergy can also involve non-protein ligands, as demonstrated by the potentiation of calcium signalling reported for CXCR2 upon co-stimulation of the PY2 nucleotide receptor (Werry et al., 2002).
1.4 Receptor trafficking

Figure 1.3 Summary of the endocytic trafficking pathways followed by agonist-stimulated chemokine receptors.
In response to prolonged agonist stimulation, chemokine receptors can be internalised via clathrin- or caveolin-dependent endocytosis, or other less well characterised independent pathways. Internalised receptors are then either sent for lysosomal degradation or recycled via one of a range of rapid or slow recycling pathways. CCP, clathrin coated pit; CCV, clathrin coated vesicle; EE, early endosome; ERC, endocytic recycling centre; LE, late endosome; LY, lysosome; MVB, multivesicular body; RE, recycling endosome.

1.4.1 Internalisation

In response to prolonged agonist stimulation, chemokine receptors can be internalised via clathrin- or caveolin-dependent endocytosis or other less well characterised independent pathways (Figure 1.3; Borroni et al., 2010). Clathrin-mediated endocytosis requires β-arrestin binding to the chemokine receptor, which interacts with the clathrin adapter molecule AP2. This enables localisation of the chemokine receptor into clathrin coated pits (CCPs), which are then cleaved from the plasma membrane by the action of dynamin to form clathrin coated vesicles (CCVs). The intracellular CCVs then deliver the chemokine receptors to early endosomes and subsequently sorting endosomes. Caveolin-mediated endocytosis involves recruitment into cholesterol-rich caveolin-containing caveosomes that once internalised also fuse with early endosomes. Interestingly, CCR2 and CCR5 have been suggested to follow both clathrin- and
caveolin-dependent pathways and the route of endocytosis could be cell type-dependent (Andjelkovic et al., 2002; Garcia Lopez et al., 2009; Ge and Pachter, 2004; Mueller et al., 2002a; Signoret et al., 2005; Venkatesan et al., 2003).

Chemokine receptors are not always internalised following agonist stimulation and the choice of ligand can influence the ability of a receptor to internalise. A range of chemokine receptors, including CCR4 (Mariani et al., 2004) and CCR7 (Byers et al., 2008), have been described to be efficiently internalised in response to some but not all of their chemokine ligands. In addition, the extent of internalisation observed for the same chemokine receptor stimulated with its different chemokine ligands can vary widely, as reported for CCR2 (Berchiche et al., 2011) and CXCR2 (Feniger-Barish et al., 2000). It appears that the strength and stability of receptor/β-arrestin interactions may be critical in determining whether or not an agonist-activated chemokine receptor is internalised, as described for CCR7 and CCR2 (Berchiche et al., 2011; Byers et al., 2008; Zidar et al., 2009). Therefore, in some cases the chemokine may influence the internalisation outcome via modulating this receptor/β-arrestin interaction. For example, CCR7 is not internalised in response to stimulation with its ligand CCL21 but it is internalised in response to another ligand CCL19, which mediates a more robust interaction with β-arrestin 2 (Byers et al., 2008; Zidar et al., 2009).

Once internalised, the intracellular path followed by a chemokine receptor determines its fate, i.e. being sent for degradation or being sequestered intracellularly before returning to the cell surface (Figure 1.3). Receptors can follow one path exclusively, such as CCR5 (Delhaye et al., 2007; Mack et al., 1998; Mueller and Strange, 2004; Signoret et al., 2000) or CXCR3 (Meiser et al., 2008), which are sent for recycling or degradation, respectively. Alternatively, they can enter either pathway depending on various factors discussed later, as reported for CXCR2 (Fan et al., 2003) and CXCR4 (Tarasova et al., 1998; Zhang et al., 2004). In contrast to CCR5, relatively little research has been carried out regarding the fate of internalised CCR2.

### 1.4.2 Recycling and resensitisation

Internalised receptors can be returned to the cell surface in an active form via the processes of recycling and resensitisation, and both fast and slow pathways have been described. Fast recycling can take place directly from the early endosome or
alternatively the receptor can be sorted to the endocytic recycling centre and return to the plasma membrane via recycling endosomes (Figure 1.3; Grant and Donaldson, 2009; Hopkins et al., 1994). Internalised CCR5 has been shown to be recycled via recycling endosomes (Signoret et al., 2000) and two reports suggested that some internalised CCR2 may be recycled (Andjelkovic et al., 2002; Wang et al., 1993a).

Most ligands are dissociated from their receptors during passage through the endosomal compartments, often due to the reduction in pH (Mellman et al., 1986). However, CCR5 agonists only slowly dissociate in a pH-independent manner and receptor/agonist complexes can undergo multiple cycles of endocytosis and recycling (Signoret et al., 2004; Signoret et al., 2000). CCR5 agonist removal requires < pH4, which is much below the physiological pH of endocytic organelles (Signoret et al., 2004). This is receptor specific, as the same agonists when bound to the atypical chemokine receptor D6 dissociate at endosomal pH (Weber et al., 2004).

1.4.3 Degradation

Alternatively, internalised receptors can be sent for degradation (Figure 1.3). For all reported chemokine receptors, with the exception of CXCR3 (Meiser et al., 2008), this degradation has been shown to be lysosome-mediated without proteasome involvement (Borroni et al., 2010). CCR5 is not degraded in response to agonist stimulation (Signoret et al., 2000). In contrast, a recent report showed some limited colocalisation of CCR2B with a lysosomal marker following agonist stimulation, suggesting that in some circumstances it may be degraded (Garcia Lopez et al., 2009).

1.4.4 Factors determining chemokine receptor fate following agonist stimulation

The decision between entry into the recycling or degradative pathway can be dependent on receptor-specific factors, as must be the case for chemokine receptors that uniquely enter one pathway. Alternatively for receptors that can enter either pathways, external factors such as the cell type and duration of ligand treatment could be responsible, as reported for CXCR2 (Fan et al., 2003) and CXCR4 (Tarasova et al., 1998; Zhang et al., 2004).
Endocytosis and subsequent sorting of internalised chemokine receptors to the recycling or degradative pathways requires complex interactions of the receptors with the machinery mediating endocytosis and movement of molecules between intracellular compartments. Some protein interactions implicated in modulating the fate of chemokine receptors have been described (Table 1.4). The C-terminal cytoplasmic tail domains of the receptors are critical for many of these interactions. Whereas, there is only one form of CCR5, two alternatively spliced isoforms of CCR2 exist (CCR2A and CCR2B) and they differ only in their cytoplasmic tails (Charo et al., 1994). CCR2B is the predominant isoform present in monocytes (Tanaka et al., 2002; Wong et al., 1997).

Adaptor proteins recognise specific determinants, mainly short peptide sequence motifs and post-translational modifications, in the cytoplasmic domains of membrane proteins and receptors including chemokine receptors (Borroni et al., 2010; Marchese et al., 2008). Some short peptide sequence motifs, such as dileucine- or tyrosine-based motifs, bind to various adaptor proteins and can regulate different steps in internalisation, intracellular trafficking and sorting to subcellular compartments (Pandey, 2010). One of the major roles of the dileucine motif is its interaction with AP2, which is a key protein for clathrin-mediated endocytosis. Several, but not all, chemokine receptors contain dileucine motifs in their cytoplasmic tails (Borroni et al., 2010; Marchese et al., 2008). The SSLKIL motif located in the CXCR4 tail has been shown to be important for ligand-independent phorbol ester-mediated internalisation of this receptor involving PKC (Orsini et al., 1999, 2000; Signoret et al., 1997; Signoret et al., 1998). Dileucine motifs lacking the preceding serines have been shown to be required for agonist-induced CXCR2 internalisation (Fan et al., 2001b) and to contribute towards the early phase of agonist-induced CCR5 internalisation (Figure 1.1; Kraft et al., 2001). No functional dileucine motifs have yet been identified in the cytoplasmic tails of either CCR2 isoform (see Figure 1.1 for CCR2B). As dileucine motifs also interact with other adaptor proteins, their potential involvement in intracellular trafficking of endocytosed chemokine receptors remains to be explored.

To date, three major types of tyrosine-based motifs, NPXY, GDAY and YXX Φ, where X is any residue and Φ is a residue with a large bulky hydrophobic side chain, have been recognised (Pandey, 2010). The tyrosine in the fourth position of NPXY and GDAY motifs is critical for receptor endocytosis via interaction with clathrin adaptor proteins
including AP2 and Dab-2 (Pandey, 2010). Due to their interaction with a wider range of adaptor proteins, YXXΦ motifs can be involved in endocytosis or lysosomal sorting, and this appears to be dependent on localisation of the motif and the identity of the X residues (Bonifacino and Traub, 2003). Although, some chemokine receptors have potential tyrosine based motifs in their cytoplasmic tails (Marchese et al., 2008), no functional roles have yet been assigned to them in this case.

Two other determinants, the PDZ ligand motif and ubiquitination, have received much interest recently, and were shown to support recycling or degradation of chemokine receptors, respectively. At least 12 chemokine receptors have been identified as containing potential PDZ ligand motifs in their extreme C-terminal cytoplasmic tail (Marchese et al., 2008). The PDZ ligand motifs are presumed to interact with PDZ domain containing proteins of the sorting machinery, but only a few of these interactions have been unveiled. CCR5 post-endocytic sorting to the recycling pathway is dependent on its PDZ ligand motif (Figure 1.1; Delhaye et al., 2007), which has been shown to interact with a protein implicated in receptor recycling called ERM Binding Protein 50 or Na+/H+ Exchanger Regulatory Factor (EBP50/NHERF-1; Hammad et al., 2010). For CXCR2 that can be both recycled following short ligand exposure and degraded following more prolonged ligand treatment (Fan et al., 2003), the PDZ ligand motif serves to delay degradation by preventing lysosomal sorting, due probably to interaction with an as yet unknown PDZ-containing protein (Baugher and Richmond, 2008). Notably, no PDZ ligand motif has been identified in the CCR2 cytoplasmic tail (Figure 1.1).

Ubiquitination has emerged as an important modification for sending the chemokine receptor CXCR4 (Marchese and Benovic, 2001) and other GPCRs (Marchese et al., 2008) to degradation. For CXCR4, CXCL12 stimulation leads to ubiquitination of cell surface receptors as well as ubiquitin-dependent endocytosis and trafficking of ubiquitinated CXCR4 to lysosomes (Marchese et al., 2003; Mines et al., 2009). However, ubiquitination does not seem to be required for the degradation of all chemokine receptors (Baugher and Richmond, 2008; Meiser et al., 2008). The ubiquitination state has recently been shown to play an important role in the intracellular trafficking of another related receptor, CXCR7, which in contrast to CXCR4 is recycled to the cell surface after internalisation (Canals et al., 2012). CXCR7 is constitutively
ubiquitinated and has been shown to be reversibly de-ubiquitinated in response to CXCL12 treatment (Canals et al., 2012).

The affinity of chemokine receptor interactions with β-arrestins can influence the fate of internalised receptors. Indeed, GPCRs that rapidly recycle (Class A) preferentially bind β-arrestin 2 with low affinity and dissociate from it upon internalisation, whereas those that slowly recycle or are degraded (Class B) bind both β-arrestins with high affinity and remain β-arrestin-bound inside the cell (Oakley et al., 2000). So far, only class B chemokine receptors have been described, with evidence for β-arrestins binding to agonist-treated CCR2 and CCR5 in internal compartments (Fox et al., 2011; Minsaas et al., 2010).

In addition to the duration of agonist treatment, the identity of the agonist itself can impact upon the fate of a receptor. For instance, with CCR5, any agonist-stimulated receptors seem to follow the recycling route but the distribution of receptors along the pathway could be agonist-specific (Figure 1.4). Following internalisation, CCR5 receptors treated with the natural chemokine CCL5 are located in recycling endosomes (RE) before re-accumulating in the plasma membrane (Signoret et al., 2000). In contrast, they keep cycling back from the cell surface to the RE after exposure to the chemically modified aminooxypentane-regulated on activation, normal T cell expressed and secreted (AOP-RANTES; Signoret et al., 2000), become trapped in the trans-Golgi network (TGN) after passage through RE with N\(^\alpha\)-(n-nonanoyl)-des-Ser\(^1\)-[L-thioprolin\(e^2\),L-α-cyclohexylglycine\(^3\)]-RANTES (PSC-RANTES; Escolà et al., 2010), and appear to bypass the RE to accumulate in the TGN with a N terminal Methionine RANTES (MET-RANTES; Kiss et al., 2009).
Table 1.4 Reported protein interactions with chemokine receptors.

<table>
<thead>
<tr>
<th>Interacting Protein</th>
<th>Chemokine Receptor</th>
<th>Suggested Function</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIP4</td>
<td>CXCR4</td>
<td>Ubiquitination/Degradation</td>
<td>(Bhandari et al., 2009)</td>
</tr>
<tr>
<td>α-catenin</td>
<td>CCR5</td>
<td>Receptor organisation at plasma membrane</td>
<td>(Schweneker et al., 2004)</td>
</tr>
<tr>
<td>AP2</td>
<td>CXCR2</td>
<td>Internalisation</td>
<td>(Fan et al., 2001b)</td>
</tr>
<tr>
<td>β-arrestins</td>
<td>Multiple including CCR2 and CCR5</td>
<td>Desensitisation/Internalisation/Signalling</td>
<td>(Aragay et al., 1998; Vila-Coro et al., 1999a) (DeFea, 2011)</td>
</tr>
<tr>
<td>DRiP78</td>
<td>CCR5, CXCR4</td>
<td>Membrane localisation/Receptor signalling complex formation</td>
<td>(Kuang et al., 2012)</td>
</tr>
<tr>
<td>EBP50/NHERF-1</td>
<td>CCR5</td>
<td>Recycling</td>
<td>(Hammad et al., 2010)</td>
</tr>
<tr>
<td>Filamin A</td>
<td>CCR2</td>
<td>Internalisation</td>
<td>(Minsaas et al., 2010)</td>
</tr>
<tr>
<td>FROUNT</td>
<td>CCR2, CCR5</td>
<td>Clustering to leading edge of lamellipodia/Link to signalling cascade leading to chemotaxis</td>
<td>(Terashima et al., 2005; Toda et al., 2009)</td>
</tr>
<tr>
<td>GASP</td>
<td>CXCR2</td>
<td>Lysosomal sorting for degradation</td>
<td>(Heydorn et al., 2004)</td>
</tr>
<tr>
<td>GRKs</td>
<td>Multiple including CCR2 and CCR5</td>
<td>Receptor Phosphorylation</td>
<td>(Aragay et al., 1998)(Oppermann et al., 1999)(Vila-Coro et al., 1999a)</td>
</tr>
<tr>
<td>Hip</td>
<td>CXCR2, CXCR4</td>
<td>Regulation of trafficking including internalisation</td>
<td>(Fan et al., 2002)</td>
</tr>
<tr>
<td>Importin 7</td>
<td>CCR2</td>
<td>Nuclear import</td>
<td>(Favre et al., 2008)</td>
</tr>
<tr>
<td>IQGAP1</td>
<td>CXCR2</td>
<td>Signalling</td>
<td>(Neel et al., 2011)</td>
</tr>
<tr>
<td>JM4</td>
<td>CCR5</td>
<td>Membrane localisation</td>
<td>(Schweneker et al., 2005)</td>
</tr>
<tr>
<td>Myosin Vb</td>
<td>CXCR2</td>
<td>Regulating recycling</td>
<td>(Fan et al., 2004)</td>
</tr>
<tr>
<td>NMMHC-IIA</td>
<td>CCR5</td>
<td>May have a role in cell migration</td>
<td>(Rey et al., 2002)</td>
</tr>
<tr>
<td>PKC</td>
<td>Multiple, including CCR2</td>
<td>Receptor phosphorylation</td>
<td>(Oppermann et al., 1999)</td>
</tr>
<tr>
<td>PP2A core enzyme</td>
<td>CXCR2</td>
<td>Receptor desphosphorylation</td>
<td>(Fan et al., 2001a)</td>
</tr>
<tr>
<td>Rab11-FIP</td>
<td>CXCR2</td>
<td>Regulating recycling</td>
<td>(Fan et al., 2004)</td>
</tr>
<tr>
<td>TRN-1</td>
<td>CCR2</td>
<td>Nuclear import</td>
<td>(Favre et al., 2008)</td>
</tr>
<tr>
<td>USP14</td>
<td>CXCR4</td>
<td>Deubiquitination/Degradation</td>
<td>(Mines et al., 2009)</td>
</tr>
</tbody>
</table>

AIP4, E3 ubiquitin ligase atrophin interacting protein 4; AP2, Adaptin 2; DRiP79, Dopamine receptor-interactin protein 78; GASP, G protein-coupled receptor-associated sorting protein; GRK, G protein receptor kinase; Hip, Hsc70-interacting protein; JM4, Jena-Muenchen4; NMMHC-IIA, nonmuscle myosin H chain-IIA; PKC, protein kinase C; Rab11-FIP, Rab11 family interacting protein; TRN-1, transportin-1; VPS4, vacuolar protein sorting-associated protein 4A
Figure 1.4 Different trafficking routes proposed for agonist-treated CCR5.
Following agonist-stimulation, CCR5 trafficks through the early endocytic pathway towards recycling and avoiding degradation. However, there are suggestions that the route followed by CCR5 may be ligand-dependent as summarised here for CCL5 (Signoret et al., 2000) and three of its chemical derivatives, AOP-RANTES (Signoret et al., 2000), PSC-RANTES (Escola et al., 2010) and MET-RANTES (Kiss et al., 2009). EE/SE, early endosome/sorting endosome; ERC, endocytic recycling centre; MVB/LE/LY, multivesicular body/late endosome/lysosome; N, nucleus; RE, recycling endosome; TGN, trans Golgi network. This figure is modified from Bennett et al., (2011).

1.5 Thesis aims

It is established that GPCRs undergo desensitisation in response to prolonged agonist stimulation, a process that often involves internalisation and intracellular transport of stimulated receptors. General intracellular trafficking routes followed by GPCRs have been described, and for some well-studied receptors details of the proteins involved are known. However, how generic these routes actually are remains to be established. Are the pathways tailored to individual receptors and does each receptor have its own unique network of interacting proteins involved in desensitisation?
This thesis aimed to add to the current knowledge of chemokine receptor regulation and to address the above questions by focusing on the case of the chemokine receptors CCR2B and CCR5. Although these receptors bind different ligands, they are both inflammatory chemokine receptors and play complementary roles in the recruitment of a subset of leukocytes to sites of infection. In addition to their ability to heterodimerise and negatively cross-regulate each other, they are both also subject to cross-desensitisation by LTA. Do these similarities imply that they may follow the same intracellular trafficking pathways? However, despite having high overall sequence homology (Figure 1.5), CCR2 and CCR5 differ in their C-terminal cytoplasmic tail (Figures 1.1 and 1.5), which is key for GPCR trafficking. Does this variation result in differing responses to agonist stimuli and if so, are these differences due to interactions with different proteins?

I took a two pronged approach to answer these questions. Chapter 3 describes initial studies carried out with the objective of determining a suitable cell system in which to tackle these questions. Then the first approach described in Chapter 4 compares and contrasts the intracellular trafficking response of CCR2B to what is known about the well-studied CCR5 showing distinct differences in the pathways followed by the two receptors and also expanding upon the current knowledge for CCR2B. During this thesis, multiple forms of CCR2B were identified and thus this chapter also aimed to characterise these forms to determine why they exist and what their individual roles are. In Chapter 5, I describe two complementary proteomic studies carried out with the objective of identifying proteins interacting with these receptors.
Figure 1.5 Schematic of CCR2B topology showing comparison of the amino acid sequence with CCR5.
The amino acid sequence of human CCR2B is depicted and residues that are identical to those in human CCR5 are indicated in grey. The probable CCR2B topology shown in this schematic is based upon a two-dimensional topology model for CCR5 (Oppermann, 2004). There is some inconsistency in the precise transmembrane domain boundaries between the published CCR2B models (Kim et al., 2011; Mirzadegan et al., 2000; Shi et al., 2002), with one recent model exhibiting some very short intra- and extra-cellular loops (Kim et al., 2011).
2 Materials and Methods

2.1 Reagents

All chemicals were from Sigma-Aldrich (Gillingham, UK) unless otherwise specified. The complete protease inhibitor cocktail and ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail were from Roche Diagnostic Ltd (Burgess Hill, UK). Chemokines were from R&D Systems (Abingdon, UK) with the exception of CCL4, which was purchased from PeproTech (London, UK). PSC-RANTES was a gift from Oliver Hartley (University of Geneva, Switzerland). Purified LTA from Staphylococcus aureus was purchased from Invivogen (Toulouse, France). TAK-779 was obtained from the NIH AIDS Research and Reference Reagent Program (Germantown, MD, USA). UCB35625 and GF109203 (bisindolylmaleimide) were purchased from Tocris Bioscience, (Bristol, UK) and Calbiochem (Nottingham, UK), respectively. All tissue culture medium and supplements were from GIBCO® (Life Technologies Ltd, Paisley, UK) with the exception of foetal bovine serum (FBS), which was from PAA (Yeovil, UK) or HyClone (Fisher Scientific UK Ltd, Loughborough, UK). All tissue culture plastic-ware was from Costar®/Corning® (Corning Life Sciences, Amsterdam, The Netherlands). 5 ml polypropylene tubes used for flow cytometry were from BD Biosciences (Oxford, UK).

2.2 Receptor agonists/antagonists

The standard and other names for the chemokine receptor agonists and antagonists used in this project are given in Table 2.1. Chemokines were used at 100 nM for down-modulation and endocytosis assays and 10 nM for calcium signalling assays unless otherwise specified.
Table 2.1 Chemokine receptor agonists and antagonists used in this project.

<table>
<thead>
<tr>
<th>Standard Name</th>
<th>Other Names</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL2</td>
<td>MCP-1, MCAF-1</td>
<td>CCR2 agonist</td>
</tr>
<tr>
<td>CCL4</td>
<td>MIP-1β</td>
<td>CCR5 agonist</td>
</tr>
<tr>
<td>CCL5</td>
<td>RANTES</td>
<td>CCR1 and CCR5 agonist</td>
</tr>
<tr>
<td>PSC-RANTES</td>
<td>N-nonanoyl, des-Ser[1-L-thioproline2, L-cyclohexylglycine3]-RANTES(2-68)</td>
<td>N-terminally modified synthetic analogue of CCL5 (Hartley 2004)</td>
</tr>
<tr>
<td>TAK-779</td>
<td>N,N- dimethyl-N-[4-[[2-(4-methylphenyl)-6,7-dihydro-5H-benzo- cyclohepten-8-yl][carbonyl]amino]benzyl]tetrahydro-2H- pyran-4-aminium chloride</td>
<td>CCR5 and, to a lesser extent, CCR2 antagonist (Baba 1999)</td>
</tr>
<tr>
<td>UCB35625</td>
<td>trans-isomer J113863</td>
<td>CCR1 and CCR3 antagonist (Sabroe 2000)</td>
</tr>
</tbody>
</table>

2.3 Buffers and solutions

Table 2.2 Buffers and their compositions.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 % NP-40 lysis buffer</td>
<td>150 mM NaCl, 20 mM Tris-HCl (pH8.0), 2 mM EDTA, 1 % Igepal (NP-40 replacement)</td>
</tr>
<tr>
<td>1 % Triton lysis buffer</td>
<td>20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1 % Triton X-100</td>
</tr>
<tr>
<td>binding medium (pH7.1)</td>
<td>1X RPMI without bicarbonate, 10 mM HEPES, 0.2 % BSA, pH7.1</td>
</tr>
<tr>
<td>coomassie solution</td>
<td>0.1% Phast Gel™ Blue K (Healthcare, Buckinghamshire UK) in 30 % methanol, 10 % acetic acid</td>
</tr>
<tr>
<td>elution medium</td>
<td>1X RPMI, 10 mM MES, 0.2 % BSA, pH3.5</td>
</tr>
<tr>
<td>FACS buffer</td>
<td>1 % BSA, 0.05 % sodium azide in PBS</td>
</tr>
<tr>
<td>glutathione elution buffer</td>
<td>20 – 50 mM reduced glutathione, 100 mM Tris-HCl (pH 8.0), 120 mM sodium chloride</td>
</tr>
<tr>
<td>homogenisation medium</td>
<td>0.25 M sucrose, 1 mM EDTA, 10 mM HEPES-NaOH, pH 7.4</td>
</tr>
<tr>
<td>LB</td>
<td>10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract</td>
</tr>
<tr>
<td>LB Agar</td>
<td>17.5 g/L agar, 10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract</td>
</tr>
<tr>
<td>PBS</td>
<td>0.14 M NaCl, 0.01 M PO₄ Buffer, 0.003 M KCl</td>
</tr>
<tr>
<td>quenching solution</td>
<td>50 mM ammonium chloride in PBS</td>
</tr>
<tr>
<td>RIPA buffer</td>
<td>150 mM NaCl, 50 mM Tris-HCl (pH8.0), 5 mM EDTA (pH8.0), 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, pH8.0</td>
</tr>
<tr>
<td>sample buffer</td>
<td>62.5 μM Tris-HCl pH 6.8, 35 % glycerol, 2 % SDS, 0.16 % bromophenol blue, +/- 5 % β-mercaptoethanol (reducing/non-reducing)</td>
</tr>
<tr>
<td>saponin staining buffer</td>
<td>0.05 % saponin, 1% BSA, 1 % FBS, in PBS</td>
</tr>
<tr>
<td>staining buffer (for intact cells)</td>
<td>1 % BSA, 1 % FBS, in PBS</td>
</tr>
<tr>
<td>stripping buffer</td>
<td>50 mM Tris-HCl, 2% SDS, 100 mM β-mercaptoethanol</td>
</tr>
<tr>
<td>TAE buffer</td>
<td>40 mM Tris-acetate, 1 mM EDTA</td>
</tr>
<tr>
<td>ubiquitination lysis buffer</td>
<td>50 mM Tris-HCl (pH8.0), 150 mM NaCl, 5 mM EDTA, 0.5 % (w/v) sodium deoxycholate, 1 % Igepal, 0.1 % (w/v) SDS, 20 mM NEM, protease inhibitor cocktail</td>
</tr>
</tbody>
</table>
2.4 Antibodies

The antibodies used in this project are described in Table 2.3 and were purchased from AbD Serotec (Kidlington, UK), BD Pharmingen™ (BD Biosciences), BD Transduction Laboratories (BD Biosciences), Covance (Princeton, NJ, USA), eBioscience Ltd (Hatfield, UK), Epitomics (Burlingame, CA, USA), GE Healthcare (Little Chalfont, UK), Invitrogen (Paisley, UK), Jackson Immuno Research Laboratories Inc (West Grove, PA, USA), Molecular Probes (Invitrogen), Novus Biologicals (Cambridge, UK), ProSci Inc (Poway, CA, USA), R&D Systems, Roche Diagnostic Ltd, Santa Cruz Biotechnology Inc (Heidelberg, Germany), Sigma-Aldrich and Stratech Scientific Ltd (Newmarket, UK) as indicated.

Table 2.3 Antibody specificities, sources and concentrations used.

<table>
<thead>
<tr>
<th>SPECIFICITY</th>
<th>Antibody</th>
<th>SPECIES &amp; ISO TYPE</th>
<th>APPLICATION &amp; CONCENTRATION</th>
<th>SOURCE &amp; CATALOGUE NUMBER (#)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR2 N-term</td>
<td>R&amp;D α-CCR2 Mouse IgG2b Rabbit monoclonal</td>
<td>FC 5 µg/ml unless otherwise stated</td>
<td>R&amp;D Systems, # MAB150</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E68      Rabbit monoclonal</td>
<td>IP 1:50</td>
<td>Epitomics # 2068-1</td>
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<tr>
<td></td>
<td>H-40     Rabbit polyclonal</td>
<td>IP 2 µg/sample WB 1 µg/ml</td>
<td>Santa Cruz Biotechnology Inc, # sc-30031</td>
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<tr>
<td>CCR2B C-term</td>
<td>CCR2 C-20 Goat polyclonal</td>
<td>WB 0.67 µg/ml</td>
<td>Santa Cruz Biotechnology Inc, # sc-6228</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MC5      Mouse IgG2a</td>
<td>FC 5 µg/ml unless otherwise stated</td>
<td>Produced in-house</td>
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<tr>
<td></td>
<td>T21/8    Mouse IgG1</td>
<td>IF 5 5 µg/ml IP 7.5 µg/sample WB 2.15 µg/ml</td>
<td>Santa Cruz Biotechnology Inc, # sc-53792</td>
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<tr>
<td>CCR5 N-term</td>
<td>CCR5 NT Rabbit polyclonal Mouse IgG1</td>
<td>IP 2 µg/sample</td>
<td>ProSci Inc, # 1112</td>
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<td>2D7      Mouse IgG2a</td>
<td>IP 5 µg/sample</td>
<td>BD Pharmingen™, # 555990</td>
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<td>3A9      Mouse IgG2a</td>
<td>IP 5 µg/sample</td>
<td>BD Pharmingen™, # 556041</td>
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<tr>
<td></td>
<td>HEK/1/85a Rat IgG2a</td>
<td>IP 5 µg/sample</td>
<td>AbD Serotec # MCA2175</td>
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<tr>
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<td>CCR5 C-20 Goat polyclonal</td>
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<td></td>
<td>R-C10 Mouse IgG1</td>
<td>WB 1 µg/ml</td>
<td>Santa Cruz Biotechnology Inc, # sc-57072</td>
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</table>
Table 2.3 Antibody specificities, sources and concentrations used.

### ANTIBODIES AGAINST TAGS

<table>
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<th>Specificity</th>
<th>Antibody</th>
<th>Species &amp; Isotype</th>
<th>Application &amp; Concentration</th>
<th>Source &amp; Catalogue Number (#)</th>
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<tbody>
<tr>
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<td>HA.11</td>
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<td>FC 5 µg/ml</td>
<td>Covance, # MMS101P/MMS-101R</td>
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<td></td>
<td></td>
<td></td>
<td>WB 4 µg/ml</td>
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<td>IF 5 µg/ml</td>
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<td></td>
<td>12CA5</td>
<td>Mouse IgG2b</td>
<td>FC as stated</td>
<td>Gift from Paul Pryor (CII,</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>WB 1 µg/ml</td>
<td>University of York, UK) and</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IP 5 - 100 µg/sample</td>
<td>bought from Roche Diagnostic</td>
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<td>Ltd, # 11583816001</td>
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<td>FLAG M2</td>
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### OTHER PRIMARY ANTIBODIES

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<td>IF 5 µg/ml</td>
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<td></td>
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<td>610456</td>
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<tr>
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<td>Mouse IgG2b</td>
<td>IF 5 µg/ml</td>
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</tr>
<tr>
<td></td>
<td>clone IB5</td>
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<td>LMCB, UCL, London, UK)</td>
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<tr>
<td>Lamp1</td>
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<td>IF 1/1000 dilution</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>WB 1/1000 dilution</td>
<td>(Department of Biochemistry,</td>
</tr>
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<td>University of Bristol, UK)</td>
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<td>Human</td>
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<tr>
<td>Rab11</td>
<td>α-Rab11</td>
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<tr>
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<tr>
<td>Nucleoporin</td>
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<td>IF 5 µg/ml</td>
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<td>Mouse IgG2b</td>
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<tr>
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<td>α-Na/K</td>
<td>Mouse IgG1 κ</td>
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<td>α-caveolin 1</td>
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<td></td>
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<td></td>
<td>610406 Clone 2297</td>
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<tr>
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<td>α-Lamp1</td>
<td>Mouse IgG2b</td>
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<td></td>
<td></td>
<td></td>
<td>WB 1 µg/ml</td>
<td>611042</td>
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<tr>
<td>BiP/GRP78</td>
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<td>β-catenin</td>
<td>Anti-β-</td>
<td>Mouse IgG1</td>
<td>WB 0.5 µg/ml</td>
<td>BD Transduction Laboratories, #</td>
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<tr>
<td></td>
<td>catenin</td>
<td></td>
<td></td>
<td>610153</td>
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<tr>
<td>GST</td>
<td>α-GST</td>
<td>Goat polyclonal</td>
<td>WB 1/2000 –1/ 5000 dilution</td>
<td>GE Healthcare, # 27-4577-01</td>
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Table 2.3 Antibody specificities, sources and concentrations used.

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<th>Antibody</th>
<th>Species &amp; Isotype</th>
<th>Application &amp; Concentration</th>
<th>Source &amp; Catalogue Number (#)</th>
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<tr>
<td>ISOTYPE CONTROL ANTIBODIES</td>
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<td>Mouse IgG1</td>
<td>FC 5 μg/ml</td>
<td>eBioscience Ltd, # 14-4714</td>
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<td></td>
<td></td>
<td>IF 5 μg/ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IP 5 μg/sample</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG1 isotype control B (MOPC-31c)</td>
<td>Mouse IgG1</td>
<td>FC 5 μg/ml</td>
<td>Sigma-Aldrich, # M9035</td>
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<tr>
<td>Mouse IgG2a isotype control</td>
<td>Mouse IgG2a</td>
<td>FC 5 μg/ml</td>
<td>eBioscience, # 14-4724</td>
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<td>IF 5 μg/ml</td>
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</tr>
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<td></td>
<td></td>
<td>IP 5 μg/sample</td>
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<td>Mouse IgG2b</td>
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<td>IF 5 μg/ml</td>
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<td>Rabbit IgG control</td>
<td>Rabbit</td>
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<td>Specificity</td>
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<td>Application &amp; Concentration</td>
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<td>Rabbit α-mouse HRP</td>
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<td>WB 0.2-0.4 μg/ml</td>
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<td>GAM-647</td>
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<td>Sheep</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Goat</td>
<td>FC 4 μg/ml</td>
</tr>
<tr>
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<td></td>
<td>Goat</td>
<td>IF 4 μg/ml</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td>Goat</td>
<td>IF 4 μg/ml</td>
</tr>
<tr>
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<td>FC 4 μg/ml</td>
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<td>Sheep</td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
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<td>WB 0.62 μg/ml</td>
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<td>Goat</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>a-rabbit HRP</td>
<td>WB 1/5000 dilution</td>
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<td></td>
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<td>DONKEY</td>
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<tr>
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<td></td>
<td>a-sheep HRP</td>
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<td>DONKEY</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>a-sheep HRP</td>
<td>IF 4 μg/ml</td>
</tr>
</tbody>
</table>

BiP, binding immunoglobulin protein; EEA1, early endosome antigen 1; FC, flow cytometry; HA, hemagglutinin; HRP, horse radish peroxidase; IF, immunofluorescence; Ig, immunoglobulin; IP, immunoprecipitation; PE, phycoerythrin; TGN, trans Golgi network; WB, western blot
2.5 Cell culture

The human monocytic cell line THP-1 was purchased directly from the American Type Culture Collection (ATCC, Manassas, VA, USA) and was cultured at 37 °C in a 5 % CO₂ in air atmosphere in RPMI medium supplemented with 10 % fetal bovine serum (FBS), 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin (all from from GIBCO®). The human monocytic cell line Mono Mac 1 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and was cultured at 37 °C in a 5 % CO₂ in air atmosphere in RPMI medium supplemented with 10 % FBS, 2 mM glutamine, 1x non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin and 0.1 mg/ml streptomycin (all from from GIBCO®). THP-1 and Mono Mac 1 cells were maintained in a non-differentiated, monocytic state at densities of between 1 x 10⁵ - 6 x 10⁵ cells/ml and 0.3 – 1 x 10⁶ cells/ml respectively, in 175 cm² tissue culture flasks (Corning Life Sciences).

The human embryonic kidney HEK293 cell line was provided by Dr Daniel Ungar (Department of Biology, University of York, UK) and originally obtained from the ATCC. The HEK293 TLR1/2 and HEK293 TLR2/6 cell lines were a gift from Dr Marika Kullberg (Centre for Immunology and Infection, University of York, UK) and originally obtained from Invivogen. All HEK293 cell lines were cultured at 37 °C in a 5 % CO₂ in air atmosphere in Dulbecco’s Modified Eagle Medium (DMEM) containing 4.5 g/L glucose and 4 mM L-glutamine supplemented with 10 % FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin and maintained at 20 – 90 % confluent in 10 cm culture dishes. HA-CCR2B and HA-CCR5 transfected cell lines were maintained under selection using 500 μg/ml G418 (Merck, Darmstadt, Germany). HEK293 TLR cell lines were maintained under selection using 10 μg/ml blasticidin (Autogen Bioclear UK Ltd, Calne, UK or Invivogen).

2.6 Production of transfected HEK293 cell lines

2.6.1 Transfection methods

For transfection of HEK293, HEK293 TLR1/2 and HEK293 TLR2/6 for transient or stable expression of HA-CCR2B or HA-CCR5, 2 x 10⁶ cells per sample were
transfected by nucleofection with 2.5 µg DNA using the Amaxa cGMP Nucleofector Kit V (Lonza, Cologne, Germany) following the manufacturer’s optimised HEK293 nucleofection protocol. pcDNA3.1+HA3-CCR2B, pcDNA3.1+HA3-CCR5 (Missouri S&T cDNA Resource Centre, Rolla, MO, USA) or control pmaxGFP (Lonza) constructs were used for the transfections.

For transient transfection of HEK293 cell lines for expression of fluorescent Rab constructs (a gift from Mark Marsh, MRC-LMCB, UCL, London, UK) or a FLAG-Ubiquitin construct (a gift from Sylvie Urbe, Institute of Translational Medicine, University of Liverpool, UK), one or more 60 % confluent wells of a 24-well plate were transfected with 0.5 µg DNA using 1 µl jetPRIME™ reagent (Polyplus-transfection SA, Ilkirch, France) following the manufacturer’s protocol. Cells were typically incubated for 24 hours at 37 °C, 5 % CO₂ to allow protein expression before being used for colocalisation, flow cytometry or co-immunoprecipitation (CO-IP) assays.

2.6.2 Production of stable cell lines

Preliminary experiments showed that transient transfection for expression of HA-CCR2B or HA-CCR5 was not suitable for this project as the transfection level was too low and so selection with 1 mg/ml G418 was used to create more stable cell lines. Immunofluorescence staining after 24 hours, and periodic immunofluorescence staining and flow cytometry over the month following transfection, showed that G418 selection resulted in mixed populations of cells with varying expression levels of HA-CCR2B or HA-CCR5. Therefore, cloning by limited dilution was carried out to obtain more homogeneous populations. Cells were seeded in a 96-well plate at a density of 10, 5, 1 or 0.1 cells/well in growth medium containing G418 and incubated at 37 °C, 5 % CO₂. The next day, after the cells had adhered, the number of cells in each well was counted by visual inspection using a brightfield microscope. The plates were visually inspected every 2 – 3 days, and when wells containing colonies originating from a single cell reached approximately 90 % confluency these cells were transferred to 24-well plates. Duplicate plates were prepared to enable characterisation of each clone for HA-CCR2B or HA-CCR5 expression by flow cytometry,
2.7 Immunofluorescence staining

2.7.1 Endocytosis assay

Cells were seeded on poly-D-lysine coated glass coverslips in a 24-well plate at 2.5 x 10^4 cells/well and incubated at 37 °C, 5 % CO₂ for approximately 36 hours. Surface HA-CCR2B or HA-CCR5 was pre-labelled using the specified antibody (usually HA.11 for HA-CCR2B and MC5 or HA.11 for HA-CCR5) at 5 μg/ml for 90 minutes at 4 °C. Unbound antibody molecules were removed by washes and cells were incubated in binding medium or growth medium containing 100 nM CCL2 or CCL5 and incubated for the specified time at 37 °C. The cells were then fixed in 3 % para-formaldehyde (PFA) in phosphate buffered saline (PBS) for 5 minutes on ice followed by 20 minutes at room temperature (RT) before free aldehyde groups were quenched by treatment with quenching solution for 20 minutes at RT. Fixed cells were subsequently permeabilised by incubation with saponin staining buffer at RT for 20 minutes under gentle shaking (40 rpm) and then incubated with 4 μg/ml Alexa Fluor-conjugated secondary antibody in saponin staining buffer at RT for 60 minutes. Initial experiments showed high background binding on HEK293 cells and so the saponin staining buffer contained both FBS and bovine serum albumin (BSA) as blocking reagents.

After washes with 0.05 % saponin in PBS and a final wash in PBS containing 1 μg/ml DAPI (4’,6-diamidino-2-phenylindole, Invitrogen), the coverslips were mounted on to glass slides using Mowiol.

For colocalisation experiments, labelling with additional primary antibodies was carried out after fixation before secondary antibody staining. When colabelling with a mouse monoclonal antibody of the same isotype as HA.11 (IgG1) or MC5 (IgG2a), the other antibody was labelled using fluorescent Zenon® Fab fragments (Molecular Probes) following the manufacturer’s instructions. Staining steps using these antibodies were carried out after secondary antibody staining and an extra 15-minute fixation step using 4 % PFA in PBS was added afterwards to avoid transfer of the non-covalently bound Zenon® fab fragment.
2.7.2 Down-modulation assay

Cells were seeded on poly-D-lysine coated glass coverslips in a 24-well plate at 2.5 x 10⁴ cells/well and incubated at 37 °C, 5 % CO₂ for approximately 36 hours. The medium was replaced with binding medium containing 100 nM chemokine and incubated at 37 °C for 60 minutes. The cells were then fixed and stained as described in Section 2.7.1. For colocalisation experiments, labelling with additional primary antibodies was carried out at the same time as labelling with HA.11, 12CA5 or MC5.

2.7.3 Microscopy

Cells were imaged using Zeiss LSM 510 or Zeiss LSM 710 confocal microscopes and either Zeiss LSM or ZEN imaging software (Zeiss, Welwyn Garden City, UK). Acquired images were subsequently analysed using Zeiss LSM Image Browser (Zeiss), ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA, http://imagej.nih.gov/ij/, 1997-2011), Photoshop CS3 (Adobe Systems Europe Ltd., Uxbridge, UK) and/or Volocity 3D Image Analysis Software (PerkinElmer, Cambridge, UK). All images presented in this thesis are confocal slices.

2.8 Flow cytometry

2.8.1 Down-modulation assay

For down-modulation assays, 1 – 2 x 10⁵ THP-1 cells per sample were re-suspended in binding medium and aliquoted into 5 ml polypropylene tubes. Binding medium alone or supplemented with chemokine to reach a final concentration of 100 nM, was added to the cells and samples were incubated at 37 °C for 60 minutes. Samples were subsequently labelled for cell-surface receptor as described in Section 2.8.2.

Initial experiments carried out on HEK293 cells treated and labelled in suspension showed that the cells did not survive the process very well and so later experiments were carried out on cells kept adhered for the treatment step and detached prior to immunolabelling, which shortened the procedure. In the suspension cell assay, cells were detached from a 10 cm plate using 10 mM EDTA in PBS and 1 x 10⁵ cells per sample were aliquoted into 5 ml polypropylene tubes and incubated in binding medium with 100 nM chemokine at 37 °C for 60 minutes. For the adhered cell assay, cells were
seeded in a 24-well plate at \(2.5 \times 10^5\) cells/well and incubated at 37 °C, 5 % CO\(_2\) for 36 hours or until they were approximately 90 % confluent. The medium was replaced with medium alone or containing 100 nM chemokine, and the cells were incubated at 37 °C for 60 minutes before washes with PBS and detachment using 10 mM EDTA in PBS. In both assays, the cells were then labelled for cell-surface receptor using either R&D α-CCR2, MC5 or HA.11 as described in section 2.8.2. Experiments were carried out in triplicate.

In order to look at the role Rab4 plays during CCR2 down-modulation, HEK HA-CCR2B cells were transiently transfected to express YFP-tagged wild type Rab4 (Rab4WT), dominant negative Rab4 (Rab4N121I), constitutively active Rab4 or, as a control, GFP as described in Section 2.6.1, and used for the down-modulation assay. For this assay only, single replicates were used.

For assays testing the effects of drugs on CCR2 down-modulation, HEK HA-CCR2B cells were incubated with the drugs described in Table 2.4 for 60 minutes prior to chemokine treatment, and the drug was maintained in subsequent steps up to fixation.

**Table 2.4 Drugs tested for their effects on CCR2 down-modulation.**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Final Concentration</th>
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</thead>
<tbody>
<tr>
<td>Ikarugamycin</td>
<td>3 µM</td>
<td>DMSO</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.4 M</td>
<td>Binding medium</td>
</tr>
<tr>
<td>Methyl-β-cyclodextrin (MβCD)</td>
<td>10 mM</td>
<td>Binding medium</td>
</tr>
<tr>
<td>Nystatin</td>
<td>50 µg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>Filipin</td>
<td>5 µg/ml</td>
<td>Binding medium</td>
</tr>
</tbody>
</table>

Down-modulation is expressed as percent reduction in cell surface expression of the receptor for chemokine-treated cells compared to cells kept in medium. For experiments using HEK HA-CCR2B cells transiently transfected to express YFP-tagged Rab4s or GFP, the ratio of down-modulation in FITC positive cells compared to FITC negative cells within the same sample was calculated.

### 2.8.2 Detection of cell-surface receptor by flow cytometry

Prior to labelling, THP-1 cells were incubated in binding medium containing 30 µg/ml human IgG for 20 minutes on ice to saturate the cells’ Fc receptors and subsequent
staining steps were carried out in the presence of 5 µg/ml human IgG to avoid non-specific binding of mouse monoclonal antibodies. Cells were labelled with primary antibody, either R&D α-CCR2, MC5 or HA.11 at 5 µg/ml (down-modulation assay) or 10 µg/ml (recycling assays), in binding medium for 90 minutes (down-modulation) or 60 minutes (recycling) before fixation with 3 % PFA in PBS and quenching. Fixed cells were labelled with 4 µg/ml Alexa Fluor-conjugated secondary antibody in FACS buffer.

For assays testing the effects of glycosylation inhibitors on cell-surface expression of CCR2, HEK HA-CCR2B cells were pretreated with growth medium containing dimethyl sulfoxide (DMSO), 2 mM benzyl α-N-Acetylgalactosamine (benzyl – GalNAC), 10 µg/ml tunicamycin or tunicamycin and benzyl α-GalNAC for 24 hours at 37 ºC, 5 % CO2 prior to labelling.

2.8.3 Flow cytometry data analysis

Most flow cytometry data was gathered on the BD FACS Array™ (BD Biosciences), using the custom software, although where indicated the Cyan flow cytometer (Beckman Coulter, High Wycombe, UK) and Summit version 4.3 (Dako, Fort Collins, CO, USA) were used instead. Data were then exported and analysed in FlowJo 7.2.2 (Tree Star Inc, Ashland, OR, USA), which was used to generate histograms. The main population of cells was gated to remove cellular debris and a cut-off was applied so that only samples with at least 1000 cells were analysed. In some experiments, other gates were applied as described. Analysis of the FlowJo-generated data was carried out in Microsoft Excel and graphs were produced using GraphPad Prism 5 (GraphPad Software Inc, La Jolla, CA, USA).

2.9 SDS-PAGE and western blot

Cell lysates and protein samples prepared as described in the results chapters were either heated to 95 ºC or incubated at RT for 5 minutes in the presence of reducing or non-reducing sample buffer as indicated. The samples were analysed by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) using a 10 % (or 8 % for GST pull-down experiments) acrylamide gel, followed by Coomassie staining or transfer to membranes for western blotting.
Proteins were transferred to nitrocellulose transfer membrane (from Geneflow Ltd, Lichfield, UK) using a Transblot SD semi dry electrophoretic transfer cell (Bio-Rad Laboratories Ltd, Hemel Hempsted, UK) at 20 V for 40 minutes in transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol). Western blotting was carried out following the PBS or TBS protocols. In the PBS protocol, the membrane was blocked with 5 % Marvel milk powder or 10 % FBS in PBS 0.1 % Tween-20 for 1 hour at RT (RT). The membrane was incubated with the primary antibody in blocking buffer overnight at 4 °C under agitation. The membrane was washed 1 – 4 times in PBS 0.5 % Tween at RT under agitation followed by 3-4 washes in PBS 0.1 % Tween. The membrane was incubated with the secondary antibody for 1 hour at RT under agitation and then washed 1 – 2 times with PBS 0.5 % Tween-20 at RT under agitation followed by 3 – 4 washes with PBS 0.1 % Tween-20. In the TBS protocol, 5 % Marvel in TBS 0.1 % Tween was used as the blocking buffer and TBS 0.1 % Tween was used as the wash buffer. The primary antibody incubation was carried out for 1 hour at RT under agitation. All other steps were as for the PBS protocol. The ECL Western Blotting Substrate, the Super Signal® West Pico kit (Pierce, Perbio Science UK Ltd, Cramlington, UK) or the EZ-ECL Chemiluminescence detection kit for horse radish peroxidase (HRP; Beit-Haemek, Israel) were used for detection. Various exposures were taken using X-ray film (Fujifilm, Milton Keynes, UK; ThermoFisher Scientific, Loughborough, UK; Amersham, GE Healthcare). Where required for re-blotting, membranes were stripped by two incubations in stripping buffer for 30 minutes at 70 °C, followed by two washes in TBS 0.1 % Tween.
3 The monocytic cell line THP-1 and transfected HEK293 cells as model cells to study chemokine receptor desensitisation

3.1 Introduction

Traditionally, there was believed to be much apparent redundancy in the chemokine system, as a single receptor may have multiple ligands and a single chemokine may bind to multiple receptors (Table 1.3). However the chemokine receptors expressed by a cell, and thus the signalling experienced within the cell, appear to be highly regulated and dependent on the cell type, differentiation state and local environment, suggesting that redundancy may not be a major factor in vivo (Allen et al., 2007). The processes of desensitisation and resensitisation enable this regulation by altering the cell’s ability to respond to receptor ligands. Desensitisation can be either agonist-induced (homologous) or a result of cross-talk with another cell surface receptor (heterologous) (Kelly et al., 2008; Salanga et al., 2009). Monocytes co-express the inflammatory chemokine receptors CCR2B and CCR5, which play complementary roles in their recruitment from the bloodstream to sites of infection and inflammation within the tissues (Boring et al., 1997; Weber et al., 2001). This essential process requires tight regulation by receptor desensitisation and resensitisation in order to prevent excessive or inappropriate immune responses.

3.1.1 Chemokine receptor desensitisation in monocytes

3.1.1.1 Agonist-induced desensitisation

Following extended agonist-stimulation, chemokine receptors, like other GPCRs, can undergo desensitisation (Bennett et al., 2011). An important step in this process is agonist-induced down-modulation, which involves removal of chemokine-activated receptor from the cell surface by endocytosis (Kelly et al., 2008). It had been shown that monocytes down-modulate CCR2 (Fox et al., 2011) and CCR5 (Mack et al., 1998) from the cell surface in response to their respective agonistic ligands CCL2 and CCL5.
Chemokine receptors can also undergo cross-regulation via stimulation of another receptor. The so-called heterologous desensitisation of chemokine receptors by bacterial compounds has been shown on various cells of the immune system (Alves-Filho et al., 2009; Fox et al., 2011; McKimmie et al., 2009; Sica et al., 1997). Down-regulation of the inflammatory chemokine receptors CCR1, CCR2, CCR5 and CXCR2 at the gene level in response to TLR2 stimulation with bacterial lipoprotein (Pam3CSK4) has been shown to occur after four hours of treatment on mouse bone marrow-derived macrophages, neutrophils, T cells and dendritic cells (DCs; Juffermans et al., 2002; McKimmie et al., 2009). Studies using a different TLR2 ligand, lipoteichoic acid (LTA), showed that rapid (within minutes) down-modulation occurs for all of these CC chemokine receptors on human monocytes (Fox et al., 2011) and CXCR2 on mouse neutrophils (Alves-Filho et al., 2009). LTA stimulation of TLR2 leads to internalisation of CCR5 on monocytes by utilising the machinery involved in agonist-induced internalisation, but following a much slower kinetic (Fox et al., 2011). The pathways downstream of TLR2 that lead to the recruitment of this machinery remain to be elucidated although several proteins that may be involved have been suggested. It is possible that TLR2 signalling feeding into homologous desensitisation pathways may be a general mechanism, as GRK2, the G protein kinase involved in agonist-induced internalisation of many chemokine receptors, has also been implicated in TLR2-mediated down-modulation of CXCR2, although further investigations are required (Alves-Filho et al., 2009).

### 3.1.2 The monocytic cell line THP-1

A range of myelomonocytic cell lines that represent different stages along the monocyte to macrophage maturation pathway have been described, and are commonly used to complement studies on human monocytes or monocyte-derived macrophages (Cassol et al., 2006). The THP-1 cell line is human monocytic leukemia cell line (Tsuchiya et al., 1980) and was chosen for the present study as its characteristics mimic best an early monocytic phenotype and it had been described as expressing both CCR2 (Charo et al., 1994; Garcia Lopez et al., 2009; Minsaas et al., 2010; Phillips et al., 2005; Tanaka et al., 2002; Van Riper et al., 1993; Wang and O, 2001; Wang et al., 1993c; Wong et al., 1997;
Xu et al., 1997; Yamamoto et al., 1999) and CCR5 (Achour et al., 2009; Cassol et al., 2006; Mueller and Strange, 2004; Wang et al., 1993c). CCR2 expression on THP-1 cells was originally shown by the ability of the cells to bind the main CCR2 ligand CCL2 (Van Riper et al., 1993; Wang et al., 1993c). Subsequent studies showed the presence of CCR2 mRNA (Charo et al., 1994; Wang and O, 2001; Xu et al., 1997; Yamamoto et al., 1999) and the recognition of CCR2 protein by specific antibodies by flow cytometry (Garcia Lopez et al., 2009; Phillips et al., 2005), immunofluorescence (Minsaas et al., 2010) and western blot (Wang and O, 2001). There are two isoforms of CCR2, namely CCR2A and CCR2B, and in THP-1 cells, like in monocytes, CCR2B is the predominantly expressed form at both the mRNA and protein level (Tanaka et al., 2002; Wong et al., 1997). For CCR5, evidence for expression on THP-1 cells was based on their ability to be infected by R5 HIV strains (Cassol et al., 2006; Kitano et al., 1990; Meylan et al., 1993), which use CCR5 as a co-receptor for entry into monocytes (Berger et al., 1998). Ligand binding studies (Van Riper et al., 1994; Van Riper et al., 1993; Wang et al., 1993b; Wang et al., 1993c) have been inconclusive due to the promiscuity of commonly used CCR5 ligands, and antibody binding studies have shown both positive (Achour et al., 2009; Mueller and Strange, 2004) and negative (Wu et al., 1997) results for CCR5 expression on THP-1 cells.

### 3.1.3 Requirement for a transfected cell line model

Previous work by our group on agonist- and LTA-induced CCR2 and CCR5 desensitisation had been carried out on primary human monocytes, however these were not suitable for the studies proposed in this project. For studying receptor intracellular trafficking, monocytes and monocytic cell lines are not ideal due to their small size coupled with the fact that their nucleus forms a large proportion of the cell, and most importantly the lack of availability of good antibodies recognising endogenous CCR2.

For proteomic studies, the main problem would be the difficulty and expense involved in obtaining sufficient quantities of cells if using monocytes. Monocytes make up only approximately 10% of the peripheral blood mononuclear cells found in whole blood (Jones et al., 1989), which results in a yield of no more than $4 \times 10^7$ monocytes per single donor sample from the UK National Blood Service. Immunoprecipitation (IP) experiments carried out to confirm the presence of a protein by western blot and
chemiluminescence, typically use a starting number of $0.5 - 2 \times 10^7$ cells per experiment (Bonifacino, 1998). For identification of novel co-immunoprecipitated proteins using mass spectrometry, it is recommended that sufficient protein is used to enable visualisation on SDS-PAGE gels by Coomassie staining, which more than 100 fold less sensitive than western blotting (Gillespie and Hudspeth, 1991). Thus in order to obtain sufficient numbers of cells, the use of multiple different donor blood samples would be required for each immunoprecipitation experiment.

Although using monocytic cell lines would appear to be the best compromise, as will be described later in this chapter, the CCR5 expression on the common monocytic cell lines does not sufficiently mimic what is observed on monocytes. Therefore, in order to study and compare both CCR2 and CCR5 a better approach would be to create stably transfected cell lines expressing tagged versions of CCR2 and CCR5. This system would have the major advantage of enabling the use of well tested antibodies against the tag instead of the endogenous receptor.

This project chose to use the human HEK293 cell line, which does not endogenously express CCR2 or CCR5, to generate transfected cell lines enabling the individual study and comparison of the two receptors, for several reasons. Firstly, HEK293 cells have been previously used successfully for trafficking studies as they are relatively large and can be easily transfected to express intracellular markers. Secondly, they have been successfully used for the identification of novel chemokine receptor-protein interactions by co-immunoprecipitation (Favre et al., 2008). Thirdly, HEK293 cells stably expressing TLR2, were available.

### 3.1.4 Overview of transfected HEK293 cell lines created

Transfected HEK293 cell lines expressing TLR2 with either HA-CCR2B or HA-CCR5 were created (Table 3.1) with the view of further dissection of the heterologous desensitisation between CCR2 or CCR5 and TLR2 uncovered in our lab (Fox 2011). TLR2 can act in conjunction with TLR1 (Wyllie et al., 2000), TLR6 (Takeuchi et al., 2001) or the less widely expressed TLR10 (Hasan et al., 2005), however it is not known which of TLR1 or TLR6 is involved in the TLR2 cross-talk with CCR2 and CCR5 in monocytes (personal communication from Nathalie Signoret, University of York, UK). Therefore, in addition to the standard HEK293 cell line, HEK293 cell lines stably
expressing mouse TLR2 with either of its heterodimerisation partners, TLR1 and TLR6 were available. The mouse and human TLR2 sequences show high sequence homology in the cytoplasmic domain TIR region (Figure 3.1), which is responsible for interaction with adaptor proteins involved in TLR2 signalling. They also both respond to the same ligands and can be recognised by the same antibodies suggesting that the mouse TLR2 should be a good substitute for human TLR2. As it is not known which heterodimer is required for LTA-induced TLR2 cross-desensitisation of the chemokine receptors, three different HEK293 cells lines (HEK293, HEK293 stably expressing TLR1 and TLR2 and HEK293 stably expressing TLR2 and TLR6) were stably transfected with either CCR2B or CCR5 giving a total of six as described in Table 3.1. Pre-made constructs encoding triple HA-tagged versions of human CCR2B and CCR5 in the pcDNA3.1+ vector, and obtained from the UMR cDNA Resource Centre (Missouri, USA) were used.

<table>
<thead>
<tr>
<th>Name</th>
<th>TLR</th>
<th>Chemokine Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK HA-CCR2B</td>
<td>N/A</td>
<td>Human HA-CCR2B</td>
</tr>
<tr>
<td>HEK HA-CCR5</td>
<td>N/A</td>
<td>Human HA-CCR5</td>
</tr>
<tr>
<td>HEK TLR1/2 HA-CCR2B</td>
<td>Mouse TLR1 &amp; TLR2</td>
<td>Human HA-CCR2B</td>
</tr>
<tr>
<td>HEK TLR1/2 HA-CCR5</td>
<td>Mouse TLR1 &amp; TLR2</td>
<td>Human HA-CCR5</td>
</tr>
<tr>
<td>HEK TLR2/6 HA-CCR2B</td>
<td>Mouse TLR2 &amp; TLR6</td>
<td>Human HA-CCR2B</td>
</tr>
<tr>
<td>HEK TLR2/6 HA-CCR5</td>
<td>Mouse TLR2 &amp; TLR6</td>
<td>Human HA-CCR5</td>
</tr>
</tbody>
</table>
Figure 3.1 Comparison of the amino acid sequence of human and mouse TLR2 using SIM (http://expasy.org/tools/sim-prot.html).

The cytoplasmic TIR domain (highlighted in yellow), which is involved in protein-protein interactions with the adaptor protein TIRAP leading to TLR signalling via MyD88, shows 91 % sequence identity. Asterisks indicate identical amino acids.
3.1.5 Objectives

The present study aimed firstly to investigate both whether we can study endogenous CCR2 on THP-1 cells and whether the CCR5 present on these cells is functional, in order to determine the utility of these cells for studying desensitisation of the receptors. The second aim was to characterise the six transfected HEK293 cells lines in order to determine how closely their CCR2 and CCR5 expression and behaviour mimics that observed in primary human monocytes. This would enable us to determine how useful THP-1 cells and the transfected HEK293 cell lines would be for studying receptor trafficking in response to agonist stimulation or LTA cross-talk and for identifying novel receptor-protein interactions.

3.2 Relevant Methodology

3.2.1 Intracellular calcium mobilisation assay

THP-1 cells, human monocytes (purified as in Fox et al., 2011) or transfected HEK293 cells were washed in PBS, loaded with 2.5 μM Fluo-8 AM (at a density of 2 x 10^6 cells/ml) for 30 minutes, then washed and resuspended in HBSS at a density of 1 x 10^6 cells/ml. 400 μl aliquots of cells were either untreated or pretreated with 400 nM TAK-779 or 100 nM UCB35625 where indicated. After obtaining a baseline fluorescence reading for 30 seconds, an identically-treated aliquot of cells were treated with PBS, 400 nM TAK-779 or 100 nM UCB35625 (as controls) or the indicated concentration of chemokine and analysed. Changes in the intracellular calcium concentration were measured as changes in the fluorescence of the cells on a CyAn flow cytometer (Beckman Coulter) using an argon laser at a wavelength of 488 nm.

The data was analysed using Summit version 4.3. Firstly, cells were gated based on FSC/SSC to exclude dead cells and cellular debris. Then additional gates were used to divide the data in to 10 second time slots and the median fluorescence at 488 nm was analysed for each gate. The average of the first 30 seconds was typically used to calculate the baseline fluorescence and all other fluorescence values were normalized to the baseline to enable comparison between samples. Due to the time needed for cells to reach the flow chamber for analysis, there was a time delay after finishing the baseline
recording before recording resumed after addition of the chemokine or control, and so data from this time frame was excluded. Graphs were plotted using GraphPad Prism 5.

3.2.2 Cell surface receptor quantification by flow cytometry

Antibody titrations were carried out by flow cytometry by incubating 2 x 10^5 transfected HEK293 cells with serial dilutions of monoclonal antibodies (20 - 0.5 μg/ml) for 90 minutes at 4 °C before fixation and labelling with secondary antibody as described in section 1.7.2. Antibody binding saturated at a concentration of 10 μg/ml, which was therefore used to quantify the cell surface receptor levels using the QIFI kit (Dako, Stockport, UK) following the manufacturer’s instructions, and analysed using a Cyan flow cytometer (Beckman Coulter).

3.2.3 Total receptor expression manipulation and detection by immunofluorescence staining

Serum starvation and treatment with cycloheximide or sodium butyrate were tested for their effect on HA-CCR2B and HA-CCR5 expression in transfected HEK293 cells. Cells were seeded on coverslips in a 24-well plate at 2.5 x 10^4 cells/well and incubated in growth medium (DMEM, 10 % FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 μg/ml blasticidin, 500 μg/ml G418) or serum-deprived medium (DMEM, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 % BSA, 10 mM HEPES, 1 mM sodium pyruvate, 10 μg/ml blasticidin, 500 μg/ml G418) at 37 °C, 5 % CO_2 for approximately 36 to 48 hours before use. When using sodium butyrate, it was added to the growth medium used in this step at a final concentration of 10 mM. When using cycloheximide, cells were first adhered to the coverslips as described and then a second incubation in growth medium containing 10 μg/ml cycloheximide was carried out for the indicated time.

The cells were then fixed, quenched and permeabilised as described in Section 2.7.1. Total HA-CCR2B or HA-CCR5 was labelled by incubation with 5 μg/ml HA.11, 12CA5 or MC5 in saponin staining buffer for 90 minutes at room temperature. The cells were washed in 0.05 % saponin in PBS, and then incubated with 4 μg/ml Alexa Fluor conjugated secondary antibody. After washes in 0.05 % saponin in PBS and a final wash
in PBS containing 1 μg/ml DAPI, the coverslips were mounted on to glass slides using Mowiol.

3.3 Results

3.3.1 Characterisation of the monocytic cell line THP-1

3.3.1.1 CCR2 and CCR5 expression

Flow cytometry analysis using R&D α-CCR2 on non-permeabilised cells confirmed the presence of cell surface CCR2 on our THP-1 cells (Figure 3.2A). However, as the antibody recognises the N-terminus, it was not able to distinguish between the CCR2A and CCR2B isoforms. An antibody against the C-terminus of CCR2B (CCR2B C-20) was available and so this antibody and others (E68, H-40 and R&D α-CCR2) were used to probe THP-1 cell lysate samples by immunoblotting (Figure 3.2B). All of the antibodies except R&D α-CCR2 showed a large amount of non-specific binding under the conditions used, and none gave a dominant band at 41 kDa, which corresponds to the predicted molecular weight of CCR2 based on its amino acid sequence. Other anti-CCR2 antibodies were also tested by our lab and did not work by western blot, which limits the use of THP-1 cells for our study.

Testing of a broad spectrum of antibodies against CCR5 by our lab has identified the presence of two forms of the receptor (the MC5 form and the CTC5 form) that show different membrane distribution characteristics, responses to agonist treatment and levels of staining on monocytes and monocytic cell lines. This investigation has found barely detectable levels of the MC5 form on THP-1 cells by immunofluorescence and flow cytometry but more staining of the CTC5 form (Figure 3.3).
**Figure 3.2 CCR2 expression in THP-1 cells.**
(A) THP-1 cells were immunolabelled for cell surface CCR2 using R&D α-CCR2 (black) and analysed by flow cytometry. Other THP-1 cells were labelled with the relevant isotype control antibody (grey). (B) $10^6$ THP-1 cells per sample were lysed in reducing sample buffer and non-boiled samples were analysed for the presence of CCR2 by SDS-PAGE and immunoblotting using the indicated α-CCR2 antibodies. Arrows show the predicted molecular weight of CCR2B, based on the amino acid sequence (41 kDa).

**Figure 3.3 CCR5 expression in THP-1 cells.**
THP-1 cells were immunolabelled for cell surface CCR5 (black) using MC5 (A) or CTC5 (B) and analysed by flow cytometry. Other THP-1 cells were labelled with the relevant isotype control antibody (grey). These histograms each show a single set of representative samples from two (A) or three (B) separate experiments each carried out in triplicate. This figure was created using data obtained by Laura Fell (University of York, UK).
3.3.1.2 Agonist-stimulated receptor signalling

Flow cytometry work carried out in this study and by other members of our lab has showed that the THP-1 cells used in this project express CCR2 and some CCR5, but are these receptors actually capable of signalling? One way of measuring chemokine receptor signalling is to monitor the intracellular calcium levels following agonist stimulation. This has been done successfully for human monocytes and has shown that both CCR2 and CCR5 respond to agonist stimulation via calcium signalling (Fox et al., 2011), and therefore we applied the same technique to study THP-1 cells.

For calcium signalling studies, THP-1 cells were loaded with Fluo-8 AM and changes in intracellular calcium concentration in response to treatment with different chemokines were determined by analysis of the changes in cell fluorescence at 488 nm. Treatment with the CCR2 agonist, CCL2, led to a spike in the intracellular calcium concentration representing receptor signalling (Figure 3.4C). This response was specific as treatment with PBS, which was used as a carrier for the chemokines, or chemokine receptor antagonists alone did not give a noticeable response (Figure 3.4A and 3.4B). The CCL2-induced calcium signalling could be inhibited by pre-treatment with TAK-779 (Figure 3.4C), which is known to antagonise CCR5 when used at low concentrations and both CCR5 and CCR2 at high concentrations (Baba et al., 1999).

Many commonly used CCR5 agonists also bind to CCR1 making it difficult to distinguish which receptor is responsible for signalling. To avoid this problem, my study used both the common agonist CCL5 in conjunction with receptor-specific antagonists and also a CCR5-specific agonist, CCL4. As CCL4 is a weaker CCR5 agonist than CCL5 (Mueller et al., 2006), a range of CCL4 concentrations from 1 nM to 40 nM was tested (Figure 3.4D). Treatment with CCL4 did not give any change in the intracellular calcium concentration at any of the concentrations tested, which suggests that the CCR5 on THP-1 cells does not signal. The CCL4 was tested by our lab on CHO cells stably transfected to express CCR5 to confirm that it was capable of eliciting a response. In contrast, treatment of THP-1 cells with CCL5 resulted in calcium signalling (Figure 3.4E). However, as pre-treatment with a CCR5 antagonist (TAK-779) only leads to an insignificant reduction in the CCL5-induced signalling (Figure 3.4E), whereas pre-treatment with a CCR1 antagonist, UCB35625 (Sabroe et al., 2000), lead to an almost
complete reduction in signalling (Figure 3.4F), it is likely that in THP-1 cells the CCL5 actually signals via its other receptor CCR1. These results were supported by those using the synthetic CCL5 analogue, PSC-RANTES, which gives a much stronger agonist response than CCL5 (Hartley et al., 2004). PSC-RANTES treatment leads to calcium signalling in both THP-1 cells (Figures 3.4G and 3.4H) and monocytes (Figure 3.4I), however whereas this signalling is partially inhibited by TAK-779 in monocytes, TAK-779 has no inhibitory effect in THP-1 cells. In addition, the PSC-RANTES-induced calcium signalling is inhibited by the CCR1 antagonist, UCB35625, in THP-1 cells (Figure 3.4H).

Taken together, these results suggest that the CCR2 present on THP-1 cells is capable of signalling via the calcium pathway, however the CCR5 either is not capable of signalling or the amount of CCR5 present that is capable of signalling is too low to give a detectable signal.

3.3.1.3 Agonist-induced receptor desensitisation

Work by other members of the lab showed that our THP-1 cells do not down-modulate CCR5 in response to its main agonistic ligand CCL5 (Figure 3.5) and I therefore concentrated on the CCR2 response in these cells. A reduction in THP-1 cell surface CCR2 epitope of 74 %, measured by flow cytometry, in response to 1 hour treatment with 100 nM CCL2 showed that the receptor is down-modulated in response to prolonged agonist stimulation (Figure 3.6). This level of down-modulation is slightly higher than previously published for CCR2 on primary human monocytes tested in comparable conditions (Fox et al., 2011).
Figure 3.4 Delineation of the receptor responsible for CCL2 and CCL5 induced calcium signalling in THP-1 cells and monocytes.

THP-1 cells (A-H) or human monocytes (I) were loaded with Fluo-8 AM. After obtaining a baseline fluorescence reading for 30 seconds, the cells were treated with PBS, 400 nM TAK-779 or 100 nM UCB35625 (as controls) or the indicated concentration of chemokine. Changes in the intracellular calcium concentration were determined by analysis of the fluorescence of the cells on a CyAn flow cytometer using an argon laser at a wavelength of 488 nm. The fluorescence was normalised to the baseline to enable comparison between samples. Where indicated (C, E, F, G, H and I), cells were either untreated (medium) or pretreated with 400 nM TAK-779 or 100 nM UCB35625 for 10 minutes prior to obtaining the baseline fluorescence reading.
Figure 3.5 Lack of CCR5 down-modulation in response to agonist treatment on THP-1 cells.
THP-1 cells were treated with medium (black) or 100 nM CCL5 (red) for 60 minutes at 37 °C then immunolabeled for cell surface CCR5 using MC5 (A) or CTC5 (B) and analysed by flow cytometry. Other medium treated THP-1 cells were labelled with an isotype control antibody (grey). These histograms each show a single set of representative samples from two (A) or three (B) separate experiments each carried out in triplicate. This figure was created using data obtained by Laura Fell (University of York, UK).

Figure 3.6 CCR2 down-modulation in response to agonist treatment on THP-1 cells.
(A) THP-1 cells were treated with medium (black) or 100 nM CCL2 (red) for 60 minutes at 37 °C then immunolabeled for cell surface CCR2 using α-CCR2 and analysed by flow cytometry. Other medium treated THP-1 cells were labelled with an isotype control antibody (grey). (A) shows a single set of representative samples from two separate experiments each carried out in triplicate. (B) represents the mean ± SD down-modulation in response to medium and CCL2 treatment for these experiments. The receptor down-modulation (reduction in cell surface epitope availability) is expressed as a percentage of that observed for medium-treated cells.
3.3.2 Characterisation of transfected HEK293 cell lines

3.3.2.1 CCR2B and CCR5 expression

Transfected HEK293 cell lines were generated by nucleofection of HEK293, HEK293 TLR1/2 and HEK293 TLR2/6 cells with constructs encoding triple-HA tagged forms of CCR2B and CCR5, followed by selection for expressing cells using G418, as described in Section 2.6. The cell surface receptor levels were tested by flow cytometry (Figure 3.7), and quantified using the QIFI kit (Dako). The number of receptors per cell is reported for each transfected HEK293 cell line in Table 3.2. These values are comparable to those published for monocytes, which fall within the range of 1000 to 20000 CCR2 and CCR5 receptors per cell (Denholm and Stankus, 1995; Grimm et al., 1998; Hladik et al., 2005; Lee et al., 1999b; Mine et al., 2006; Wang et al., 1993a; Wang et al., 1993c; Yoshimura and Leonard, 1990; Zhang et al., 1994). Clonal populations were obtained for all cell lines with the exception of HEK TLR1/2 HA-CCR5. The presence of faster growing HA-CCR5 non-expressing cells meant that repeated passaging lead to an overall gradual loss of HA-CCR5 expression in this cell line over time.

Table 3.2 Number of HA-CCR2B or HA-CCR5 receptors per cell estimated for the transfected HEK293 cells using the QIFI kit.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>HA-CCR2B Receptors Per Cell</th>
<th>HA-CCR5 Receptors Per Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK HA-CCR2B</td>
<td>4718</td>
<td></td>
</tr>
<tr>
<td>HEK HA-CCR5</td>
<td>10496</td>
<td></td>
</tr>
<tr>
<td>HEK TLR1/2 HA-CCR2B</td>
<td>7159</td>
<td>16527</td>
</tr>
<tr>
<td>HEK TLR1/2 HA-CCR5</td>
<td>8659</td>
<td>6525</td>
</tr>
<tr>
<td>HEK TLR2/6 HA-CCR2B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEK TLR2/6 HA-CCR5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.7 HA-CCR2B and HA-CCR5 expression in transfected HEK293 cells.
The indicated transfected HEK293 cells were immunolabelled for cell surface receptor using HA.11 (A), α-CCR2 (B, CCR2-transfected cells) or MC5 (B, CCR5-transfected cells) and analysed by flow cytometry. Other aliquots of transfected HEK293 cells were labelled with an isotype control antibody (grey).
However, immunofluorescence imaging revealed a large accumulation of receptors in the biosynthetic pathway of transfected HEK293 cells as shown by intercalation of the receptors with TGN46 (Figure 3.8). Treating the cells with cycloheximide for up to 6 hours, to block de novo protein synthesis (Kerridge, 1958) and enable transport of existing receptors through the biosynthetic pathway, reduced but did not completely remove the intracellular receptor accumulation (Figure 3.9). The presence of serum has been shown to increase the rate of protein synthesis in cells leading to accumulation of protein (Ballard, 1982; Kaminskas, 1972). Therefore, serum-deprivation was tested as a method of reducing protein synthesis to allow time for the backlog of receptors to traffic through the biosynthetic pathway. However, it had little effect on the intracellular accumulation of the receptor. Sodium butyrate is an inhibitor of histone deacetylase (Boffa et al., 1978; Candido et al., 1978; Sealy and Chalkley, 1978) and has been shown to inhibit cell growth whilst repressing the expression of some genes and enhancing the expression of others (Davie, 2003). Treatment with sodium butyrate enhanced the receptor expression both at the cell surface and intracellularly (data not shown) and so was not useful for reducing the biosynthetic pathway accumulation. Although intracellular accumulation of the expressed transfected receptor does not appear to impact on the growth rate or morphology of transfected cells, it does mean that assays need to be designed to selectively study the fate of cell surface receptors.
Figure 3.8 Constitutive intracellular accumulation of HA-CCR2B and HA-CCR5 in transfected HEK293 cells.
The cells were co-labelled with HA.11 (HA-CCR2B, red) or MC5 (HA-CCR5, red) and TGN46 (green), and counterstained with DAPI (blue). Image settings are optimised for the intracellular receptor. Scale bar = 10 μm. Arrows indicate the location of inset 2X zoom panels.

Figure 3.9 Treatment with cyclohexamide partially reduces HA-CCR2B intracellular accumulation in transfected HEK293 cells.
Cells were untreated or treated with 10 μg/ml cycloheximide for 6 hours before fixation and permeabilisation. HA-CCR2B was labelled using HA.11 (grey) and cells were counterstained with DAPI (blue). Scale bar = 10 μm. Arrows indicate intracellular accumulation of HA-CCR2B.
3.3.2.2 Comparative analysis of CCR2B and CCR5 internalisation in response to agonist treatment

Both receptors are internalised in all of the transfected HEK293 cell lines in response to 1 hour agonist treatment as shown by flow cytometry and immunofluorescence assays (Figures 3.10, 3.11A, 3.12). The reduction in cell surface epitope measured by flow cytometry was used as a measure of the receptor down-modulation. In response to 1 hour agonist treatment, between 51% and 80% down-modulation was observed for CCR2B and CCR5 in the different cell lines (Table 3.3, Figure 3.10). These down-modulation levels are comparable to those published for primary human monocytes (Fox et al., 2011) and similar to the CCR2 down-modulation level measured on THP-1 cells.

Binding of some chemokines to their receptors can mask the epitope recognised by the antibody (Klasse et al., 1999; Navenot et al., 2001). N-terminal residues of CCR2 and CCR5 have been reported to contribute to high affinity binding of their chemokine ligands (Bannert et al., 2001; Blanpain et al., 1999; Datta-Mannan and Stone, 2004; Hemmerich et al., 1999; Monteclaro and Charo, 1996, 1997). The antibodies used in my experiments (R&D α-CCR2 and MC5) bind to the N-termini of the two receptors. Therefore, to confirm that binding of the ligands used in this experiment (CCL2 and CCL5) does not affect the binding of these antibodies, the assay was repeated using HA.11, a monoclonal antibody recognising the triple HA tag added at the N-terminus, and which should not be implicated in ligand binding. Indeed with HA.11, very similar down-modulation values, 69% for CCR2B and 45% for CCR5, were observed (Figure 3.10B) implying that epitope masking is not an issue.

Any replenishment of cell surface receptor from internal stores could reduce the level of down-modulation observed by flow cytometry. An endocytosis immunofluorescence assay, in which cell surface receptors were pre-labelled with HA.11 antibody prior to treatment of the cells with chemokine, enabled us to only follow the fate of cell surface receptors. This assay shows that the majority of both cell surface receptors are internalised in response to 60 minutes of agonist treatment, however we observed a difference in the localisation of internalised receptors (Figure 3.11A and 3.12). HA-
CCR5 is seen clustered in the perinuclear area, as previously described for human monocytes (Fox 2011) and other transfected cells (Signoret et al., 2000). Conversely, internalised HA-CCR2B appears to be present in vesicles spread out in the cytoplasm and does not accumulate into perinuclear structures or colocalise with TGN46 as has been reported for CCR5 (Escola et al., 2010).

**Figure 3.10 Down-modulation of cell surface receptors in response to agonist treatment.**
The indicated transfected HEK293 cells were treated with medium or 100 nM agonist (CCL2 for HA-CCR2B and CCL5 for HA-CCR5) for 60 minutes at 37 °C then immunolabeled for cell surface receptor expression using antibodies to the receptor itself [R&D α-CCR2 or MC5, (A)] or to the HA-tag [HA.11, (B)] and analysed by flow cytometry. The receptor down-modulation (reduction in cell surface epitope availability) is expressed as a percentage of that observed for medium-treated cells. (A) represents the means ± SD of the indicated number of separate experiments each carried out in triplicate. (B) represents the means ± SD of single experiments carried out in triplicate.
Table 3.3 Level of down-modulation in response to 60 minute agonist (CCL2 or CCL5) stimulation measured by flow cytometry for the different transfected HEK293 cell lines.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>% Down-modulation (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK HA-CCR2B</td>
<td>72 ± 6 n=3</td>
</tr>
<tr>
<td>HEK HA-CCR5</td>
<td>51 ± 6 n=3</td>
</tr>
<tr>
<td>HEK TLR1/2 HA-CCR2B</td>
<td>66 ± 7 n=2</td>
</tr>
<tr>
<td>HEK TLR1/2 HA-CCR5</td>
<td>80 ± 3 n=2</td>
</tr>
<tr>
<td>HEK TLR2/6 HA-CCR2B</td>
<td>67 ± 14 n=3</td>
</tr>
<tr>
<td>HEK TLR2/6 HA-CCR5</td>
<td>78 ± 8 n=3</td>
</tr>
</tbody>
</table>

Figure 3.11 (A) Differential localisation of internalised HA-CCR2B and HA-CCR5 upon agonist treatment in transfected HEK293 cells. (B) Lack of colocalisation of internalised HA-CCR2B with TGN46.

(A) The receptors were pre-labeled using HA.11 (HA-CCR2B, grey) or MC5 (HA-CCR5, grey) and the cells were either untreated or treated with 100 nM CCL2/CCL5 for 1 hour at 37 °C. Scale bar = 10 μm.
(B) HA-CCR2B was pre-labeled using HA.11 (red) and the cells were treated with 100 nM CCL2 for 60 minutes at 37 °C. After fixation the cells were co-labelled with TGN46 (green) and counterstained with DAPI (blue). Scale bar = 10 μm.
Figure 3.12 Internalisation of HA-CCR2B and HA-CCR5 upon agonist or LTA treatment in transfected HEK293 cells.
The receptors were pre-labeled using HA.11 (HA-CCR2B, grey) or MC5 (HA-CCR5, grey) and the cells were either untreated or treated with 100 nM CCL2/CCL5 or 10 µg/ml LTA as indicated for 1 hour at 37 °C. Cells were counterstained with DAPI. Scale bar = 10 µm.
3.3.2.3 Cross-talk between TLR2 and CCR2B/5

The effect of LTA on HA-CCR2B and HA-CCR5 cell surface expression in transfected HEK293 cells was investigated to determine if these cells could be used to further dissect the TLR2/CC chemokine receptor cross-talk pathway discovered in monocytes (Fox et al., 2011). Some limited internalisation in response to 1 hour LTA treatment is observed for all of the transfected HEK293 TLR2 cell lines using the immunofluorescence endocytosis assay (Figure 3.12). However, unlike in monocytes, where the LTA-induced CCR5 internalisation is similar to that following agonist-stimulation (Fox et al., 2011), the LTA-induced changes in HA-CCR2B and HA-CCR5 receptor distribution are discrete with only few vesicles observed inside the transfected HEK293 cells. Although in monocytes the LTA-induced CCR2/5 internalisation follows a slower kinetic than agonist-induced internalisation (Fox et al., 2011), in transfected HEK293 cells the LTA-induced CCR2/5 internalisation is not increased by longer (2 hour) treatment (data not shown). Additionally, CCR5 internalised in response to 1 hour LTA treatment appears to be in vesicles in the cytoplasm (Figure 3.12), not clustered at the perinuclear area as is described in monocytes (Fox et al., 2011). The LTA-mediated CCR2 and CCR5 down-modulation observed in human monocytes was concentration dependent, with 10 µg/ml LTA resulting in 30 – 40 % receptor down-modulation (Fox et al., 2011). Therefore, flow cytometry was used to assess the level of down-modulation in the presence of different concentrations of LTA in the transfected HEK293 cells. In contrast to the immunofluorescence data, there was no down-modulation of cell surface HA-CCR2B or HA-CCR5 levels following a 1 hour treatment with a range of LTA concentrations (0.5 µg/ml – 50 µg/ml) (Figure 3.13B). To check if the lack of cross-talk is TLR2 ligand specific, another receptor agonist, Pam3CSK4, was tested on HEK TLR1/2 HA-CCR2B and HEK TLR2/6 HA-CCR2B. Unlike for LTA, dose-dependent down-modulation of CCR2 was observed at relatively high Pam3CSK4 concentrations in both cell lines (Figure 3.13C) with an average of 43 % (HEK TLR1/2 HA-CCR2B) or 25 % (HEK TLR2/6 HA-CCR2B) down-modulation following treatment with 20 µg/ml Pam3CSK4. These results suggest that the TLR2 is functional and that a TLR2-CCR2B cross-talk pathway is present but it may be ligand-dependent and may not fully reproduce what is seen in monocytes.
Figure 3.13 Comparison of cell surface receptor down-modulation in response to agonist, LTA and Pam3CSK4 treatment.
The indicated transfected HEK293 cells were treated with medium, 100 nM agonistic ligand (A), varying concentrations of LTA (B) or varying concentrations of Pam3CSK4 (C) as indicated for 60 minutes at 37 °C then immunolabeled for cell surface receptor using antibodies to the receptor (α-CCR2 or MC5) or isotype control antibodies and analysed by flow cytometry. The histograms shown are representative of at least two experiments (except HEK TLR1/2 HA-CCR2B treated with LTA) carried out in triplicate, with the exception of treatment with 40 μg/ml Pam3CSK4, for which a single sample was used.

3.3.2.4 Receptor signalling

As LTA did not appear to cross-talk with CCR2 and CCR5 on the transfected HEK293 TLR2 cell lines, experiments were carried out to check if the TLR2 is functional for calcium signalling using the calcium flux technique that was successfully employed for
THP-1 cells. Each cell line was tested at least twice, often with multiple replicates in each test, however, the results were inconclusive. The HA-CCR2B expressing cell lines gave some response to LTA stimulation but the HA-CCR5 expressing versions did not. Additionally, both transfected HEK293 TLR2 cell lines expressing HA-CCR2B responded to CCL2 stimulation in some but not all replicates, however, no response to CCL5 was detected for either HEK293 TLR2 cell line expressing HA-CCR5. This variability and inconsistency mean that no conclusions about the calcium signalling abilities of these cells can be drawn from this study. Work carried out during this thesis has shown that the large, adherent HEK293 cells are less suited to flow cytometry than the smaller non-adherent THP-1 cells, as they are often easily damaged during the procedure leading to a higher proportion of dead cells and so calcium imaging may be a better technique. HEK293 cells are often easily damaged by the mechanical stresses introduced by the procedure, such as detachment and declumping by manual pipetting, and the physical forces on the cell created by the flow rate necessary for detection using a flow cytometer. This is evidenced by the relatively high proportion of dead cells and the leak of the Fluo-8 AM dye over time.

3.4 Discussion

Neither of the two cell options discussed here, the monocytic cell line THP-1 or transfected HEK293 cells, offer a complete solution for studying the aims of this thesis, however they can each be used to answer specific questions.

Based on the calcium signalling study, the CCR5 present on our THP-1 cells does not appear to be functional. This is in agreement with other studies carried out in our group showing that our THP-1 cells do not migrate in response to CCR5 specific stimulation. These findings suggest that our THP-1 cells are not suitable for studying processes downstream of CCR5 agonist stimulation or cross-talk of this receptor with TLR2. In the limited studies carried out by our group, another monocytic cell line, MonoMac1, was tested and also appears to have low CCR5 functionality suggesting that monocytic cell lines may not be good models for studying CCR5. However, based upon the positive CCR2 expression, calcium signalling and down-modulation results, THP-1 cells can be used to study some aspects of CCR2 down-modulation/desensitisation in
response to agonist stimulation using flow cytometry but not immunofluorescence or immunoblotting methods with the antibodies currently available.

THP-1 cells have been cultured and used by many groups since their original development over thirty years ago. THP-1 cells can be differentiated towards more macrophage-like phenotypes by stimulation with phorbol myristate acetate (PMA) or vitamin D (Daigneault et al., 2010). However, it has also been shown that continuous culture of THP-1 cells can lead to changes in the cellular characteristics, such as adherence (Tominaga et al., 1998; Tsuchiya et al., 1982), suggesting that the THP-1 cells used in different laboratories may not be exactly the same. This potential heterogeneity of THP-1 cells may give an explanation for the conflicting reports on CCR5 expression on THP-1 cells detailed in the introduction to this chapter. Our THP-1 cells were purchased directly from the ATCC and were not subjected to any conditions known to induce uncontrolled differentiation.

Transfected HEK293 cells can be used to compare the agonist-induced desensitisation and associated intracellular trafficking of both receptors. The intracellular localisation of internalised CCR5 agrees with what has been published for monocytes. Internalised CCR2B localises differently to CCR5 however this appears to be a general phenomenon, not just a result of expression in HEK293 cells, as a similar pattern has been observed in the nerve astrocyte cell line A7 (Minsaas et al., 2010). This group also reported an agonist-induced change from uniform cell surface to punctate CCR2 staining in their THP-1 cells suggestive of internalisation, but the nature of these intracellular vesicles has not been determined.

HEK293 cells are more suitable than THP-1 for further immunofluorescence studies of receptor intracellular trafficking due to their larger size, volume of cytoplasm and cleaner staining using the α-HA antibody HA.11 than some of the anti-CCR2 antibodies available commercially. The initial chemokine receptor transfections showed that the HEK293 cells give a reproducible sufficient transfection efficiency of approximately 30% and so dominant negative and consitutively active forms of proteins involved in intracellular trafficking, such as Rabs, can be easily expressed to investigate their involvement in trafficking of the receptors.
This study also showed that whilst HEK293 cells can be analysed by flow cytometry, they are not well suited for this technique. Therefore, for studies of CCR2 it would be useful to use THP-1 that endogenously express CCR2 for flow cytometry studies to provide complementary information.

Neither the THP-1 cell line, due to the lack of CCR5 functionality, nor the transfected HEK293 cells are suitable for the study of LTA-induced TLR2 cross-talk with both CCR2 and CCR5. There could be multiple reasons for the different LTA response observed in the transfected HEK293 TLR2 cells lines and monocytes. Firstly, transfected HEK293 TLR2 cells may be missing a part of the LTA-induced TLR2-CCR2/5 cross-talk pathway as the pathway is yet to be fully identified but has been shown to be independent of the canonical TLR2 signalling via TIRAP and instead to involve PLC and Rac1 (Fox et al., 2011). As a non-moncytic cell line, HEK293 cells, do not endogenously express either TLR2 or chemokine receptors and so may not express all the members of the signalling pathways associated with the receptors and required for cross-talk.

As is observed for agonist-stimulated cells, CCR5 is also phosphorylated on the GRK-specific phosphorylation site S349 in response to LTA-stimulation, however, the phosphorylation follows a slower kinetic (Fox et al., 2011). This suggests that one or more GRK is involved in the LTA-induced cross-desensitisation of the chemokine receptor. Overexpression of various GRKs can lead to agonist-induced phosphorylation of CCR5, however, it is thought that GRK2 and GRK3 are the main GRKs that phosphorylate CCR5 in cells where they are endogenously expressed (Oppermann, 2004). Additionally, CCR2 is also phosphorylated by GRK2 during agonist-induced desensitisation (Aragay et al., 1998) and GRK2 has been implicated in the TLR2-mediated down-modulation of CXCR2 (Alves-Filho et al., 2009). GRK2 and GRK3, but not GRK5 or GRK6, are highly expressed in human monocytes (Olbrich et al., 1999). In contrast, a recent study (Atwood et al., 2011) carried out microarray analysis of mRNA levels of GPCR related signalling proteins in HEK293 cells and showed that they express detectable mRNA for GRK3, GRK4 and GRK5 but not for GRK1 and GRK2. Therefore, the potential involvement of GRK2 may explain the lack of LTA-induced cross-desensitisation of CCR2B and CCR5 in transfected HEK293 cells.
The ratio of chemokine receptor to TLR2 molecules present in monocytes may be a limiting factor for cross-desensitisation and this is unlikely to be conserved in the transfected HEK293 cell lines. Indeed, the chemokine receptor level does differ slightly between the cell lines. Additionally, CCR2 has been shown to hetero-oligomerise with CCR5, CXCR4 and various other GPCRs (see Bennett et al., 2011 for review) that are not expressed in HEK293 cells and such hetero-oligomerisation could be relevant to cross-desensitisation by TLR2. It is possible that the spatial arrangement of the transfected receptors in the HEK293 cell plasma membrane differs from that of endogenous receptors in monocytes.

Secondly, mouse TLR2, despite displaying good homology to human TLR2 and binding the same ligands, may not be capable of cross-desensitisation of human chemokine receptors. Thirdly, it is possible that the LTA-induced TLR2-CCR2/5 cross-talk pathway is present but less efficient in transfected HEK293 TLR2 cells than in monocytes. If this is the case, it is possible that rapid replenishment of the internalised cell surface receptor from the large intracellular accumulation of receptors at the trans-golgi network could provide an explanation for the conflicting results seen by immunofluorescence endocytosis experiments and flow cytometry down-modulation experiments. However, this is unlikely, as in transfected HEK293 cells agonist-induced CCR2 and CCR5 down-modulation levels are similar to or higher than those observed in monocytes, suggesting that replenishment of cell surface receptor from the intracellular stores does not have a major impact. However, as LTA-induced internalisation of CCR2 and CCR5 follows a slower kinetic than that of agonist-induced internalisation, replenishment from intracellular stores could have more influence, although this is probably not the full explanation.

Although no down-modulation was observed in response to LTA by flow cytometry, some down-modulation of CCR2 was observed when using high concentrations (≥ 20 μg/ml) of Pam3CSK4, a ligand that has been suggested to cross-talk with CCR2 via two pathways that differ from that described for LTA cross-regulation on monocytes (Fox et al., 2011). These are down-regulation of CCR2 at the RNA transcript level (McKimmie et al., 2009) and induction of autocrine chemokine production that results in down-modulation of CCR2 (Parker et al., 2004). It is possible that HEK293 cell express the machinery for one but not all of these pathways.
4 Characterisation of agonist-induced CCR2B trafficking

4.1 Introduction

4.1.1 Desensitisation of chemokine receptors as a consequence of agonist activation

Following agonist binding, the intracellular signalling cascade results in rapid phosphorylation of the receptor’s cytoplasmic tail, usually by a member of the G protein receptor kinase (GRK) family, which uncouples the G protein from the receptor and prevents further activation. Phosphorylated receptors interact with one of the β-arrestins acting as a scaffold targeting receptors for internalisation via clathrin-mediated endocytic pathways. Caveolin-mediated and other clathrin-independent endocytic pathways have also been reported (Borroni et al., 2010). The internalised receptors then traffic through the endocytic pathway and are either sent for degradation or recycled to the cell surface. Different chemokine receptors follow either uniquely one route or different pathways depending on a number of influencing factors. Although the trafficking trend appears conserved between chemokine receptors, the mechanisms involved vary and thus cannot be considered generic.

4.1.2 Overview of CCR5 trafficking pathways following chemokine agonist treatment

As CCR5 acts as a HIV co-receptor, a wide range of tools are available and so its trafficking in response to ligand stimulation has been well studied. It has been shown that CCR5 is internalised via clathrin-mediated endocytosis to early endosomes (Signoret et al., 2005), although in some cell types there may be a contribution of clathrin-independent internalisation pathways (Mueller et al., 2002a; Venkatesan et al., 2003). Once internalised, CCR5 enters recycling endosomes from which it returns to the plasma membrane without entering the degradative pathway (Signoret et al., 2000).

4.1.3 Current knowledge of CCR2B trafficking pathways

CCR2 has been shown to be internalised in response to stimulation with its main ligand CCL2 in monocytes (Fox et al., 2011), astrocytes (Andjelkovic et al., 2002) and several transfected cell lines (Minsaas et al., 2010). In the previous chapter, I showed that HA-
CCR2B expressed in transfected HEK293 cell lines also internalised in response to CCL2, as expected. It has been suggested that CCR2 internalisation involves both clathrin-mediated and caveolar endocytic pathways, and that dynamin (Garcia Lopez et al., 2009) and filamin A (Minsaas et al., 2010) are important for the endocytosis. Unfortunately, the antibodies and tools available for studying CCR2B are more limited than for CCR5 and thus relatively little is known about post-endocytic trafficking of CCR2B. However, the differential localisation of internalised HA-CCR2B and HA-CCR5 observed in transfected HEK293 cells, as reported in the previous chapter, suggests that the intracellular routes followed by these two receptors may not be comparable. A study using Cherry-CCR2B showed colocalisation with the early endosome marker, early endosome antigen 1 (EEA1) and the lysosomal marker Lamp1 (Garcia Lopez et al., 2009), however this receptor was tagged with the large cherry tag on the C-terminus and it has been shown that the C-terminal cytoplasmic tail plays an important role in the intracellular trafficking of chemokine receptors (see Bennett et al., 2011 for review). This highlights the requirement for further study of CCR2B using complementary conditions.

4.1.4 Objectives

The main objective of this chapter was to characterise the intracellular trafficking of CCR2B in response to stimulation with CCL2, and to compare it to that of CCL5-treated CCR5. These objectives were carried out using HEK293 cells transfected to stably express HA-CCR2B or HA-CCR5.

4.2 Relevant Methodology

4.2.1 Transferrin/EGF uptake

Fluorescent transferrin and epidermal growth factor (EGF; both from Molecular Probes) were used for colocalisation assays. 25 μg/ml transferrin-488 or transferrin-594 or 2 μg/ml EGF-488 was added to the binding medium at the same time as the chemokine.

4.2.2 Recycling assay

Recycling assays using THP-1 and HEK HA-CCR2B cells were carried out in triplicate whereas recycling assays using HEK HA-CCR2B cells transiently transfected to express
GFP or Rab4s used single replicates. HEK HA-CCR2B cells were either used directly or transiently transfected to express YFP-tagged wild type Rab4 (Rab4WT), dominant negative Rab4 (Rab4N121I), constitutively active Rab4 or, as a control, GFP as described in Section 2.6.1. Adhered HEK HA-CCR2B cells were treated with either binding medium alone or in the presence of 100 nM CCL2 for 60 minutes at 37 °C. The cells were then subjected to an acid-strip wash to remove cell-surface bound CCL2. The acid strip wash consisted of two rinses in ice-cold pH3.5 elution medium followed by two 3-minute washes in pH3.5 elution medium and then neutralisation using binding medium at pH7.1. At this point, samples of CCL2-treated (i) and non-chemokine-treated (ii) cells were detached and immunolabelled for cell-surface CCR2 using R&D α-CCR2 or using an irrelevant mouse IgG2b as an isotype control. Some CCL2-treated samples (iii) were further incubated in binding medium at 37 °C for 60 minutes, to enable cell-surface CCR2 recovery, prior to immunolabelling as described in Section 2.8.2. THP-1 cells were treated with either either binding medium alone or in the presence of 100 nM CCL2 for 60 minutes at 37 °C. The cells were then subjected to an acid-strip wash to remove cell-surface bound CCL2. The acid strip wash consisted of two 3-minute washes in pH3.5 elution medium and then neutralisation using binding medium at pH7.1. For non-stripped cells, the same washes were carried out using binding medium. Non-chemokine-treated cells were then incubated again in binding medium at 37 °C for 60 minutes (ii). CCL2 treated cells were further incubated in 100 nM CCL2 (i), binding medium (iii) or with 400 nM TAK-779 at 37 °C for 60 minutes. Cells were immunolabelled for cell surface CCR2 using R&D α-CCR2 as described above.

The cell surface expression for samples (i) and (iii) was expressed as a percent of the expression in samples (ii), and the recycling was expressed as fold recovery of cell-surface CCR2 expression during the recycling period, i.e., (cell surface expression of [iii])/(cell surface expression of [i]). For experiments using HEK HA-CCR2B cells transiently transfected to express YFP-tagged Rab4s or GFP, the ratio of recycling in FITC positive cells compared to FITC negative cells within the same sample was calculated.
4.2.3 CCR2 degradation assay

HEK HA-CCR2B cells were seeded in a 24-well plate at 7 x 10^5 cells/well and incubated at 37 °C, 5 % CO_2 for 24 hours. The medium was replaced with growth medium containing 10 µg/ml cycloheximide and the cells were incubated at 37 °C, 5 % CO_2 for 4 hours. A sample was taken after four hours for T0 and then the medium was replaced with binding medium containing 10 µg/ml cycloheximide with or without 100 nM CCL2 and the cells were incubated for the indicated time. Cells were then lysed in non-reducing sample buffer and analysed by SDS-PAGE and western blotting using HA.11 as described in Section 2.9. Densitometry analysis of the three HA-CCR2B bands and a loading control was carried out using ImageJ following the protocol from Luke Miller (http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/). Briefly, the relative density profile plots of identically sized boxes containing the loading control bands were measured. After removal of background noise by closing off peaks, the peak area of each band was calculated as a percentage of the total area of all measured peaks. The T0 peak was used as the standard and assigned a relative density of 1. All other peaks were assigned relative densities based on comparison of their peak area to that of T0. This analysis was repeated with the HA-CCR2B bands. Finally, each HA-CCR2B band relative density was divided by the appropriate loading control relative density to give an adjusted density, which was then expressed as a percentage showing the percentage of the initial receptor present at T0 that remained at the timepoint tested.

4.2.4 Testing for post-translational modifications of HA-CCR2B

To test for PKC-mediated phosphorylation, HEK HA-CCR2B cells from one 90 % confluent well of a 24 well plate were either untreated or pre-treated with 5 µM GF109203X (bisindolylmaleimide, PKC inhibitor) or 100 ng/ml PMA for 60 minutes at 37 °C, 5 % CO_2. The cells were washed in PBS and lysed by scraping into 1 % NP-40 lysis buffer containing a protease inhibitor cocktail, left to incubate for 20 minutes on ice and centrifuged at 13000 xg to remove unbroken cells. Samples were analysed by SDS-PAGE and western blotting using HA.11 to detect HA-CCR2B as described in Section 2.9.
To test for glycosylation, HEK HA-CCR2B cells from one 70 % confluent well of a 24 well plate were either untreated or pretreated with DMSO, 10 µg/ml tunicamycin or 2 mM benzyl α-N-Acetylgalactosamine (GalNAc) for 24 hours at 37 °C, 5 % CO₂. The cells were then lysed as described above and, where indicated, the cell lysate was incubated with 50 mU neuraminidase for 20 hours at 37 °C. Samples were analysed by SDS-PAGE and western blotting using HA.11.

4.2.5 Detection of ubiquitinated chemokine receptors

The immunoprecipitation (IP) protocol for detection of ubiquitinated chemokine receptors was adapted from a protocol by Marchese et al. (Marchese 2009), using an antibody against the FLAG tag on the transiently expressed ubiquitin. HEK and HEK HA-CCR2B cells were plated in a 24-well plate at 2 x 10⁵ cells/well and incubated at 37 °C, 5 % CO₂ for 24 hours before transfection with pcDNA3.1 FLAG-Ub using jetPRIME™ reagent to give HEK, HEK HA-CCR2 and FLAG-Ub and HEK FLAG-Ub cells. 24 hours later, 4 wells per sample were washed in PBS and scraped into 400 µl ubiquitination lysis buffer and incubated at 4 °C for 30 minutes followed by 10 s sonication at 20 % amplitude and centrifugation to removed unbroken cells and debris. The cell lysate was pre-incubated with 10 µg of the FLAG M2 antibody for 1 hour at 4 °C, then a 10 µl bed volume of Amintra protein G-coated agarose beads (Expedeon, Harston, UK) was added and incubated for a further 1 hour at 4 °C. Following two washes with lysis buffer, bound proteins were eluted by incubation with reducing sample buffer for 30 minutes and then analysed by SDS-PAGE and western blotting with both HA.11 and FLAG M2.

4.2.6 Subcellular fractionation

Two 70 % confluent 10 cm plates of HEK HA-CCR2B were used for each experiment. The cells were either untreated, to study the normal state of the receptor, or treated with 100 nM CCL2 in binding medium for 30 minutes at 37 °C, to study the agonist-stimulated receptor. The cells were washed in ice-cold PBS and homogenization medium containing a protease inhibitor cocktail and then scraped into homogenization medium and lysed by 35 - 40 passes through a ball bearing homogenizer (Isobiotech, Heidelberg, Germany) with a 12 µm clearance. A 10.5 ml 1 – 22 % continuous Optiprep gradient was prepared by over-layering equal volumes of 1 %, 8 %, 15 % and 22 %
Optiprep solutions into a thinwall polyallomer tube (Beckman Coulter) and incubating at 4 °C for 14 hours to enable diffusion. The cell lysate was loaded on to the top of this gradient and centrifuged at 200000 xg, 3 hours, 4 °C in an Ultra Beckman X100 (Beckman Coulter). 1 ml or 0.5 ml fractions were harvested manually by aspiration from the meniscus using a 1 ml Gilson pipette. Non-reducing sample loading buffer was added to 100 µl aliquots to lyse the organelles and the samples were frozen at −80 °C and later analysed by SDS-PAGE on a 10 % acrylamide gel followed by western blotting with antibodies against the HA-tag and various organelle and intracellular trafficking pathway markers. Up to four replicate gels were run for each experiment and the membranes were cut into sections for analysis with different antibodies. Densitometry analysis of the marker and HA-CCR2B bands present in each fraction was carried out as described in Section 4.2.3, except that no loading control was used and the most intense band for each set of marker/HA-CCR2B bands was used as the standard.

An identical control Optiprep gradient loaded with homgenisation medium instead of sample was harvested in the same way and used to measure the refractive index of each fraction using an Abbe 5 refractometer (Bellingham and Stanley Ltd, Kent, UK). This data was plotted using GraphPad Prism 5 to check the linearity of the gradient.

4.3 Results

4.3.1 Agonist-induced CCR2B internalisation

4.3.1.1 Time course of CCR2B internalisation

The agonist-induced internalisation of CCR2B and CCR5 was described in the previous chapter. An initial time course experiment was carried out to assess the time taken for internalisation and how the internalised CCR2B intracellular localisation changes over time. Pre-labelling of cell surface HA-CCR2B on HEK HA-CCR2B cells with HA.11 was used to follow the internalisation of cell surface receptor in response to treatment with 100 nM CCL2 for up to 60 minutes (Figure 4.1). In untreated cells only cell surface HA-CCR2B staining is observed. After 5 minutes of treatment, vesicular staining at the edge of the cell starts to become apparent. At 15 minutes many of these vesicles can be seen further inside the cell and by 30 and 60 minutes of treatment the majority of the HA-CCR2B is internalised. The kinetics of CCR2 internalisation on monocytes have not
been fully described, however, the internalisation time course shown in Figure 4.1 is comparable to that observed for CCR2 in other cell types including THP-1 (Andjelkovic et al., 2002; Dzenko et al., 2001; Garcia Lopez et al., 2009; Wang et al., 1993a) and for CCR5 on monocytes (Fox et al., 2011).

**Figure 4.1 Internalisation time course of HA-CCR2B in HEK HA-CCR2B cells.**
HA-CCR2B was pre-labeled using HA.11 (grey) and the cells were either untreated or treated with 100 nM CCL2 for the indicated time at 37 °C. Scale bar = 10 μm.

### 4.3.1.2 Route of CCR2B internalisation

Immunofluorescence colocalisation studies and flow cytometry down-modulation assays coupled with various drug and inhibitor treatments were used to address the clathrin or lipid dependency of the HA-CCR2B down-modulation process.

The HA-CCR2B down-modulation level of cells pre-treated with the clathrin-mediated endocytosis inhibitor ikarugamycin (Luo et al., 2001) was measured by flow cytometry and found to be significantly reduced compared to the DMSO control (Figure 4.2). This result agrees with the reduction in the down-modulation level observed following pre-treatment with 0.4 M sucrose (Figure 4.2), which has been previously used to show clathrin-dependency for endocytosis of various chemokine receptors (Bruhl et al., 2003; Luker et al., 2010; Otero et al., 2006; Rose et al., 2004). Transferrin uptake has been
extensively studied and has been shown to proceed mainly via clathrin-mediated endocytosis of the molecule and its receptor (Harding et al., 1983). The immunofluorescence endocytosis assay, used previously for the time course in Section 4.3.1.1, was repeated and the cells were treated with fluorescent transferrin in addition to CCL2. Partial colocalisation of internalised HA-CCR2B with fluorescent transferrin was observed at early time points following agonist stimulation (Figure 4.3), supporting the suggestion that endocytosis of HA-CCR2B may be clathrin-dependent.

However, flow cytometry assays using inhibitors of the cholesterol-dependent pathways showed different impacts of the drugs on the level of HA-CCR2B down-modulation, and results can be interpreted in two different ways. Pre-treatment with the cholesterol-extracting drug methyl-β-cyclodextrin (Klein et al., 1995) leads to a large reduction in the down-modulation in response to agonist treatment (Figure 4.2). A smaller reduction is also observed following treatment with nystatin, which binds to and sequesters cholesterol (Smart and Anderson, 2002). However, pretreatment with filipin, another cholesterol sequestering drug (Smart and Anderson, 2002), had no effect on the down-modulation. These results could be due to the difference in the stringency of the inhibitors. Filipin and nystatin are less stringent than methyl-β-cyclodextrin as they disrupt but do not completely remove cholesterol from the membrane and so they may not have a sufficient effect on the lipid composition to impact much on the receptor down-modulation. The concentration of cholesterol modulating drugs requires careful optimisation for each cell type (Smart and Anderson, 2002) and thus the conditions used for filipin treatment may have been less suitable than those used for methyl-β-cyclodextrin and nystatin. The removal or oxidation of cholesterol can impact on the conformation of receptors and thus has been shown to affect ligand binding to some chemokine receptors including CCR5 (Nguyen and Taub, 2002; Nguyen and Taub, 2003a, b; Signoret et al., 2005). The reduction in agonist-induced down-modulation observed following methyl-β-cyclodextrin treatment could be an indirect effect due to a reduction in agonist-binding.
Figure 4.2 Effect of endocytosis inhibitors on receptor down-modulation.
Stably transfected HEK HA-CCR2B cells pretreated with the indicated drug for 60 minutes at 37 °C were treated with medium (black histogram) or 100 nM CCL2 (coloured histogram) in the presence of the drug for 60 minutes at 37 °C then immunolabeled for cell surface HA-CCR2B using R&D α-CCR2 or the relevant isotype control antibody and analysed by flow cytometry. The % of receptor down-modulation (reduction in cell surface epitope availability) is expressed as a percentage of that observed for medium-treated cells. (A) shows histograms of individual replicates from a single experiment performed in triplicate and all of the mean down-modulations ± SD from that experiment are summarised in (B). (C) represents the means ± SD of two separate experiments each carried out in triplicate. A 1-way ANOVA followed by a Bonferroni multiple comparisons test was used to compare drug and control treated cells. *, p<0.05.
Figure 4.3 Colocalisation study using HEK HA-CCR2B cells and fluorescent transferrin. HA-CCR2B was pre-labeled using HA.11 (red) and the cells were treated with 100 nM CCL2 and 25 µg/ml transferrin-488 (green) for the indicated time at 37 °C. The cells were counterstained with DAPI (blue). Scale bar = 10 μm. Open arrow heads indicate examples of colocalisation.
4.3.1.3 CCR2B trafficking to early endosomes

Immunofluorescence colocalisation studies were carried out using two different markers, EEA1 and transferrin, to investigate how HA-CCR2B traffics through the endocytic pathway following agonist stimulation. The majority of the internalised HA-CCR2B colocalises with EEA1 at 5 minutes after agonist stimulation (Figure 4.4) suggesting that internalised HA-CCR2B traffics to early endosomes. Additionally, at 5 minutes a large proportion of the internalised HA-CCR2B colocalises with internalised fluorescent transferrin (Figure 4.3) confirming that the receptor is in the early endocytic pathway. Partial colocalization of HA-CCR2B and EEA1/transferrin is seen at later time points and this gradually decreases over time with the HA-CCR2B and EEA1/transferrin dots moving from being completely overlapping to being adjacent to each other (Figures 4.3 and 4.4).

4.3.2 Agonist-induced CCR2B recycling

4.3.2.1 Colocalisation with transferrin pathway and Rab4

CCR5 is known to recycle via the recycling endosome to the cell surface following internalisation induced by its natural ligands. As the intracellular localisation of internalised CCR2B is different to that of CCR5 as described in the previous chapter, experiments were carried out to see what impact this has on the eventual fate of the receptor. Endocytosed transferrin and its receptor are recycled by both a rapid and a slow recycling pathway (Hopkins et al., 1994) and therefore fluorescent transferrin is commonly used as a marker for receptor recycling. In this study HEK HA-CCR2B cells were treated with both CCL2 and fluorescent transferrin. Some partial colocalisation with transferrin remains even after 60 minutes of agonist treatment (Figure 4.3). The presence of HA-CCR2B in early endocytic/recycling structures may indicate that the receptor can recycle.
Figure 4.4 Colocalisation study using HEK HA-CCR2B cells and the early endosome marker EEA1.

HA-CCR2B was pre-labeled using HA.11 (red) and the cells were treated with 100 nM CCL2 for the indicated time at 37 °C. After fixation the cells were co-stained with an antibody against EEA1 (green) and counterstained with DAPI (blue). Scale bar = 10 μm. Open arrow heads indicate examples of colocalisation.
Endocytosed receptors are delivered to the early/sorting endosome. Fast recycling can take place directly from the early/sorting endosome or alternatively the receptor can be sorted to the endocytic recycling centre and be recycled to the plasma membrane via recycling endosomes (Grant and Donaldson, 2009; Hopkins et al., 1994). This slower recycling has been shown to be Rab11-dependent for many receptors (Fan et al., 2003; Grimsey et al., 2011; Moore et al., 2004; Ullrich et al., 1996). Although Rab11 is found in multiple different types of endosomes, a large proportion is found marking the endocytic recycling compartment localised near to the microtubule organising centre and Golgi complex in most cell types (Grant and Donaldson, 2009; Sonnichsen et al., 2000). Thus Rab11 normally shows predominantly perinuclear staining, which contrasts with the vesicular staining of internalised receptors observed in our transfected HEK293 cells and so the involvement of Rab11 in HA-CCR2B recycling was not investigated.

Another Rab protein that is key for receptor recycling is Rab4, which is found at both the early/sorting endosome and the recycling endosome (Sonnichsen et al., 2000). Rab4 is involved in rapid recycling from the early/sorting endosome (Sheff et al., 1999; Sonnichsen et al., 2000), and via its sorting function also regulates the recycling endosome pathways (van der Sluijs et al., 1992). The potential role of Rab4 in recycling of internalised HA-CCR2B was investigated using transient over-expression of wild-type and mutant fluorescently labelled Rab4 proteins.

Some colocalisation of internalised HA-CCR2B with fluorescent wild-type Rab4 is observed (Figure 4.5), and is more common in peripheral vesicles than in the main endocytic recycling area. Treatment with the microtubule disrupting agent nocodazole was tested to investigate if the apparent colocalisation represented true localisation in the same vesicles, however the results were inconclusive (data not shown).
Figure 4.5 Colocalisation study using HEK HA-CCR2B cells and fluorescent Rab4.
HEK HA-CCR2B cells transfected to transiently express Rab4-YFP (green) were labelled for HA-CCR2B using HA.11 (red) and then treated with 100 nM CCL2 for 60 minutes at 37 °C. Scale bar = 10 μm.

4.3.2.2 Recycling Assay

The partial colocalisation of HA-CCR2B with Rab4 and transferrin could indicate some recycling of HA-CCR2B. Therefore, recycling assays were carried out using a 60 minute agonist stimulation followed by acid stripping and a 60 minute recovery period in medium before flow cytometric analysis of cell surface CCR2 staining. Acid stripping using elution buffers of pH < 4.0 is employed as a technique to remove any molecules of chemokine remaining on cell surface receptors, which could interfere with antibody binding or lead to re-internalisation of the receptor (Signoret et al., 2000). The required pH is dependent on the receptor and ligand being studied, as pH 2.0 removes more than 80 % of surface bound CCR5 ligands CCL4 and CCL5 (Signoret et al., 2004) whereas pH 3.0 can be used to remove the CXCR4 ligand CXCL12 (Amara et al., 1997). The highest effective pH should be used in order to reduce cellular damage and thus pH 3.5 was chosen for this study as it had been used previously for CCL2 removal (Ge et al., 2008). The same cell surface expression level for untreated cells and the same level of down-modulation for CCL2-treated cells was observed for stripped and non-stripped THP-1 cells. This suggests that the acid strip has no effect on the R&D α-CCR2 antibody binding.

Partial recycling was observed with the cell surface receptor level increasing after the recovery period to on average 1.4 times the level observed after agonist treatment (Figure 4.6).
Figure 4.6 HA-CCR2B recycling in HEK HA-CCR2B cells.
HEK HA-CCR2B cells were either treated with medium (black histogram) or 100 nM CCL2 (red histogram) for 60 minutes at 37 °C, then acid stripped and immunolabelled for cell surface HA-CCR2B using R&D α-CCR2. Other medium treated cells were labelled with the relevant isotype control antibody (grey histogram). A separate sample of cells were treated with 100 nM CCL2 for 60 minutes at 37 °C, then acid stripped and treated with medium at 37 °C for a 60 minute recovery period before labelling cell surface HA-CCR2B (blue histogram). The cells were analysed by flow cytometry. (A) shows a single set of samples representative of five separate experiments. (B) shows all five experiments, using the average receptor surface expression where multiple replicates were performed in the same experiment. % receptor surface expression is expressed as the percentage of the expression observed for medium treated cells.

This level of recycling is quite low compared to what has been observed for some other chemokine receptors (Signoret et al., 2004). To address the possibility of the increase in cell surface expression being due to replenishment from intracellular stores rather than internalised and recycled CCR2B, I carried out the same experiment using THP-1 cells, which endogenously express CCR2 and do not show massive accumulation of CCR2 in the biosynthetic pathway like observed in HEK HA-CCR2B. In these cells more cell surface CCR2 recovery, to an average of 3.4 times the level observed after agonist treatment, was observed, which confirms CCR2’s ability to recycle (Figure 4.7). The greater cell surface recovery may reflect a more efficient recycling pathway in THP-1 cells. The level of recycling observed is much higher on stripped THP-1 cells (Figure 4.7A) compared to those that were washed in a neutral pH medium (Figure 4.7B). Recycling levels could be increased by the use of a CCR2 antagonist, TAK-779, that blocks recycled CCR2 at the plasma membrane (Figure 4.7). This suggests under the conditions used here, the pH3.5 elution buffer used for the acid strip may not remove all CCL2, resulting in re-internalisation/re-endocytosis of some recycled receptors.
Figure 4.7 CCR2 recycling in THP-1 cells.
THP-1 cells were either treated with medium (black histogram) or 100 nM CCL2 (red, blue and green histograms) for 60 minutes at 37 °C, then acid stripped (A) or washed in neutral pH medium (B) and treated with medium (black and blue histograms), 400 nM TAK-779 (green histogram) or 100 nM CCL2 (red histogram) at 37 °C for 60 minutes. Cells were then immunolabelled for cell surface HA-CCR2B using R&D α-CCR2. Other medium treated cells were labelled with the relevant isotype control antibody (grey histogram). The cells were analysed by flow cytometry. The histograms each show a single set of samples representative of a single experiment carried out in triplicate. The graphs show the mean ± SD of all replicates. % receptor surface expression is expressed as the percentage of the expression observed for medium treated cells.

As some colocalisation of the internalised HA-CCR2B is observed with Rab4 (Figure 4.5), further work was carried out to determine if Rab4 plays a role in the HA-CCR2B recycling. Three fluorescently labelled Rab4 constructs, wild-type Rab4 (Rab4WT), dominant negative Rab4 (Rab4N121I) and constitutively active Rab4 (Rab4Q67L), were transiently transfected into the HEK HA-CCR2B cells. As the Rab4 constructs are
tagged with YFP, cells expressing the construct and non-expressing cells could be
differentiated by flow cytometry using a 488 nm laser and detection in the fluorescein
isothiocyanate (FITC) channel. The transfection efficiency was approximately 28 %
without lethal effects on the cells. Therefore, by gating on either FITC positive or FITC
negative cells, Rab4 expressing and non-expressing cells could be compared within the
same sample. The recycling assay used previously for HEK HA-CCR2B cells was
repeated on these Rab4 transfected HEK HA-CCR2B cells. The level of recycling seen
for transfected cells, both Rab4 expressing and non-expressing, was generally higher
than previously observed for the non-transfected HEK HA-CCR2B cells but was
slightly inconsistent between the different Rab4 constructs. Cells transfected with GFP
used as a control also showed higher levels of recycling, suggesting that the transfection
process may impact on the endocytic and intracellular trafficking pathways. Therefore,
to enable analysis the recycling level observed in Rab4 expressing cells was normalised
to the Rab4 non-expressing cells in the same sample and compared to GFP transfected
cells not non-transfected cells. The ratio of recycling in FITC positive (Rab4 expressing)
compared to FITC negative (Rab4 non-expressing) cells was slightly greater than 1 for
all Rab4 constructs (Figure 4.8). However, no significant difference was observed
between the HA-CCR2B recycling in GFP transfected and all of the Rab4 construct
transfected cell samples (Figure 4.8) suggesting that Rab4 does not play a role in HA-
CCR2B recycling.
Figure 4.8 Involvement of Rab4 in HA-CCR2B recycling in HEK HA-CCR2B cells.
(C) HEK HA-CCR2B cells were transfected to express YFP-tagged wild type Rab4 (Rab4WT), dominant negative Rab4 (Rab4N121I), constitutively active Rab4 or, as a control, GFP. The cells were either treated with medium (i) or 100 nM CCL2 (ii) for 60 minutes at 37 °C then acid stripped and immunolabelled for cell surface HA-CCR2B using R&D α-CCR2 or treated with 100 nM CCL2 for 60 minutes at 37 °C then acid stripped and treated with medium at 37 °C for a 60 minute recovery period (iii) before labelling cell surface HA-CCR2B. The cells were analysed by flow cytometry. The cell surface expression for (ii) and (iii) is expressed as a percentage of that observed in (i) and the recycling is expressed as (cell surface expression of [iii])/(cell surface expression of [i]). The transfection efficiency is approximately 28 % and so each sample contains both Rab4/GFP expressing cells (FITC positive) and cells not expressing Rab4/GFP (FITC negative). As the transfection process itself appears to result in higher apparent levels of recycling, the ratio of recycling in FITC positive cells compared to FITC negative cells is shown on the graph. Graphs show the means ± SD of 2 to 4 experiments each carried out in triplicate. Comparison of all three Rab4s to GFP using a one-way ANOVA followed by a Dunnett’s multiple comparison tested show no significant difference. Comparison of all three Rab4s to GFP using a one-way ANOVA followed by a Dunnett’s multiple comparison tested show no significant difference.

As only partial recycling was observed for CCR2, this suggests that receptors may also follow another fate. Endocytosed endothelial growth factor (EGF) and its receptor can undergo different fates in a ligand concentration-dependent manner (Sigismund et al., 2008). At low EGF concentrations (typically 1.5 ng/ml) most of the internalised EGF ligand-receptor complex is recycled to the cell surface (Sigismund et al., 2008). However, at higher, but still physiological (reviewed in Sigismund et al., 2005), EGF concentrations (typically 20 – 100 ng/ml), the level of recycling is reduced and instead the majority of the complex enters the lysosomal degradation pathway (Sigismund et al., 2008). The epidermal growth factor receptor (EGFR) is constitutively expressed on many cell lines including low levels on HEK293 cells (Lin et al., 2006). Therefore high
concentrations of fluorescent EGF are commonly used as a marker for the degradative pathway. In this study HEK HA-CCR2B cells, pre-labelled for cell surface HA-CCR2B, were treated with both CCL2 and 2 µg/ml fluorescent EGF before being studied by immunofluorescence following the endocytosis assay. Partial colocalisation of internalised HA-CCR2B with EGF is observed following the endocytosis assay at all time points tested up to 60 minutes suggesting that at least a proportion of HA-CCR2B may follow the same pathway as EGF (Figure 4.9). Co-labelling with fluorescent EGF and fluorescent transferrin showed that HA-CCR2B that does not colocalise with EGF often colocalises with transferrin (Figure 4.10) confirming that CCR2 can enter both the recycling and degradative pathways.
**Figure 4.9 Colocalisation study using HEK HA-CCR2B cells and fluorescent EGF.**

HA-CCR2B was pre-labeled using HA.11 (red) and the cells were treated with 100 nM CCL2 and 2 µg/ml fluorescent EGF (green) for the indicated time at 37 °C. Cells were counterstained with DAPI (blue). Scale bar = 10 µm. Open arrow heads indicate examples of colocalisation.
Figure 4.10 Colocalisation study using HEK HA-CCR2B cells, fluorescent transferrin and fluorescent EGF.
HA-CCR2B was pre-labeled using HA.11 (red) and the cells were treated with 100 nM CCL2, 25 µg/ml transferrin-594 (green) and 2 µg/ml EGF-488 (white) for the indicated time at 37 °C. Cells were counterstained with DAPI (blue). Scale bar = 10 µm. Arrows indicate the location of inset 2X zoom panels.

4.3.3 Agonist-induced CCR2B degradation

4.3.3.1 Colocalisation with late endosomal and lysosomal markers

Two late endosome/lysosome markers, CD63 and Lamp1, were used to investigate if internalised HA-CCR2B enters the lysosomal degradation pathway. After 5 and 15 minutes of agonist treatment almost no colocalisation of pre-labelled HA-CCR2B with CD63/Lamp1 is observed (Figures 4.11 and 4.12). At later time points HA-CCR2B dots can be observed adjacent to CD63 dots and this is greatest at 60 minutes (Figure 4.11). However, as visual inspection of these images showed very little overlap of the adjacent dots, they were further analysed out using Volocity 3D Image Analysis Software to investigate if the dots were actually colocalised or just in close proximity. A single line was drawn through the middle of both of the dots and the intensity profiles of the red (HA-CCR2B) and green (CD63) fluorescence were measured at regular frequent intervals along this line. The most intense red and green points mark the centres of intensity of the two dots and the distance between these centres was measured. This distance was typically greater than 200 nm, which is above the 70 – 100 nm threshold commonly used to define colocalisation (Anantharam et al., 2011; Barysch et al., 2009; Koyama-Honda et al., 2005). This suggests that the HA-CCR2B and CD63 proteins are not in the same vesicles, but in adjacent structures.

The lack of colocalisation of HA-CCR2B with lysosomal degradation pathway markers contrasts with the colocalisation observed with EGF (Figure 4.9). This could be due to slight technical differences in the assays. EGF marks the entire trafficking pathway
whereas CD63 and Lamp1 only mark the later part of the pathway. The endocytosis assay is dependent on a maintained antibody-HA-CCR2B interaction to visualise HA-CCR2B in the later part of the pathway.

To address the possibility of pH change-induced antibody dissociation during trafficking through the pathway, the effect of ammonium chloride, used to neutralise endosomal pH, was tested (data not shown). The results were inconclusive and highly variable from cell to cell. However, the general view was that there was no major increase in the level of colocalisation of HA-CCR2B and CD63 after 30 or 60 minutes of agonist stimulation, compared to cells without ammonium chloride treatment.
Figure 4.11 Colocalisation study using HEK HA-CCR2B cells and the late endosome marker CD63.

HA-CCR2B was pre-labeled using HA.11 (red) and the cells were treated with 100 nM CCL2 for the indicated time at 37 °C. After fixation the cells were co-stained with an antibody against CD63 (green) and counterstained with DAPI (blue). Scale bar = 10 μm. Open arrow heads indicate examples of colocalisation. Closed arrow heads indicate examples of adjacent dots.
Figure 4.12 Colocalisation study using HEK HA-CCR2B cells and the lysosomal marker Lamp1.
HA-CCR2B was pre-labeled using HA.11 (red) and the cells were treated with 100 nM CCL2 for the indicated time at 37 °C. After fixation the cells were co-stained with an antibody against Lamp1 (green) and counterstained with DAPI (blue). Scale bar = 10 μm. Open arrow heads indicate examples of colocalisation.
Another possibility is that the antibody is rapidly degraded upon entry into the lysosome. This was addressed by pretreatment of the cells with the lysosomal-specific protease inhibitor leupeptin. As prolonged inhibition of lysosomal degradation can lead to enlargement of lysosomes and result in a block of entry into the lysosomal degradative pathway, both overnight and 90 minute pretreatment with leupeptin were tested. No increase in colocalisation of HA-CCR2B with CD63 is observed in cells pretreated with leupeptin overnight (Figure 4.13). Some limited colocalisation is observed when pretreating with leupeptin for 90 minutes only (Figure 4.13).

![Figure 4.13 Colocalisation study using leupeptin-treated HEK HA-CCR2B cells and the late endosome marker CD63.](image)

HEK HA-CCR2B cells were treated with 100 µM leupeptin overnight or for 90 minutes during pre-labelling as indicated. HA-CCR2B was then pre-labeled using HA.11 (red) and the cells were treated with 100 nM CCL2 and 100 µM leupeptin for 60 minutes at 37 °C. After fixation the cells were co-stained with an antibody against CD63 (green) and counterstained with DAPI (blue). Scale bar = 10 μm. Arrows indicate the location of inset 2X zoom panels.

Taken in combination the ammonium chloride and leupeptin treatment results did not give a clear answer and so a different complementary down-modulation assay was employed to assess the distribution of total CCR2 molecules. The down-modulation assay has the advantage of not being dependent on a maintained antibody-HA-CCR2B interaction, however it does visualise all HA-CCR2B, both receptors trafficking from the cell surface and those from the biosynthetic pathway and so is only useful to
compare treated and untreated cells. An increase in the proportion of HA-CCR2B that colocalises with CD63 is observed in agonist-treated cells compared to medium-treated cells (Figure 4.14). This indicates that a proportion of the internalised HA-CCR2 may enter the degradative pathway.

**Figure 4.14 Colocalisation study using HEK HA-CCR2B cells and the late endosome marker CD63 following the down-modulation assay.**

HEK HA-CCR2B cells treated with medium or 100 nM CCL2 for 60 minutes at 37 °C. After fixation the cells were co-labelled with HA.11 (HA-CCR2B, red) and α-CD63 (green) and counterstained with DAPI (blue). Images represent single sections cut through the CD63 compartment. Scale bar = 10 μm. Arrows indicate the location of inset 2X zoom panels.

### 4.3.3.2 Biochemical analysis of agonist-induced degradation

As some colocalisation with degradative pathway markers was observed, a biochemical assay was carried out to check if HA-CCR2B is actually degraded following agonist-induced internalisation. Cell lysates, from HEK HA-CCR2B cells treated with cycloheximide and either medium or agonist for various time points (up to 8 hours), were analysed for the presence of HA-CCR2B by immunoblotting. Three different forms of the receptor were identified, here termed the low, “normal” and high molecular weight forms based on comparison to the expected molecular weight of CCR2 (Figure 4.15). Treatment of the cells with cycloheximide, to stop *de novo* protein synthesis,
showed that there is a basal level of degradation of HA-CCR2B over the 4 or 8 hours for which the experiments were carried out, even for cells that were treated with medium only (Figure 4.15). The half-lives of the three different forms of HA-CCR2B appear to vary, but are within the range of hours (Figure 4.15) rather than days in accordance with what has been reported for several other chemokine receptors including CCR5 (Percherancier et al., 2001; Signoret et al., 2000; Weber et al., 2004) and CXCR4 (Marchese and Benovic, 2001). However the high molecular weight form of HA-CCR2B, unlike the other two forms, is degraded faster in response to agonist treatment (Figure 4.15). After 4 hours of treatment there is a significant difference in the percentage of the high molecular weight HA-CCR2B receptor remaining in medium- (80 ± 14 %) and agonist- (32 ± 21 %) treated cells (Figure 4.15C). In contrast, this is not the case for the “normal” (medium, 22 ± 12 %; agonist, 34 ± 18 %) or low (medium, 21 ± 22 %; agonist, 29 ± 23 %) molecular weight forms (Figure 4.15C). Treatment with either leupeptin or ammonium chloride resulted in 22 % or 90 %, respectively of the high molecular weight HA-CCR2B band remaining after 4 hours of CCL2 stimulation, compared to only 13 % for untreated cells in the same experiment (Figure 4.15D). This reduction in the level of degradation suggests that the high molecular weight HA-CCR2B form undergoes lysosomal degradation.
Figure 4.15 Degradation of HA-CCR2B in response to agonist treatment.

(A) Cell lysates, from HEK HA-CCR2B cells treated with 10 µg/ml cycloheximide and either medium (BM) or 100 nM CCL2 for 0, 1, 4 or 8 hours were analysed for the presence of HA-CCR2B by SDS-PAGE and immunoblotting using HA.11. β-catenin and the transferrin receptor were used as loading controls. Cell lysates from untreated HEK HA-CCR5 cells were analysed in the same way. (B) Densitometry analysis of the percentage of receptor remaining after 0, 1, 2, 3 or 4 hours of medium (black) or 100 nM CCL2 (red) treatment was carried out for each band identified in (A) using ImageJ. Graphs show the means ± SD of 2 experiments. A 2-way ANOVA was carried out to test for interaction. *, p<0.05; ns, non-significant. (C) Densitometry analysis of the percentage of receptor remaining after 4 hours of medium (black) or 100 nM CCL2 (white) treatment was carried out for each band identified in (A) for 4 experiments using ImageJ. A 2-way ANOVA followed by a Bonferroni multiple comparisons test was used to check for differences between medium and agonist treatment. ****, p<0.0001; ns, non-significant. (D) Comparison of cell lysates from HEK HA-CCR2B cells treated with either medium (BM) or 100 nM CCL2 and 10 µg/ml cycloheximide alone (untreated) or in combination with 100 µM leupeptin or 50 mM ammonium chloride for 4 hours. Arrows indicate the high (red), “normal” (green) and low (blue) molecular weight forms of HA-CCR2B.
4.3.3.3 Identification and characterisation of the different forms of CCR2B

Figure 4.15 identifies at least three forms of HA-CCR2B present in HEK HA-CCR2B cells. The predicted molecular weight for HA-CCR2B is 44 kDa, which corresponds to the middle band, here termed the “normal” molecular weight band. Two lines of evidence suggest that the other bands are different forms of HA-CCR2B and not just non-specific bands. Firstly both the low and high molecular weight bands are detected using the α-HA antibody, HA.11, in cell lysate from HEK HA-CCR2B cells but not in cell lysate from the same parent cells transfected to express HA-CCR5 (Figure 4.15A). Secondly, the high molecular weight band responds differently to treatment with medium and the CCR2 agonist CCL2 (Figure 4.15).

Subcellular fractionation of HEK HA-CCR2B cells shows that the three forms of the HA-CCR2B receptor are localised differently within the cell. The subcellular fractionation profile of the high molecular weight form has two peaks. The major peak represents to fractions co-labelling for the plasma membrane markers CD49b and Na/K ATPase α-1, whereas the minor peak encompasses intracellular marker-containing fractions and corresponds best to that of the ER marker binding immunoglobulin protein (BiP; Figure 4.16).

The “normal” and low molecular weight forms are not found in plasma membrane positive fractions. The low molecular weight form corresponds best to BiP-containing fractions but also partially overlaps with fractions positive for Lamp1 and TGN46. The “normal” molecular weight form shows a similar pattern but with greater overlap with Lamp1- and TGN46-containing fractions (Figure 4.16).

Upon agonist-stimulation, the high molecular weight form, but not the other two forms, significantly changes its distribution within the fractions. The major peak that previously fractionated with plasma membrane markers is no longer easily detectable and a single peak that corresponds to intracellular markers, in particular Lamp1, is observed (Figure 4.17). This suggests that the high molecular weight plasma membrane HA-CCR2B form is internalised and degraded in response to agonist treatment.
Figure 4.16 Subcellular fraction of the HA-CCR2B forms in untreated HEK HA-CCR2B cells.
HEK HA-CCR2B cells were lysed using a ball bearing homogeniser and used for subcellular fractionation. Samples were loaded onto 0 – 22 % continuous Optiprep gradients and centrifuged at 200000 xg for 3 hours. Twelve 1 ml fractions were collected and probed for the presence of different organelle markers by SDS-PAGE and immunoblotting. Densitometry analysis of the marker and HA-CCR2B bands present in each fraction was carried out using ImageJ and presented as a percentage of the most intense band for that marker.
Figure 4.17 Subcellular fraction of the HA-CCR2B forms in CCL2-treated HEK HA-CCR2B cells

HEK HA-CCR2B cells, treated with 100 nM CCL2 for 30 minutes at 37 °C, 5 % CO2, were lysed using a ball bearing homogeniser and used for subcellular fractionation. Samples were loaded onto 0 – 22 % continuous Optiprep gradients and centrifuged at 200000 xg for 3 hours. Twenty-four 0.5 ml fractions were collected and probed for the presence of different organelle markers by SDS-PAGE and immunoblotting. Densitometry analysis of the marker and HA-CCR2B bands present in each fraction was carried out using ImageJ and presented as a percentage of the most intense band for that marker.

In summary, it is the high molecular weight form that is present at the plasma membrane of the cells and that is internalised following agonist treatment and then degraded. Therefore, this is the form that is of interest for studying intracellular trafficking following agonist-induced internalisation.

The high molecular weight form is likely to be a post-translationally modified form of HA-CCR2B. By western blotting it is observed as a large band that appears to actually consist of several bands with spacing between some that could be consistent with mono- and poly-ubiquitinated forms of the receptor (Figure 4.18A). Immunoprecipitation experiments carried out with HEK HA-CCR2B cells transfected to express FLAG-ubiquitin show no co-immunoprecipitation of HA-CCR2B with FLAG-ubiquitin (Figure
This suggests that the post-translational modification is not ubiquitination, however no positive control was available.

Figure 4.18 Testing for post-translational modifications of HA-CCR2B.
(A) Long and short exposures of the same immunoblot of HEK HA-CCR2B cell lysates probed using HA.11 to show the high molecular weight form of HA-CCR2B as a single or multiple bands. (B) HEK293 cells transiently transfected to express FLAG-ubiquitin analysed by immunofluorescence using M2 (α-FLAG) to show the expression pattern. Cells were counterstained with DAPI (blue). Scale bar = 10 µm 
(C) HEK293 cells transfected to express HA-CCR2B and/or FLAG-ubiquitin were used for immunoprecipitation by M2 (α-FLAG) and the cell lysates and immunoprecipitated protein were analysed by SDS-PAGE and immunoblotting with HA.11 to detect HA-CCR2B. The membrane was stripped and re-blotted with M2 to detect proteins that had incorporated FLAG-ubiquitin. (D) Lysates from HEK HA-CCR2B cells, untreated or pretreated with 5 µM GF109203X (PKC inhibitor) or 100 ng/ml PMA for 60 minutes at 37 °C, 5 % CO2, analysed by SDS-PAGE and immunoblotting using HA.11 to detect HA-CCR2B. Arrows indicate the high (red), “normal” (green) and low (blue) molecular weight forms of HA-CCR2B.
A common post-translational modification observed for chemokine receptors is phosphorylation of the cytoplasmic tail, usually in response to specific ligand binding. The increase in molecular weight due to the addition of phosphate groups can be detected on a western blot as a band shift as reported for CCR5 (Signoret et al., 2000). Therefore it is possible that the “normal” and modified forms of HA-CCR2B could represent non-phosphorylated and phosphorylated forms of the receptor respectively, however the results of several different experiments suggest that this is unlikely. The high molecular weight band is present on western blots using cell lysate from both untreated and agonist-treated HEK HA-CCR2B cells, and no increase in band intensity is observed following agonist treatment (Figure 4.15A). This suggests that unlike typical chemokine receptor phosphorylation, the modification resulting in the high molecular weight form of HA-CCR2B is probably not agonist-induced. Treatment of HEK HA-CCR2B cells with a PKC inhibitor to prevent PKC-mediated phosphorylation, did not appear to result in a significant reduction in intensity of the high molecular weight HA-CCR2B band. Accordingly, treatment with PMA, which activates PKC, did not lead to an increase in the intensity of the high molecular weight band (Figure 4.18D). These results suggest that the post-translational modification is unlikely to be phosphorylation.

Glycosylation can also result in an increase in protein molecular weight that can be observed on a western blot. Pretreatment of HEK HA-CCR2B cells with tunicamycin, an N-glycosylation inhibitor, resulted in a change in band pattern suggesting that the receptor is N-glycosylated (Figure 4.19A). A reduction in intensity of the “normal” band and the appearance of a slightly lower molecular weight band is observed suggesting that the “normal” form of HA-CCR2B is partially N-glycosylated. The high molecular weight band appears to be mainly replaced by intermediate bands. This result differs slightly from the discrete band shift from a fully N-glycosylated to a non-glycosylated form that would be typically expected. As there is only one consensus NXS/T potential N-glycosylation site motif on HA-CCR2B, this suggests that the high molecular weight band is due to elaboration of the N-glycan present on the “normal” form carried out in the Golgi and that the intermediate bands represent different stages in the glycosylation process. Tunicamycin blocks the transfer of N-acetylglucosamine-1-phosphate from uridine diphosphate N-acetylglucosamine to dolichol phosphate leading to a reduction in formation of the dolichol pyrophosphate N-acetylg glucosamine precursor (Esko and Bertozzi 2009). This precursor donates its entire glycan to the protein during the first
step of N-glycosylation, which is carried out in the endoplasmic reticulum and subsequent remodeling steps are carried out in the endoplasmic reticulum and in the Golgi. The required tunicamycin concentration for effective inhibition of N-glycosylation is cell type dependent (Esko and Bertozzi, 2009) and the total cellular expression level of HA-CCR2B is relatively high. It is therefore likely that the intermediate bands are present due to incomplete inhibition of precursor formation, allowing some limited partial N-glycosylation to take place resulting in a small amount of the “normal” form, which can be further remodelled in the Golgi. Higher concentrations of tunicamycin or longer treatment were not tested due to the toxic effect on the cells that was observed.

Two different approaches were used to test for O-glycosylation: treatment of cell lysate with neuraminidase, an enzyme that cleaves off sialic acids from the Gal-β(1-3)-GalNAc core, and pretreatment of cells with benzyl-α-GalNAc, an inhibitor of part of the O-glycosylation pathways. Both treatments gave a band pattern change when used individually suggesting that HA-CCR2B is O-glycosylated (Figure 4.19A). When used in combination with tunicamycin, neuraminidase treatment resulted in the complete absence of the high molecular weight band and also the intermediate bands (Figure 4.19A). Absence of the high molecular weight form was also observed using benzyl-α-GalNAc (Figure 4.19A). These results suggest that the high molecular weight form of HA-CCR2B is both N- and O-glycosylated.
Figure 4.19 Glycosylation of HA-CCR2B

(A) HEK HA-CCR2B cells were untreated or pretreated with DMSO, 10 µg/ml tunicamycin or 2 mM benzyl α-GalNAC for 24 hours at 37 °C, 5 % CO₂. The cells were then lysed and where indicated the cell lysate was incubated with 50 mU neuraminidase for 20 hours at 37 °C. Cell lysates were analysed by SDS-PAGE and immunoblotting using HA.11 to detect HA-CCR2. Arrows indicate the high (red), “normal” (green) and low (blue) molecular weight forms of HA-CCR2B. (B) HEK HA-CCR2B cells were pretreated with DMSO, 2 mM benzyl α-GalNAC, 10 µg/ml tunicamycin or tunicamycin and benzyl α-GalNAC for 24 hours at 37 °C, 5 % CO₂, and then the cell surface expression was measured by flow cytometry using α-CCR2 and compared to untreated cells. The means ± SD of 1 representative experiment with 3 replicates is shown.

The high molecular weight form of HA-CCR2B is glycosylated and was shown by subcellular fractionation to be localised at the plasma membrane. The importance of glycosylation for trafficking to the plasma membrane was tested by pretreating with glycosylation inhibitors for 24 hours before measuring the cell surface expression level of HA-CCR2B by flow cytometry. Compared to untreated cells, pretreatment with
benzyl-α-GalNAc appears to have no effect, however pretreatment with tunicamycin reduced the cell surface HA-CCR2 expression level dramatically (Figure 4.19B). This suggests that glycosylation aids trafficking of HA-CCR2B to the cell surface.

4.4 Discussion

4.4.1 Comparison of CCR2B trafficking to CCR5 trafficking and overview of pathway

In response to CCL2 ligand stimulation, CCR2B is internalised in early endosomes and then a proportion is degraded and a proportion is recycled to the cell surface (Figure 4.20). Unlike CCR5, which undergoes 50% recovery in 60 min in CHO-CCR5 cells (Signoret et al., 2000) and almost 100% recovery in 120 minutes (Mueller et al., 2002a), in HEK HA-CCR2B cells the surface CCR2 level only partially recovers after 60 min. Unlike CCR5, but similar to several other chemokine receptors, a proportion of CCR2B appears to undergo lysosomal degradation following agonist-induced internalisation.

4.4.2 CCR2B internalisation

The limited research published on CCR2 internalisation has been mainly carried out on brain tissue or astrocytes with a single study using HEK293 and THP-1 cells. These studies suggest that the receptor can be internalised in response to ligand-stimulation via clathrin or caveolae-dependent endocytosis (Figure 4.20) and that the route followed may be cell type specific. Endocytosis studies in the past have suffered from the limited specificity or off-target effects of the drugs used to inhibit these two pathways. This study attempted to use a broader panel of inhibitors to enable greater certainty about the pathways involved.

The observed colocalisation of internalised receptor with transferrin and the inhibitory effect of ikarugamycin and hypertonic sucrose on receptor down-modulation support a clathrin-mediated endocytosis pathway for HA-CCR2B in HEK293 cells, which is in agreement with complementary studies using clathrin shRNA and colocalisation with Lamp1 (Garcia Lopez et al., 2009). However, none of these assays showed a complete block in receptor down-modulation suggesting that either they were not efficient or specific enough or that there could be a second pathway that functions to some extent.
Other approaches to test the first possibility include using the new more specific PitStop inhibitors, which act on the clathrin terminal domain to immobilise CCPs (von Kleist et al., 2011) or using siRNA against clathrin or the crucial adaptor protein AP2. Acting on AP2 instead of on clathrin directly should only affect clathrin-mediated endocytosis and not other important roles played by clathrin including sorting of cargo within the early/sorting endosomes for entry into the degradative pathway (Raiborg et al., 2002).

An alternative recently developed approach, the knocked sideways technique (Robinson et al., 2010), involves rapid inactivation of AP2 via rerouting it to the mitochondria, a subcellular compartment that is not involved in the endocytic pathway. This technique exploits the ability of the drug rapamycin to cause dimerisation of rapamycin-domain containing proteins. Cells are firstly stably transfected to express two proteins: a siRNA-resistant form of the AP-2 α subunit with a FKBP tag that can bind rapamycin, and a protein containing a mitochondrial localisation domain and a FRB domain that can bind rapamycin and FKBP. The endogenous AP2 α subunit is then knocked down using siRNA and is compensated for by the overexpressed form until the addition of rapamycin, which results in dimerisation of the two proteins and thus relocation of AP2 to the mitochondria. The knocked sideways technique has the advantage of acting much more rapidly than siRNA-mediated knockdown as following the addition of rapamycin you do not need to wait for existing protein to be degraded. For transferrin the AP2 knocked sideways inhibition of uptake is as efficient as siRNA knockdown but takes place on a time scale that is three to four orders of magnitude faster. However, this is a labour-intensive approach that could not be carried out within the time frame of this study.

The inhibitory effect of methyl-β-cyclodextrin and nystatin on receptor down-modulation support the existence of an additional lipid-dependent, possibly caveolar, pathway of internalisation previously described for astrocytes using filipin and caveolin-1 siRNA. Contrary to these results, the single experiment using filipin showed no effect on HA-CCR2 down-modulation, however several possible explanations exist for this. This could just be due to a difference in stringency of the efficiency of the inhibitors used in this experiment. Alternatively, it could be the result of the differing mode of action of the inhibitors. The extraction of cholesterol by methyl-β-cyclodextrin could impact on the ligand binding to the receptor and indirectly affect its down-modulation. Further experiments would be required to reach a definitive conclusion on the contribution of
membrane lipids to agonist-induced CCR2 internalisation. A radiolabelled ligand binding study in the presence and absence of methyl-β-cyclodextrin could be used to detect the influence of the drug on ligand binding. To determine the involvement of the caveolar pathway in HA-CCR2B internalisation, caveolin-1 siRNA treatment could be used as it has been used for astrocytes (Ge and Pachter, 2004). C-terminally tagged caveolin-1-GFP and N-terminally tagged GFP-caveolin-1 constructs have been generated and exhibit wild-type and dominant-negative inhibitor behaviour in the caveolar-dependent endocytosis pathway (Pelkmans et al., 2001). Transient expression of these constructs in the transfected HEK293 cells could be used to further investigate the potential caveolin-1 dependence of HA-CCR2B endocytosis in these cells. Attempts to detect caveolin-1 by immunofluorescence on cells co-stained for HA-CCR2B and on immunoblots of subcellular fractions from HEK HA-CCR2B cells showed no signal. Other caveolin-1 antibodies are commercially available and have been used successfully on astrocytes by immunofluorescence (Andjelkovic et al., 2002) and mink lung endothelial cells by electron microscopy (Signoret et al., 2005) and could therefore be tested to check the expression level of caveolin-1 on HEK293 cells and if it colocalises with HA-CCR2B.

4.4.3 CCR2B degradation

A proportion of CCR2 appears to be degraded following agonist-induced internalisation (Figure 4.20). This study showed colocalisation of internalised HA-CCR2B with EGF and an increase in the proportion of total HA-CCR2B that colocalised with CD63 in agonist-treated as compared to untreated cells. Another study (Garcia Lopez et al., 2009) reported partial colocalisation of a Cherry-tagged CCR2B with Lamp1. Although almost no colocalisation with Lamp1 was observed in this study when using the endocytosis assay, it was probably a result of the problems with using antibodies to label CCR2B instead of a fluorescent tag. This possibility could be addressed by using the down-modulation assay to compare the level of colocalisation of total HA-CCR2B with Lamp1 in untreated and agonist-stimulated cells as done for CD63.

In support of the immunofluorescence colocalisation studies, biochemical studies showed that CCR2B is degraded in response to agonist stimulation. Preliminary studies using ammonium chloride and leupeptin indicate that internalised CCR2B undergoes
lysosomal degradation as is the case for the majority of degraded chemokine receptors studied so far (Borroni et al., 2010) with the exception of CXCR3 (Meiser et al., 2008). CXCR3 has been suggested to undergo proteasomal degradation instead. Repeat of the degradation experiments using proteasome inhibitors such as MG132 would be required to exclude this route for CCR2. Additional lysosomal inhibitors, such as chloroquine and concanamycin A, are also available to complement the leupeptin and ammonium chloride treatment.

4.4.4 CCR2B recycling

Partial colocalisation with the transferrin receptor and the results of the recycling assays suggest that a proportion of the internalised HA-CCR2B is recycled in HEK HA-CCR2B cells. As these cells have an accumulation of HA-CCR2B in their biosynthetic pathway, the possibility of replenishment from internal stores cannot be ruled out. However, recycling experiments carried out on THP-1 cells suggest that this is not the case as recycling appears to occur in THP-1 cells, which express endogenous levels of CCR2. Indeed, early binding studies using $^{125}$I-CCL2 on THP-1 cells showed rapid replenishment of ligand binding sites within 20 minutes that was not dependent on de novo synthesis of the receptor suggesting recycling (Wang et al., 1993a). However, in that study the level of recycling was not quantified and so cannot be compared to that observed in HEK HA-CCR2B cells.

Despite partial colocalisation of internalised HA-CCR2B with Rab4, over-expression of Rab4s had no effect on recycling of the receptor. This suggests that like for CCR5 (personal communication from Nathalie Signoret, University of York, UK), HA-CCR2B recycling is not Rab4-dependent. In addition to its role in recycling endosomes, Rab4 is also present at the early/sorting endosome (Sonnichsen et al., 2000). Therefore this colocalisation could represent an earlier point in the endocytic pathway where HA-CCR2B transits through the sorting endosome on its way to the degradative pathway or back to the cell surface (Figure 4.20). Recycling could potentially take place from the sorting endosome in Rab4-independent vesicles.

The level of recycling observed in HEK HA-CCR2B cells is quite low suggesting that this is a minor pathway following internalisation. There is also quite a lot of variability
in the level of recycling observed suggesting that an unknown factor or factors may influence the recycling potential.

### 4.4.5 Choice of recycling or degradation

Internalised CCR2B can undergo one of two fates; either recycling back to the cell surface where it can potentially be restimulated by new ligand or alternatively being sent to the lysosome for degradation halting its ability to signal (Figure 4.20). Several other chemokine receptors, such as CXCR2 and CXCR4, have been shown to enter either pathway depending on cell type and duration of ligand treatment respectively. In common with at least eleven other chemokine receptors, CXCR2 contains a PDZ ligand motif at its extreme C-terminus (Marchese et al., 2008). For CXCR2 it appears that this PDZ ligand motif is involved in determining the fate of the receptor as it serves to delay degradation by preventing lysosomal sorting, probably due to an interaction with an as yet unknown PDZ-containing protein. CCR2 however, does not have a C-terminal PDZ ligand motif and it appears to be able to undergo, at least to some extent, both fates in the same cell type suggesting that there may be more currently undetermined factors influencing the post-internalisation trafficking and that they may be receptor specific. In fact it has been observed for CCR5 that the choice of agonist itself can impact on the fate of the receptor by causing the receptor to accumulate at various points along the recycling pathway (Bennett et al., 2011). The experiments presented here have been carried out using the main CCR2 ligand, CCL2, however the receptor does have several other ligands and it would be interesting to observe the influence of these on the trafficking of the receptor.

### 4.4.6 Different forms of CCR2B

Three different forms of HA-CCR2B have been described in this study: high, “normal” and low molecular weight. Based on subcellular fractionation studies, the high molecular weight form localises both to the plasma membrane and to an intracellular pool whereas the other two forms are localised intracellularly. This study supports previous work, which proposed that CCR2 is N-glycosylated (Preobrazhensky et al., 2000) and suggests that CCR2 also undergoes O-glycosylation. The involvement of glycosylation in GPCR trafficking to the cell surface is thought to vary between receptors (Dong et al., 2007). For some GPCRs, such as the AT1a angiotensin receptor,
glycosylation appears to be essential for cell surface expression (Jayadev et al., 1999) yet for other receptors, such as the M2-muscarinic receptor, glycosylation appears to play no role (van Koppen and Nathanson, 1990). There is another group of GPCRs for which glycosylation may play an important but non-essential role in the receptor trafficking to the cell surface. An example of this group of GPCRs is the β-adrenergic receptor, where mutation of the two N-terminal N-glycosylation sites reduced the percentage of the receptor present at the cell surface by approximately 50% (Rands et al., 1990). Both my study and previously published work (Preobrazhensky et al., 2000) showed partial but incomplete reduction in cell surface CCR2 expression following tunicamycin treatment suggesting that CCR2 may belong to this group. However mutation studies would be required to confirm that it is glycosylation of CCR2 and not any other trafficking machinery proteins involved in its export that is responsible. HA-CCR2B appears to also be O-glycosylated but this modification does not seem to play a role in its cell surface expression.

Observing the fractions from most to least dense, the low molecular weight form of HA-CCR2B appears first followed by the “normal” form and then the intracellular peak of the high molecular form. All three forms overlap to some degree with the ER (BiP), Golgi (TGN46) and lysosome (Lamp1) markers, which appear in that order from most to least dense. However, the separation between the markers is not very good and there is quite a lot of overlap. To determine where precisely the intracellular high, “normal” and low molecular weight forms are localised a modified fractionation protocol and narrower gradient would be required.

These results could suggest that the “normal” molecular weight form of HA-CCR2B is a non-mature form that is produced and N-glycosylated in the ER and then traffics to the Golgi for N-glycan remodelling and O-glycosylation before trafficking to the plasma membrane. The identity of the low molecular weight form is unclear although it is likely to be either a truncation or degradation product. It is usually present but the level varies from experiment to experiment. It shows more colocalisation with BiP than with Lamp1 and does not accumulate following agonist-induced degradation of the high molecular weight form, which suggests that it is more likely to be a truncation product.
**Figure 4.20 Trafficking of internalised CCR2B.**

In response to agonist stimulation, CCR2 can be internalised via clathrin and caveolin-dependent pathways. It can then either be recycled back to the cell surface or be sent to the lysosome for degradation. Arrows show the proposed paths followed by internalised CCR2.
5 Identification of interacting partners

5.1 Introduction

The previous chapters show that internalised CCR2B and CCR5 localise differently within the cell and that whereas CCR5 is recycled to the cell surface, CCR2B appears to be both recycled and sent for degradation. This difference could be due to interactions with different elements of the trafficking machinery.

Multiple examples of interactions with specific proteins being important for the intracellular trafficking of agonist stimulated chemokine receptors have been demonstrated. Interaction of the chemokine receptor with kinases is required for phosphorylation of the cytoplasmic tail, which is involved in the initial step of clathrin-mediated endocytosis (Neel et al., 2005). Subsequently, sorting of internalized chemokine receptors to the recycling or degradative pathways requires complex interactions with the machinery mediating movement of molecules between intracellular compartments. For CCR5, post-endocytic sorting to the recycling pathway is dependent on interaction of its PDZ ligand motif with a protein implicated in receptor recycling called EBP50/NHERF-1 (Hammad et al., 2010). The post-translational modification ubiquitination has emerged as being important for sending CXCR4, but not other chemokine receptors, to degradation presumably due to modulation of protein-protein interactions.

In addition, specific protein-protein interactions involving the cytoplasmic domains of chemokine receptors can influence the signalling activity of these receptors. Indeed, it has emerged that GPCRs can also elicit G protein-independent signals through interaction with the scaffolding proteins β-arrestins, linking activated receptors to various signalling pathways that act independently of, in synergy with or in opposition to, G protein-mediated signals.

Therefore, identification of novel common and/or distinct interacting partners of CCR2B and CCR5 could help to further our understanding of receptor specific trafficking and signalling mechanisms.
5.1.1 Choice of techniques

Other groups have used various techniques to try to identify proteins interacting with the two chemokine receptors. Several proteins have been found (Table 5.1) but it is likely that many remain to be identified. Indeed recent work on another chemokine receptor, CXCR2, has shown that there are still novel interacting proteins to be found using proteomics approaches (Neel et al., 2009).

This project employed two different but complementary techniques with the aims of identifying novel interacting proteins for CCR2B and CCR5 under resting and chemokine or LTA stimulated conditions. The GST pull down technique, using the cytoplasmic tails of the receptors fused to GST, was chosen to target proteins whose binding sites are within the tail, whereas, receptor co-immunoprecipitation, targets proteins that bind to any region of the receptor. This is important as it has been shown that although many proteins interact with the cytoplasmic tail of GPCRs (Heydorn et al., 2004), some proteins require other parts of the receptors, such as the intracellular loops, for binding (Luttrell, 2006).

The cytoplasmic tail of CCR5 has been shown to interact with α-catenin (Schweneker et al., 2004), β-arrestin (Huttenrauch et al., 2002), DRiP78 (Kuang et al., 2012), EBP50/NHERF-1 (Hammad et al., 2010), FROUNT (Toda et al., 2009) and Jena-Muenchen 4 (Schweneker et al., 2005), whereas the cytoplasmic tail of CCR2B has been shown to bind to filamin A (Minsaas et al., 2010) and FROUNT (Terashima et al., 2005). Both receptors can be phosphorylated on their cytoplasmic tails by GRKs or second messenger-activated kinases. Four phosphorylation sites (S336, S337, S342, S349) have been identified for CCR5 (Oppermann et al., 1999; Pollok-Kopp et al., 2003) and the CCR2B cytoplasmic contains four serines and six threonines that can be phosphorylated (Franci et al., 1996). In addition, the last four amino acids (SVGL) of the CCR5 cytoplasmic tail form a PDZ ligand (Delhaye et al., 2007) although no equivalent sequence has been identified for CCR2B. As chemokine receptors are 7 trans-membrane receptors, it is not feasible to express the entire CCR2B or CCR5 protein fused to GST in bacteria. However, the importance of the cytoplasmic tail for protein interactions and the success of yeast-2-hybrid screens using the cytoplasmic tails suggest that this part of the receptors is suitable to address the aims of this project.
Table 5.1 Cellular proteins identified as interacting with CCR2B or CCR5 in previously published work.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Protein Identified</th>
<th>Identification Technique</th>
<th>Cells</th>
<th>Validation Technique</th>
<th>Cells</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR2</td>
<td>β-arrestin</td>
<td>CO-IP</td>
<td>MonoMac1 (E)</td>
<td></td>
<td></td>
<td>(Aragay et al., 1998)</td>
</tr>
<tr>
<td>CCR2B</td>
<td>Filamin A</td>
<td>Y2H</td>
<td>Human leukocyte cDNA library</td>
<td>Pull down</td>
<td>A7, M2</td>
<td>(Minsaas et al., 2010)</td>
</tr>
<tr>
<td>CCR2B</td>
<td>FROUNT</td>
<td>Y2H</td>
<td>THP-1 cell cDNA library</td>
<td>Pull down, CO-IP</td>
<td>HEK293 (O)</td>
<td>(Terashima et al., 2005)</td>
</tr>
<tr>
<td>CCR2</td>
<td>GRK2</td>
<td>CO-IP</td>
<td>MonoMac1 (E)</td>
<td></td>
<td></td>
<td>(Aragay et al., 1998)</td>
</tr>
<tr>
<td>CCR2B</td>
<td>Importin 7</td>
<td>SEC-IP then MS/MS</td>
<td>HEK293 (O)</td>
<td></td>
<td></td>
<td>(Favre et al., 2008)</td>
</tr>
<tr>
<td>CCR2B</td>
<td>Importin 9</td>
<td>SEC-IP then MS/MS</td>
<td>HEK293 (O)</td>
<td></td>
<td></td>
<td>(Favre et al., 2008)</td>
</tr>
<tr>
<td>CCR2B</td>
<td>Importin β1 subunit</td>
<td>TCP-1γ</td>
<td>HEK293 (O)</td>
<td>SEC-IP then WB GST pull down, SEC-IP then WB CO-IP</td>
<td>HEK293 (O)</td>
<td>(Favre et al., 2008)</td>
</tr>
<tr>
<td>CCR2B</td>
<td>TRN-1</td>
<td>SEC-IP then MS/MS</td>
<td>HEK293 (O)</td>
<td>SEC-IP then WB GST pull down, SEC-IP then WB CO-IP</td>
<td>HEK293 (O)</td>
<td>(Favre et al., 2008)</td>
</tr>
<tr>
<td>CCR5</td>
<td>α-catenin</td>
<td>Y2H</td>
<td>Human B cell cDNA library</td>
<td>CO-IP</td>
<td></td>
<td>(Schweneker et al., 2004)</td>
</tr>
<tr>
<td>CCR5</td>
<td>β-arrestin</td>
<td>CO-IP</td>
<td>HEK-293 (O)</td>
<td></td>
<td></td>
<td>(Vila-Coro et al., 1999a)</td>
</tr>
<tr>
<td>CCR5</td>
<td>DRiP78</td>
<td>BiFc-BRET</td>
<td>HEK-293 (O)</td>
<td>CO-IP GST pull down</td>
<td>HEK293 (O)</td>
<td>(Kuang et al., 2012)</td>
</tr>
<tr>
<td>CCR5</td>
<td>EBP50/NH ERF-1</td>
<td>BiFc-BRET</td>
<td>HEK-293 (O)</td>
<td>CO-IP GST pull down</td>
<td>HEK293 (O)</td>
<td>(Hammad et al., 2010)</td>
</tr>
<tr>
<td>CCR5</td>
<td>FROUNT</td>
<td>Y2H</td>
<td>cDNA encoding FROUNT aa 500-656</td>
<td>CO-IP</td>
<td>HOS (O)</td>
<td>(Toda et al., 2009)</td>
</tr>
<tr>
<td>CCR5</td>
<td>GRK2</td>
<td>CO-IP</td>
<td>HEK293 (O)</td>
<td></td>
<td></td>
<td>(Opperman n et al., 1999)</td>
</tr>
<tr>
<td>CCR5</td>
<td>JM4</td>
<td>Y2H</td>
<td>Human pre-B cell cDNA library</td>
<td>CO-IP</td>
<td>C2Th (O), HEK293 (O)</td>
<td>(Vila-Coro et al., 1999a)</td>
</tr>
<tr>
<td>CCR5</td>
<td>NMMHC-IIA</td>
<td>GST pull down</td>
<td>Peer T cells</td>
<td>CO-IP</td>
<td></td>
<td>(Schweneker et al., 2005)</td>
</tr>
</tbody>
</table>

cDNA, complementary deoxyribonucleic acid; CO-IP, co-immunoprecipitation; E, cells endogenously expressed the chemokine receptor; HOS, human osteosarcoma cell line; JM4, Jena-Muenchen4; N/A, not applicable; NMMHC-IIA, nonmuscle myosin H chain-IIA; O, cells were transfected to over-express the chemokine receptor; SEC-IP, size exclusion chromatography followed by immunoprecipitation; TCP-1γ , T-complex protein 1 γ subunit; TRN-1, transportin-1; WB, western blotting; Y2H, yeast-2-hybrid screen
Previous approaches have concentrated on finding novel binding partners of CCR2 and CCR5 in their un-stimulated state, either by yeast-two-hybrid assays or by co-immunoprecipitation, followed in some cases by investigation into whether there is any change in the binding of these proteins following chemokine agonist-stimulation (Favre et al., 2008; Schweneker et al., 2004, 2005; Terashima et al., 2005). However, there is no published work carried out to specifically identify novel proteins that interact with the receptors following ligand-stimulation in cells endogenously expressing both receptors, such as monocytes. Therefore, this project initially aimed to use monocytic cell lines to identify interacting partners for the receptors. However, due to problems identified with the CCR5 expression on monocytic cell lines described in Chapter 3, transfected HEK293 cells were also used.

5.1.1.1 GST pull down

The GST pull down technique is commonly used to confirm suspected protein-protein interactions. A recombinant protein consisting of GST fused to a part or the whole of one protein of interest is produced in bacteria and then incubated with either a purified form of the other protein of interest or extracts containing this protein. The GST tag provides a simple way for “pulling out” the tagged protein and its interacting partners using glutathione coated beads. The technique can however also be used to fish for novel interacting proteins when using total lysate from the cells or tissue of interest. The present study aimed to produce and purify fusion proteins consisting of GST fused to the cytoplasmic tail of either CCR2B or CCR5 and then to use these proteins in a pull down assay with lysates from a monocytic cell line to identify novel endogenous interacting partners for the two receptors. Note that these experiments were planned before the lack of functional CCR5 on these cell lines (reported in Chapter 3) was fully investigated.

The initial stages of this project, including the production of a construct coding for the cytoplasmic tail of CCR2B fused to GST and two initial pull down experiments, were carried out during my undergraduate final year research project in the Signoret Laboratory at the Department of Biology, University of York. These experiments led to the detection of five potential CCR2B-interacting proteins as bands on an SDS-PAGE gel showing the potential of using this GST pull down technique.
5.1.1.2 Immunoprecipitation

Co-immunoprecipitation has also been widely used to confirm suspected protein-protein interactions. However, there have been some recent examples of co-immunoprecipitation being used to identify novel interacting partners of chemokine receptors, including CXCR2 (Neel et al., 2009) and CCR2 (Favre et al., 2008), demonstrating the potential for this technique.

Although the chemokine receptor cytoplasmic tail has been found to be essential for interaction with several proteins, some other receptor domains have also been shown to be involved in protein binding. Both CCR2B and CCR5 contain a DRY motif in their second intracellular loop, which is involved in the interaction of the receptor with its G protein (Lagane et al., 2005; Mellado et al., 1998). Additionally, β-arrestin can bind to both the DRY motif and the phosphorylated cytoplasmic tail of the receptor (Huttenrauch et al., 2002; Marion et al., 2006). This highlights the importance of using co-immunoprecipitation, in addition to the GST pull down, to identify proteins that fall into this category.

Co-immunoprecipitation offers some technical advantages over the GST pull down. Firstly, the GST pull down experiment is carried out on cell lysate and so false interactions or fewer interactions may be experienced due to the loss of spatial organization and addition of detergent during lysis. This risk is reduced when using the co-immunoprecipitation technique as the protein-protein interactions happen in the cell itself, prior to lysis, where the proteins should be in their correct spatial environment. Secondly, interactions of some proteins, such as kinases, with chemokine receptors may be transient and/or weak and so could be lost during the washing steps of the GST pull down experiment, but the co-immunoprecipitation technique offers the potential to use various crosslinking methods to fix protein-protein interactions before lysis.

Co-immunoprecipitation experiments can be carried out using either two purified proteins, a purified protein and a cell lysate containing the second protein of interest or alternatively a cell lysate containing both proteins of interest. The proteins used can be either endogenously expressed or over-expressed tagged or untagged versions of the proteins of interest, and the choice is often dependent on the availability of antibodies.
targeting the protein. The use of lysates from cells endogenously expressing the proteins of interest provides greater confidence in the physiological relevance of the interactions.

Therefore, as an antibody with a previously demonstrated ability to immunoprecipitate CCR5 (MC5; Signoret et al., 2004) and a panel of antibodies against CCR2 were available, the initial focus of this study was to use monocytic cell lines. However, recent observations about the lack of functionality of CCR5 expressed on these cells, as discussed in Chapter 3, lead to a change in the cell type used.

HEK293 cell lines expressing TLR2 were transfected to express HA-tagged forms of CCR2B and CCR5 with the aim of enabling co-immunoprecipitation assays to be carried out on resting, chemokine agonist and/or LTA stimulated cells. Unfortunately, as discussed in chapter 3, the LTA cross-desensitisation of CCR2 and CCR5 discovered in monocytes was not sufficiently replicated in these cells. Nevertheless, the transfected HEK293 cells did provide several potential advantages over the monocytic cell lines in regards to agonist-induced down-modulation and so therefore were used for the immunoprecipitation set up experiments. Firstly, as both of the receptors had a HA-tag the same antibody could be used for co-immunoprecipitation experiments for each receptor. This would enable a fairer comparison of the proteins identified as interacting with each receptor by reducing influencing factors dependent on the antibody used. The system has extra advantages for CCR2, as using antibodies against the N-terminus of endogenous CCR2 in monocytic cells lines targets both isoforms of CCR2 present (CCR2A and CCR2B), whereas using the transfected cell lines would enable the study of CCR2B only. Additionally, as discussed in Chapter 3, the antibodies that I had available to target CCR2 were not very good, whereas the anti-HA antibody 12CA5 has been previously used for successful immunoprecipitation (Schweneker et al., 2005). This antibody could be produced in-house thus we could produce sufficient quantities for the scale of immunoprecipitation required.

5.1.2 Objectives

The initial objective of this chapter was to develop the GST pull down and co-immunoprecipitation techniques to be used in combination with MS/MS for the identification of novel protein interactions for the two receptors. The second objective was to then use these techniques to compare the protein interactions between the two
receptors and under different conditions including resting, chemokine agonist or LTA stimulation.

5.2 Relevant methodology

5.2.1 Molecular biology techniques

The CCR2B and CCR5 cDNA, with a N-terminal triple HA tag, cloned into the pcDNA3.1+ vector were purchased from the UMR cDNA Resource Centre. Constructs summarized in Table 5.2 were created encoding the cytoplasmic tails of the two receptors (residues 309 – 360 for CCR2B and residues 302 – 352 for CCR5) by standard molecular cloning techniques.

Table 5.2 Constructs created or used in this project.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Encoded protein</th>
<th>Cleavage site after GST</th>
<th>How it was generated</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEX-4T-1</td>
<td>GST</td>
<td>Thrombin</td>
<td>Purchased from GE Healthcare.</td>
</tr>
<tr>
<td>pGEX-4T-1-CCR2Bct</td>
<td>GST-CCR2Bct</td>
<td>Thrombin</td>
<td>Created during undergraduate project.</td>
</tr>
<tr>
<td>pGEX-4T-1-CCR2Bct-HIS₆</td>
<td>GST-CCR2Bct-HIS₆</td>
<td>Thrombin</td>
<td>cDNA encoding CCR2Bct/CCR5ct amplified by PCR from pGEX-4T-1-CCR2Bct/CCR5ct with addition of a HIS₆ tag, stop codon and EcoRI (CCR2) or XhoI (CCR5) site to the 3’ end. Cloned into pGEX-4T-1 following BamHI and EcoRI/XhoI.</td>
</tr>
<tr>
<td>pGEX-4T-1-CCR5ct-HIS₆</td>
<td>GST-CCR5ct-HIS₆</td>
<td>Thrombin</td>
<td></td>
</tr>
<tr>
<td>pGEX-6P-1</td>
<td>GST</td>
<td>Prescission Protease</td>
<td>A gift from Gareth Evans (Department of Biology, University of York, UK). Originally purchased from GE Healthcare.</td>
</tr>
<tr>
<td>pGEX-6P-1-CCR2Bct</td>
<td>GST-CCR2Bct</td>
<td>Prescission Protease</td>
<td>cDNA encoding CCR2Bct/CCR5ct was excised from pGEX-4T-1-CCR2Bct/CCR5ct using BamHI and XhoI/EcoRI and cloned into pGEX-6P-1 vector digested with the same enzymes.</td>
</tr>
<tr>
<td>pGEX-6P-1-CCR5ct</td>
<td>GST-CCR5ct</td>
<td>Prescission Protease</td>
<td></td>
</tr>
<tr>
<td>pGEX-6P-1-CCR2Bct-HIS₆</td>
<td>GST-CCR2Bct-HIS₆</td>
<td>Prescission Protease</td>
<td>cDNA encoding CCR2Bct/CCR5ct-HIS₆ was excised from pGEX-4T-1-CCR2B/CCR5ct-HIS₆ using BamHI and EcoRI/XhoI and cloned into pGEX-6P-1 vector digested with the same enzymes.</td>
</tr>
<tr>
<td>pGEX-6P-1-CCR5ct-HIS₆</td>
<td>GST-CCR5ct-HIS₆</td>
<td>Prescission Protease</td>
<td>Two complementary oligos encoding a glycine-rich linker (PGISGGGGG) were annealed and ligated into the BamHI site in pGEX-6P-1-CCR2Bct/CCR5ct-HIS₆.</td>
</tr>
<tr>
<td>pGEX-6P-1-gly-CCR5ct-HIS₆</td>
<td>GST-gly-CCR5ct-HIS₆</td>
<td>Prescission Protease</td>
<td></td>
</tr>
</tbody>
</table>

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5.2.1.1 Oligos

Custom oligos were purchased from Sigma-Aldrich. Oligos used for sequencing were provided by the relevant sequencing service.

Table 5.3 Oligos used in this project.

<table>
<thead>
<tr>
<th>Oligo Name</th>
<th>Oligo Sequence (5’ to 3’)</th>
<th>Application</th>
<th>Extra Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEX 5’ cloning forward primer</td>
<td>GCC TTT GCA GGG CTG GCA AGC CAC GTT TGG T</td>
<td>Amplification of the cDNA encoding CCR2Bct from pGEX-4T-1-CCR2Bct with addition of a HIS₆ tag, stop codon and EcoRI site to the 3’ end.</td>
<td>Binds pGEX at 861, upstream of the BamHI site used for cloning</td>
</tr>
<tr>
<td>CCR2Bct HIS₆ reverse primer</td>
<td>GCG AAT TCC TCA CAC TAC TAC CAC CAC TAC TAA ACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEX 5’ cloning forward primer</td>
<td>GCC TTT GCA GGG CTG GCA AGC CAC GTT TGG T</td>
<td>Amplification of the cDNA encoding CCR5ct from pGEX-2T-CCR5ct with addition of a HIS₆ tag, stop codon and XhoI site to the 3’ end.</td>
<td>Binds pGEX at 861, upstream of the BamHI site used for cloning</td>
</tr>
<tr>
<td>CCR5ct HIS₆ reverse primer</td>
<td>AGA CTC GAG TTA CAC TAC TAC CAC CAC TAC CAA GCC CAC AGA TAT TTC CTG C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BamHI glycine linker 1</td>
<td>GA TCC CCA GGT ATT TCC</td>
<td>Addition of DNA encoding a glycine-rich linker between GST and CCR2B/5ct</td>
<td>Contains a BsaWI site</td>
</tr>
<tr>
<td>BamHI glycine linker 2</td>
<td>GA TCC TCC ACC ACC ACC ACC GGA AAT ACC TGG G</td>
<td></td>
<td>Contains a BsaWI site</td>
</tr>
<tr>
<td>pGEX 5’/forward primer</td>
<td>GGG CTG GCA AGC CAC GTT TGG TG</td>
<td>Sequencing pGEX constructs.</td>
<td>Binds 869-891 of pGEX</td>
</tr>
<tr>
<td>pGEX 3’/reverse primer</td>
<td>CCG GGA GCT GCA TGT GTC AGA GG</td>
<td></td>
<td>Binds downstream of the multiple cloning site in pGEX</td>
</tr>
</tbody>
</table>

5.2.1.2 PCR

The pGEX-4T-1-CCR2Bct construct was created during my undergraduate project.

The PCR reagents used for amplification of the cDNA encoding CCR2Bct or CCR5ct and addition of a HIS₆ tag are described in Table 5.4. Table 5.5 shows the PCR programme used.
5.2.1.3 Annealing oligos

As the DNA encoding the glycine linker was short, two complementary oligos encoding the glycine linker with a BamHI site at either end were annealed together instead of using PCR. 0.5 pmol/µl of each oligo were incubated together in DNA ligase buffer in a thermocycler at 95 °C for 5 minutes. The temperature was then reduced by 1 °C every minute prior to incubation at the calculated nearest neighbour melting temperature (56 °C) for 30 minutes and subsequent temperature reduction of 1 °C per minute until 4 °C was reached.

5.2.1.4 Restriction enzyme digests

Restriction enzyme digests of PCR products and recipient vectors were carried out using BamHI, EcoRI and XhoI (all from New England BioLabs [NEB], Hitchin, UK) prior to ligation steps to create the constructs described in Table 5.2. Typically the maximum possible volume of DNA was digested using 1 µl of each restriction enzyme using the appropriate buffer, and BSA if required, in a total volume of 20 µl by incubation at 37 °C for 2 hours.

### Table 5.4 PCR reagents.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration in reaction mixture (total volume of 20 µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA</td>
<td>10 ng</td>
</tr>
<tr>
<td>Forward primer</td>
<td>1 µM</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>1 µM</td>
</tr>
<tr>
<td>dNTPs (Invitrogen)</td>
<td>200 µM of each dNTP</td>
</tr>
<tr>
<td>Buffer (Minerva, Berlin, Germany)</td>
<td>1 X</td>
</tr>
<tr>
<td>Taq polymerase (Minerva)</td>
<td>2 Units</td>
</tr>
<tr>
<td>Magnesium chloride (Minerva Biolabs)</td>
<td>1.5 mM</td>
</tr>
</tbody>
</table>

### Table 5.5 PCR programme used in this project.

<table>
<thead>
<tr>
<th>PCR steps</th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Denature</td>
<td>94 °C</td>
<td>3 minutes</td>
</tr>
<tr>
<td>b. Anneal</td>
<td>47 °C</td>
<td>1 minute</td>
</tr>
<tr>
<td>c. Repeat the following cycles 36 times</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extend</td>
<td>72 °C</td>
<td>1 minute (2 minutes for first cycle only)</td>
</tr>
<tr>
<td>Denature</td>
<td>94 °C</td>
<td>1 minute</td>
</tr>
<tr>
<td>Anneal</td>
<td>64 °C</td>
<td>1 minute</td>
</tr>
<tr>
<td>d. Extend</td>
<td>72 °C</td>
<td>8 minutes</td>
</tr>
<tr>
<td>e. Store</td>
<td>4 °C</td>
<td>Overnight</td>
</tr>
</tbody>
</table>
Restriction enzyme digests of constructs were carried out using BamHI, EcoRI, XhoI, BsaWI (NEB), Aat II (NEB), SwaI (NEB) and AfeI (SibEnzyme, Novosibirsk, Russia) to check the presence and orientation of inserts. Enough mini-prep or midi-prep DNA to visualise all of the digest fragments by gel electrophoresis, at least 200 ng DNA in most cases, was digested as described above using different temperature conditions or sequential incubations where advised by the manufacturer for specific enzymes.

5.2.1.5 Ligation

Prior to ligation, restriction enzyme-digested DNA fragments were run on an agarose gel, the correct bands were excised and the DNA was purified from the gel using the QIAquick gel extraction kit (Qiagen, Crawley, UK). Ligations were carried out using insert: vector ratios of 2:1 and 1:1 in a total volume of 10 µl using 3 units of T4 DNA ligase (Promega, Southampton, UK) for 1 hour at room temperature.

5.2.1.6 Transformation

Chemically competent DH5α, BL21, BL21 pLysS and BL21 Rosetta cells were prepared and transformations were carried out as follows. To prepare competent cells, a bacterial culture grown in 2xTY, with an OD$_{600}$ of $0.4 – 0.8$, was pelleted and the cells resuspended in 100 mM CaCl$_2$, incubated on ice for 60 minutes before spinning down again and resuspending in 100 mM CaCl$_2$ containing 20 % glycerol and freezing at –80 °C. For transformation, an aliquot of bacterial cells was mixed with 20 ng DNA, incubated at 4 °C for 30 minutes before being subjected to a 70 second 42 °C heat shock, then chilled and incubated in SOC medium at 37 °C for 60 minutes, plated on to LB agar with the appropriate antibiotic and grown overnight at 37 °C. BL21 and variants were selected using 25 µg/ml chloramphenicol. Ampicillin was used at 50 µg/ml to select for bacteria containing the plasmid of interest.

5.2.1.7 Purification of plasmid DNA

Plasmid DNA was purified from transformed DH5α using either minipreparations following the alkaline lysis protocol from Molecular Cloning (Sambrook and Russell, 2001) or mini, midi and maxi preparations (using the Plasmid kits from Qiagen).
5.2.1.8 DNA quantification

DNA concentration of solutions was assessed by measuring the absorbance at 260 nm using the a spectrophotometer (6505 UV/Vis spectrophotometer, Jenway, Stone, UK) or the NanoDrop 1000 (NanoDrop Products, Thermo Scientific, Wilmington, DE, USA). In some cases, concentration was estimated from the band intensity of linearized plasmid (following restriction enzyme digest as described in Section 5.2.1.4) in comparison to bands of known mass in the 1 kb and 100 bp DNA ladders (NEB).

5.2.1.9 Agarose gel electrophoresis

1 - 1.5 % agarose gels containing SYBR Safe™ (Invitrogen) or 0.5 µg/ml ethidium bromide in TAE buffer were used for most analytical gels. NuSieve GTG low-melting point agarose (Cambrex Bio Science Wokingham, Ltd, Wokingham, UK) was used for preparative gels. Gels were migrated at 50 V for 40 minutes and then visualised using UV light.

5.2.2 DNA sequencing

DNA sequencing of plasmid constructs was carried out either by the University of York Technology Facility or Geneservice (Source BioScience, Nottingham, UK) using pGEX 5'/forward and/or pGEX 3'/reverse primers (Table 5.3), which binds either side of the insert. Sequences were aligned using Align (http://xylian.igh.cnrs.fr/bin/align-guess.cgi).

5.2.3 GST fusion protein production and purification

Constructs were transformed into BL21 DE3 pLysS, plated on LB Agar containing 50 µg/ml ampicillin and 25 µg/ml chloramphenicol and incubated at 37 °C overnight. BL21 DE3 (not chloramphenicol resistant) and BL21 Rosetta were also tested. One colony was used to inoculate LB containing 50 µg/ml ampicillin and 25 µg/ml chloramphenicol (LB amp+ cam+) and incubated at 37 °C overnight with agitation. This overnight preculture was then diluted 100 fold in LB amp+ cam+ and grown until the specified OD₆₀₀ (typically OD₆₀₀=0.9). Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM and the culture was incubated at 37 °C for the specified induction time (optimised to 15 minutes). The bacteria were harvested by centrifugation at 3500 rpm for 30 minutes at 4 °C. The bacterial pellet was resuspended in 45 ml (per
lire of culture) PBS containing a complete protease inhibitor cocktail and lysed using either a french press at 25 kpi or sonication (4 - 6 cycles of 30 seconds at 20 % amplitude with 45 seconds resting between each) as indicated, followed by the addition of 1 % Triton X-100 and incubation on ice for 30 minutes. Alternatively, where indicated the pellet was resuspended in 1/5th culture volume of BugBuster™ Protein Extraction Reagent (Merck) containing a complete protease inhibitor cocktail and 25 U/ml DNase, and incubated for 30 min at RT. Following centrifugation at 10000 x g for 15 minutes, the supernatant was used for purification of the fusion proteins. Fusion protein purification was trialled using both glutathione sepharose 4B beads (GE Healthcare) and cobalt resin (Pierce, Perbio Science UK Ltd). For purification using glutathione sepharose 4B beads, the supernatant was incubated with a 500 µl bed volume (per litre of culture) of beads at 4°C under rotation for between 1 and 16 hours as specified. The beads were washed 4 – 8 times with PBS 0.1 % Triton X-100 containing a complete protease inhibitor cocktail. The bead-bound fusion protein was either used directly for the pull down or eluted with glutathione elution buffer and dialysed prior to the pull down.

Where indicated, thrombin cleavage was carried out by incubation with 1 U thrombin per 100 µg fusion protein at 37 °C for 2 hours. Alternatively, Prescission Protease cleavage was carried out by incubation with one bed volume of 320 U/ml Prescission Protease at 4 °C overnight with end-over-end rotation and the eluate was kept for analysis.

For purification using cobalt resin, the bacterial cell lysate supernatant was produced as mentioned above but using an EDTA-free protease inhibitor cocktail. The sodium chloride concentration was adjusted to 300 mM, 10 mM imidazole was added and the pH was adjusted to pH 7.4. The fusion protein from a 0.5 l culture was bound to a 0.5 or 1 ml bed volume of cobalt resin either by applying the filtered supernatant to a column twice or by batch purification for 30 min at 4 °C with end-over-end rotation. The resin was washed with wash buffer (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, EDTA-free protease inhibitor cocktail; pH 7.4) and the fusion protein was eluted three times with a 2X bed volume of elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazole, EDTA-free protease inhibitor cocktail; pH 7.4) and analysed by SDS-PAGE.
5.2.4 GST pull down technique

1.8 x 10^8 THP-1 cells per pull down were washed in PBS and then lysed at 2 x 10^7 cells/ml in 1 % Triton lysis buffer containing a protease inhibitor cocktail at 4 °C for 30 minute with end-over-end rotation followed by mild sonication. After centrifugation, at 2970 x g. for 5 minutes, the supernatant was kept and pre-cleared by two 30 minute incubations with 50 μl glutathione sepharose beads/ 10^8 cells at 4 °C with end-over-end rotation. The indicated amount of fusion protein bound beads, or already eluted fusion protein and glutathione sepharose beads was incubated with the pre-cleared lysate at 4 °C with end-over-end rotation for the indicated time. The beads were then washed twice with 1% Triton lysis buffer. Proteins were eluted using reducing sample buffer. The same amount of GST-bound beads normalised to the same bead bed volume was used as a control. The same amount of fusion protein-bound beads was analysed to allow elimination of contaminant proteins. Samples were analysed by SDS-PAGE followed by mass spectrometry (MS) of individual bands where relevant.

5.2.5 Protein identification by mass spectrometry

Unique bands were excised from gels and destained, then subjected to in gel tryptic digest and analysed by MALDI-MS and MS/MS in the University of York Technology Facility (http://www.york.ac.uk/biology/technology-facility/proteomics/services/protein-id/). A Mascot (Perkins et al., 1999) search of the NCBI_nr database was then carried out to identify proteins.

5.2.6 Immunoprecipitation

Initial immunoprecipitation assays were carried out using protein A/G-coated agarose beads (Pierce, Perbio Science UK Ltd). 1 x 10^7 MonoMac1 cells were lysed in RIPA buffer containing a complete protease inhibitor cocktail on ice with regular vortexing and mechanical disruption by pipetting. Lysis was confirmed by visual inspection using a brightfield microscope. Alternatively, 2 plates of approximately 70 % confluent HEK HA-CCR2B or HEK HA-CCR5 cells were harvested by scrapping in RIPA buffer containing a complete protease inhibitor cocktail. The cells were lysed by sonication using three 10-second cycles at 40 % amplitude with 30 seconds rest between cycles. Cell lysates were centrifuged to remove unbroken cells. In some experiments a pre-
clearing step consisting of a 20 minute incubation of the cell lysate with an aliquot of protein A/G agarose was carried out at 4 °C with end-over-end mixing. The immunoprecipitation assay was then carried out following one of two protocols. In the original method, the cell lysate was first incubated with antibody, typically 5 µg/ml, for 4 or 16 hours at 4 °C with end-over-end mixing, followed by a second incubation with 20 µl bed volume of protein A/G agarose for 1.5 or 16 hours at 4 °C with end-over-end mixing. Alternatively the antibody was pre-bound to the beads before being incubated with the cell lysate. A 15 µl bead volume of beads was washed in PBS 1% BSA and incubated with the stated quantity of antibody (typically 5 µg) diluted in PBS containing protease inhibitors for 1 hour with end-over-end mixing. The antibody bound-beads were then washed in RIPA buffer containing 1 % BSA and incubated with the cell lysate containing 0.1 % BSA for 2 hours at 4 °C with end-over-end mixing. In both cases the beads were then washed four times in RIPA buffer followed by a final wash in 150 mM NaCl/20 mM TRIS before eluting bound proteins by addition of non-reducing sample buffer and a 5-minute incubation at 95 °C. Samples were analysed by SDS-PAGE followed by western blotting.

Immunoprecipitation using protein A/G agarose leads to contamination of immunoprecipitated proteins with large amount of immunoglobulin and this can interfere with subsequent analysis by mass spectrometry as it may mask the presence of other less abundant proteins. Therefore two alternatives based on covalent immobilization of the antibody to a support were tested: BioMag® Amine (Bangs Laboratories, Indiana, USA) and AminoLink® Plus Coupling Resin (Pierce, Perbio Science UK Ltd).

When testing BioMag Amine, each antibody was coupled to pre-activated BioMag Amine at a ratio of 100 µg antibody and 150 µg BSA carrier protein to 100 µl BioMagAmine following the manufacturer’s standard protocol. The coupling efficiency was estimated by measuring the absorbance at 280 nm using a spectrophotometer (6505 UV/Vis spectrophotometer) of the pre and post-coupling solutions. The BioMag Amine particles were washed with RIPA buffer followed by RIPA buffer containing 1 % BSA and protease inhibitors before use. Typically four 70 % confluent 10 cm plates of transfected HEK293 cells cells per sample were washed with PBS and harvested in 0.35 ml RIPA buffer containing a complete protease inhibitor cocktail per plate using a cell
The cells were lysed by sonication using three 10-second cycles at 40 % amplitude with 30 seconds resting between each, and then centrifuged to remove unbroken cells and an aliquot was taken for analysis. The cell lysate was pre-cleared by a 20-minute incubation with 10 µl BioMag Amine at 4 °C with end-over-end mixing. BSA was added to the pre-cleared cell lysate at a final concentration of 1 % and this was incubated with 10 µg antibody coupled to BioMag Amine particles for 2 hours at 4 °C with end-over-end mixing. Four washes were carried out over 30 minutes in RIPA buffer followed by a final wash in 150 mM NaCl/20 mM TRIS. Immunoprecipitated proteins were removed from the antibody and BioMag Amine by a two stage acid elution process, either using 0.1 M glycine pH2.4 for the first 1-minute and second 10-minute steps or by using 0.1 M glycine pH2.8 followed by pH2.4. After addition of sample buffer, the acid pH was neutralised using 1M TRIS and eluted immunoprecipitation samples, but not cell lysate samples, were incubated at 95 °C for 5 minutes before analysis by SDS-PAGE and western blotting.

When testing AminoLink® Plus Coupling Resin, each antibody was coupled to beads at a ratio of 1 µg antibody to 1 µl beads following the manufacturer’s standard protocol. The coupling was checked by Coomassie (0.1 % Phast Gel™ Blue K from GE Healthcare) staining of the antibody solution used for coupling and the flow-through from the coupling column spotted onto filter paper. A plate of transfected HEK293 cells was washed twice in PBS and the cells were scraped into 1 ml 1 % NP-40 lysis buffer, incubated on ice or at 4 °C with end-over-end rotation for 10 to 20 minutes and then centrifuged to remove the unbroken cells and debris and an aliquot taken for analysis. Typically the cell lysate from half of a 70 % confluent 10 cm plate was incubated with 100 µg antibody coupled to beads for 2 hours at 4 °C with end-over-end mixing. The beads were then transferred to a column and washed four times in 1 % NP-40 lysis buffer followed by a wash in Pierce conditioning buffer. Two sequential elutions were carried out; first an incubation with 300 µl Pierce elution buffer for 1 min at 4 °C, then a second incubation with 700 µl Pierce elution buffer for 10 minutes at room temperature. The eluate was neutralised by addition of 1 M Tris-HCl, pH8 until approximately pH7 was reached. After addition of non-reducing sample buffer, aliquots of the eluted immunoprecipitation samples, but not the cell lysate samples, were incubated at 95 °C for 5 minutes before analysis by SDS-PAGE and western blotting.
Elutions using 30 µl then 70 µl of the following elution buffers were also tested using 12.5 % of the beads from a single immunoprecipitation experiment: 100 mM triethylamine pH 11.5, 100 mM glycine pH 2.5, 5 M LiCl/10 mM phosphate pH7.2 and 3.5 M MgCl₂/10 mM phosphate pH7.2.

When required, eluate was concentrated using a Vivaspin 500 with a 5 kDa molecular weight cut off (Generon, Maidenhead, UK) and the sample was centrifuged for 110 minutes at 15000 xg, 4 °C. Dialysis against Aquacide II (Calbiochem) using a dialysis cassette with a 10 kDa molecular weight cut off (Pierce, Perbio Science UK Ltd) for 40 minutes at 4 °C was also tested. Alternatively, samples were subjected to trichloracetic acid (TCA) precipitation for 30 minutes at 4 °C using a 20 % w/v trichloroacetic acid solution and then pelleted by centrifugation for 15 minutes at 22000 xg. The pellet was washed twice with cold acetone and pelleted again by centrifugation for 5 minutes at 22000 xg, 4 °C, dried by incubation at 95 °C for 10 minutes, resuspended in sample buffer and incubated at 95 °C for 5 minutes.

5.3 Results

5.3.1 GST pull down: Optimization of GST-fusion protein production and purification

The starting point for this project was to use the previously created constructs encoding GST fused to the cytoplasmic tail of CCR2B or CCR5 (GST-CCR2Bct and GST-CCR5ct) to improve the assay for the production and purification of these fusion proteins.

5.3.1.1 GST fusion proteins are produced but are contaminated with GST

The GST-CCR2Bct and GST-CCR5ct fusion proteins were expressed in the BL21 DE3 strain of *E.coli*, as this is deficient in *Ion* and OmpT proteases, and purified from the bacterial cell lysates using glutathione sepharose beads. Expression of the fusion proteins was confirmed by the western blot detection of bands of the expected molecular weights (32 kDa) in bacterial cell lysates and/or bound to the beads following purification, using antibodies against both GST and the receptor cytoplasmic tails (Figure 5.1). However, probing with the α-GST antibody (Figure 5.1) also showed the
presence of a couple of significant contaminating lower molecular weight products of approximately the same size as GST (~26 kDa).

Figure 5.1 Production and purification of GST-CCR2B/5ct fusion proteins. GST-CCR2Bct (A) and GST-CCR5ct (B) fusion proteins were produced in BL21 DE3 following a 1 hour induction at OD$_{600}$ = 0.6 (CCR2) or 0.8 (CCR5) using IPTG. Following lysis by sonication, the bacterial cell lysate was centrifuged to give the pellet (insoluble, I) and supernatant (soluble, S) fractions. The fusion protein was purified from the supernatant by a 2 hour incubation with glutathione sepharose beads. Equal fractions of the pellet and supernatant and a portion of the beads were analysed by SDS-PAGE followed by Coomassie staining or western blotting using α-GST, α-CCR2 (CCR2 C-20) or α-CCR5 (RC-10) antibodies, to enable identification of the fusion protein and contaminants.

After confirming the lack of contamination with parental plasmid DNA by enzyme digests, the question of whether the GST contamination was due to the production of a truncated fusion protein during translation or due to degradation of the intact fusion protein was addressed. As nearly 10% of the DNA encoding the CCR2B cytoplasmic tail is made up of codons that are rarely used in E.coli, it was hypothesized that translation of the fusion protein may be halted after translating the GST. Production using E.coli BL21 Rosetta, which is optimised for translation of DNA containing rare codons, did not reduce the amount of GST (Figure 5.2), thus suggesting that the GST is not a truncated product due to premature termination of translation. Finally, western blots have shown that the GST is present at the end of the induction step before lysis and that the degradation increases with induction time (Figure 5.3). Taken together these
experiments suggest that the fusion proteins are unstable and that the GST is a degradation product produced by C-terminal degradation by exoproteases during the production in *E. coli*.

**Figure 5.2 Production and purification of GST-CCR2B in different strains of *E. coli*.** GST-CCR2Bct (A) and GST-CCR5ct (B) fusion proteins were produced in BL21 DE3, BL21 Rosetta and BL21 pLysS as indicated following a 1 hour induction at $\text{OD}_{600} = 0.5$ – 0.8 using IPTG. Following lysis by sonication, the bacterial cell lysate was centrifuged and the fusion protein was purified from the supernatant by a 2 hour incubation with glutathione sepharose beads. An equal fraction of the beads from each experiment were analysed by SDS-PAGE followed by Coomassie staining.
The GST-CCR2Bct fusion protein is degraded during production.

(A) GST-CCR2Bct and GST-CCR5ct fusion proteins were produced in BL21 pLysS following a 15 minute induction at $\text{OD}_{600} = 0.9$ using IPTG. A sample of bacteria was taken before lysis and lysed in SDS-PAGE loading buffer (BL). Following lysis using BugBuster, the bacterial cell lysate was centrifuged to give the pellet (insoluble, I) and supernatant (soluble, S) fractions. Equal fractions of I, S and BL were analysed by SDS-PAGE followed by western blotting using $\alpha$-GST. 

(B) GST-CCR2Bct fusion proteins were produced in BL21 pLysS following induction for the indicated time at $\text{OD}_{600} = 0.65$ using IPTG. Following lysis using Bug Buster, the bacterial cell lysate was centrifuged to give the pellet (insoluble, I) and supernatant (soluble, S) fractions. Equal fractions of I and S were analysed by SDS-PAGE followed by western blotting using $\alpha$-GST.

5.3.1.2 Approaches tested to reduce GST contamination

The use of mass spectrometry as the downstream analysis method following GST pull downs requires the removal of the large amounts of contaminating GST (Daulat 2009). Three different approaches were trialled: (a) to reduce the level of degradation during production, (b) performing extra purification steps to enable isolation of the GST fusion protein only after production, (c) to remove the contaminating GST at a later stage.
(a) Various inductions times were tested to try to reduce the degradation during production. Shorter induction times were shown to lead to less GST contamination (Figure 5.3B). The optimal conditions were found to be a 15 minute induction starting with bacteria at OD$_{600}$=0.9.

(b) Isolation of the intact fusion protein from the degradation product would lead to a large reduction in the amount of GST present. Doubly-tagged constructs coding for the CCR2B/CCR5 cytoplasmic tail with the original N-terminal GST tag and an extra C-terminal poly-histidine (HIS$_6$) tag were produced. A second version of each fusion protein was created with a glycine linker (Guan and Dixon, 1991; Hakes and Dixon, 1992) between GST and CCR2Bct/CCR5ct. As the fusion protein is degraded from the C-terminus, purification using cobalt resin to bind to the C-terminal HIS$_6$ tag should enable isolation of only intact fusion proteins. However, very little or no fusion protein was successfully isolated (Figure 5.4A). Isolation of the fusion protein using glutathione beads instead showed that the fusion protein and the degradation product were still present (Figure 5.4B). Western blots confirmed the presence of the fusion protein (Figure 5.4B) but not the HIS$_6$ tag (data not shown). These results suggest that the HIS$_6$ tag is partially degraded during the fusion protein production. However, it does appear to have a protective effect in reducing the level of degradation product (Figure 5.7).

(c) An alternative approach is to carry out the GST pull down using the fusion protein in the presence of the contaminating GST and then to remove the GST prior to downstream analysis. The GST-CCR2Bct fusion protein contains a thrombin cleavage site between GST and CCR2Bct and so thrombin was tested for its ability to cleave off the CCR2Bct part of the fusion protein leaving the GST bound to the beads. Following a 2 hour incubation at 37 °C, the ratio of fusion protein:GST remaining on the beads was compared to a controls incubated without thrombin at 4 °C and 37 °C (Figure 5.5). No difference was observed, showing that the cleavage was not successful. Longer incubation times were not tested due to the tendency of the fusion protein to be degraded. Instead a different enzyme, Precission Protease, which is optimally active at 4 °C, was tested. This enzyme has the added advantage of a GST tag, which should facilitate its removal from the sample, thus reducing the amount of contaminating proteins. CCR2Bct was cloned into an alternative vector, pGEX6P1, which contains the
Precission Protease site after GST. Overnight incubation at 4°C with at least 320 U/ml beads of Precission Protease was sufficient for cleavage (Figure 5.6).

**Figure 5.4 Production and purification of GST-CCR2B/5ct-HIS$_6$.**

(A) The indicated fusion proteins were produced in BL21 pLysS following a 1 hour induction at OD$_{600} = 0.6 – 0.7$ (GST-CCR2Bct-HIS$_6$ and GST-CCR5ct-HIS$_6$) or a 15 minute induction at OD$_{600} = 0.9$ (GST-GLY-CCR5ct-HIS$_6$) with IPTG. Following lysis by sonication (GST-CCR2Bct-HIS$_6$ and GST-CCR5ct-HIS$_6$) or french press (GST-GLY-CCR5ct-HIS$_6$), the bacterial cell lysate was centrifuged and the fusion protein was purified from the supernatant by batch purification (GST-CCR2Bct-HIS$_6$ and GST-CCR5ct-HIS$_6$) or on a column (GST-GLY-CCR5ct-HIS$_6$) using cobalt resin. Following elution, the purified protein was analysed by SDS-PAGE and Coomassie staining. (B) GST-CCR2Bct-HIS$_6$ and GST-CCR5ct-HIS$_6$ were produced in BL21 pLysS following a 1.5 hour induction at OD$_{600} = 0.6 – 0.7$ using IPTG. Following lysis by sonication the fusion proteins were purified from the supernatant by an 18 hour incubation with glutathione sepharose beads. An equal fraction of the beads from each experiment was analysed by SDS-PAGE followed by Coomassie staining or western blotting using α-GST, α-CCR2 (CCR2 C-20) or α-CCR5 (RC-10) antibodies as indicated.
Figure 5.5 Testing thrombin cleavage of fusion proteins.
GST-CCR2Bct fusion protein bound glutathione sepharose beads were incubated with or without (control) thrombin at 37 °C or 4 °C (control) as indicated for 2 hours. The beads and eluate were then analysed by SDS-PAGE and Coomassie staining.

Figure 5.6 Testing Prescission Protease cleavage of fusion proteins.
GST-CCR2Bct fusion protein bound glutathione sepharose beads were incubated with the indicated concentration of Prescission Protease for 15 hours at 4 °C. The beads were then analysed by SDS-PAGE and Coomassie staining.

5.3.1.3 The GST fusion proteins are partially insoluble
In addition to being rapidly degraded, the fusion proteins were also partially insoluble. For GST-CCR2Bct the ratio of insoluble to soluble protein was approximately 2:1 when using a 2 hour induction (Figure 5.3B), however this was even higher for GST-CCR5ct. The ratio was improved by shortening the induction time to 15 minutes (Figures 5.3B and 5.7). The HIS₆-tagged constructs were less soluble than their non-tagged versions (Figure 5.7), and for the CCR2 constructs this resulted in an approximately 10 fold lower yield after purification (Table 5.6).
Figure 5.7 Comparison of the solubility of the different GST fusion proteins for CCR2B (A) and CCR5 (B).
The indicated CCR2B (A) or CCR5 (B) fusion proteins were produced in BL21 pLysS following a 15 minute induction at OD$_{600}$ = 0.9 using IPTG. Following lysis using Bug Buster, the bacterial cell lysate was centrifuged to give the pellet (insoluble, I) and supernatant (soluble, S) fractions. Equal fractions of I and S were analysed by SDS-PAGE followed by western blotting using α-GST.

Several production conditions that are known to influence fusion protein solubility were tested. Changing the growth temperature, induction time and bacterial density at the time of induction gave no significant improvement in the fusion protein solubility (Figure 5.8). However, improving the lysis conditions used did increase the fusion protein solubility (data not shown). Producing the fusion protein in BL21 pLysS, which contains bacteriophage T7 lysozyme, improved the GST-CCR2Bct insoluble:soluble ratio from ~2:1 to ~1:1. Using a French press instead of sonication also gave better solubility.
Figure 5.8 Changing the growth temperature (A) or bacterial density at the time of induction (B) gave no significant improvement in the fusion protein solubility.

(A) The GST-CCR2Bct fusion protein was produced in BL21 pLysS following an induction at the indicated temperature for the indicated time at OD600 = 0.7 (37 and 30 °C) or 0.6 (26 or 18 °C) using IPTG. Following lysis using Bug Buster, the bacterial cell lysate was centrifuged to give the pellet (insoluble, I) and supernatant (soluble, S) fractions. Equal fractions of I and S were analysed by SDS-PAGE followed by western blotting using α-GST. (B) The GST-CCR2Bct fusion protein was produced in BL21 pLysS following an induction for the indicated time at the indicated OD600 using IPTG. Following lysis using Bug Buster, the bacterial cell lysate was centrifuged to give the pellet (insoluble, I) and supernatant (soluble, S) fractions. Equal fractions of I and S were analysed by SDS-PAGE followed by western blotting using α-GST.

5.3.1.4 GST fusion protein yields

Following various optimization experiments, a successful assay has been developed for production of the GST-CCR2Bct fusion protein in E.coli BL21 DE3 pLysS followed by purification on glutathione sepharose beads. Although not all of the fusion protein binds to the beads, using a bed volume of 0.5 ml beads per litre of culture, this assay yields 2 – 8 µg fusion protein/µl beads (Table 5.6). This yield is suitable for use in the GST pull down assay. Lower yields were obtained using other GST-CCR2Bct fusion proteins. The CCR5 fusion proteins appeared to be less stable and less soluble that their CCR2B equivalents. The maximum concentration of CCR5 fusion protein that could be obtained under the conditions used was at least 20 fold lower than for CCR2B and was
insufficient for GST pull down assays (Table 5.6). Therefore, this technique could not be used to identify and compare interacting partners of CCR2B and CCR5.

Table 5.6 Yields of GST fusion proteins following production in *E.coli* BL21 and purification on glutathione sepharose beads.

<table>
<thead>
<tr>
<th>Fusion Protein</th>
<th>Glutathione sepharose bead-bound Yield (µg/µl beads)</th>
<th>Yield (mg/L culture produced) when purified on glutathione sepharose beads</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST-CCR2Bct (1)</td>
<td>2 – 8</td>
<td>1 – 4</td>
</tr>
<tr>
<td>GST-CCR2Bct-HIS6 (2)</td>
<td>0.2 - 0.8</td>
<td>0.1 – 0.4</td>
</tr>
<tr>
<td>GST-gly-CCR2Bct-HIS6 (3)</td>
<td>0.3</td>
<td>0.15</td>
</tr>
<tr>
<td>GST-CCR5ct (4)</td>
<td>0.08</td>
<td>0.042</td>
</tr>
<tr>
<td>GST-CCR5ct-HIS6</td>
<td>Not estimated but &lt; for GST-CCR2Bct-HIS6</td>
<td></td>
</tr>
<tr>
<td>GST-gly-CCR5ct-HIS6 (5)</td>
<td>0.1</td>
<td>0.05</td>
</tr>
</tbody>
</table>


5.3.2 GST pull-down: Identification of interacting proteins

The GST-CCR2Bct fusion protein was used for an initial set of four pull down experiments using cell lysate from monocytic THP-1 cells. Following separation of bound proteins by SDS-PAGE, eleven bands (Figure 5.9) were identified as specific to the GST-CCR2Bct pulldown and were analysed by MALDI-MS and MS/MS.

The majority of the proteins were identified as GST due to the large amount of fusion protein and contaminant GST loaded on the gel. However, two proteins of interest, human β-tubulin and importin 7, were identified through a Mascot search using the NCBI nr database with probability based Mowse scores of 450 and 201 respectively.

Due to technical difficulties in the production of GST-contaminant free fusion proteins, no further work was carried out using this technique to identify CCR2B interacting partners in chemokine agonist or LTA-stimulated cells.
A GST pull down experiment was carried out using equal amounts of GST-CCR2Bct bound glutathione sepharose beads (pull down), GST bound glutathione sepharose beads (control 1) or just glutathione sepharose beads (control 2) incubated with THP-1 cell lysate. These pull down samples, a sample of the GST-CCR2ct bound glutathione sepharose beads and a sample of the THP-1 cell lysate used for the pull down were analysed by SDS-PAGE and Coomassie staining. Arrows 1 – 11 mark bands that are unique to the pull down and are not found in either of the controls. These bands were submitted for analysis by MALDI-MS and MS/MS. Bands A and B were identified as human β-tubulin and importin 7 through a Mascot search using the NCBI nr database with probability based Mowse scores of 450 and 201 respectively.
5.3.3 Immunoprecipitation

5.3.3.1 Immunoprecipitation of receptors from monocytic cell lines

A selection of anti-CCR2 antibodies (E68, H-40) and anti-CCR5 antibodies (2D7, 3A9, CCR5 NT, HEK/1/85a, MC5, T21/8) were tested for their ability to immunoprecipitate the receptors from the monocytic cell-line MonoMac 1. None of the antibodies tested were successful in the conditions used for immunoprecipitation (Figure 5.10).

![Figure 5.10](image-url)

**Figure 5.10** Testing a selection of α-CCR2 (E68, H-40) and α-CCR5 (2D7, 3A9, CCR5 NT, HEK/1/85a, MC5, T21/8) antibodies for immunoprecipitation of the receptors from the monocytic cell line MonoMac 1.

For each antibody tested, pre-cleared MonoMac cell lysate from 1 x 10^7 cells was incubated with 2 μg (H-40, T21/8), 5 μg (2D7, 3A9, CCR5 NT, HEK/1/85a), 7.5 μg (MC5) or a 1:50 dilution (E68) of the indicated antibody for 16 hours at 4 °C, followed by a 1.5 hour incubation at 4 °C with protein A/G agarose beads. After washing, the beads were boiled in non-reducing loading buffer for 5 minutes to eluate any bound proteins and this was analysed by SDS-PAGE followed by western blotting using α-CCR2 (CCR2 C-20) or α-CCR5 (CCR5 C-20) as indicated. The red arrow indicates the expected molecular weight (41 kDa) for CCR2B and CCR5 based on their primary amino acid sequences.

5.3.3.2 Immunoprecipitation of receptors from transfected HEK293 cells

Due to the lack of functional CCR5 expressed on the monocytic cell-lines THP-1 (Chapter 3) and MonoMac1, subsequent immunoprecipitation assay development was carried out for transfected HEK293 cells.
This work was performed prior to the identification of the existence of multiple forms of HA-CCR2B in transfected HEK293 cells. The ‘normal’ forms of HA-CCR2B and HA-CCR5 were successfully immunoprecipitated from transfected HEK293 cells using the anti-HA antibody 12CA5 (HA-CCR2B) and MC5 (CCR5) (Figure 5.11). However, the higher molecular weight modified forms of HA-CCR2B were either not immunoprecipitated at all or only immunoprecipitated in very low amounts that could not be detected by western blot under the conditions used. At the time it was not known that the plasma membrane form of CCR2B is the higher molecular weight glycosylated form and further work was carried out into immunoprecipitation of the intracellular ‘normal’ molecular weight form. In retrospect this was unlikely to yield any interacting proteins of interest to this project.

An experiment comparing the amount of HA-CCR2B and HA-CCR5 remaining in cell lysates incubated with protease inhibitors at 4°C overnight, showed that the receptors are rapidly degraded (Figure 5.12). Therefore, optimisation of the immunoprecipitation conditions was vital.
Figure 5.12 Degradation of HA-CCR2B and HA-CCR5 in cell lysate over time.
HEK HA-CCR2B and HEK HA-CCR5 cells were lysed in RIPA buffer containing a complete protease inhibitor cocktail, sonicated and centrifuged to remove unbroken cells. Cell lysate samples from before (time = 0 h) and after (time = 23 h) an incubation at 4 °C for 23 hours were analysed by SDS-PAGE followed by western blotting using α-HA (HA.11). Arrows mark the high (red) and “normal” (green) molecular weight forms of HA-CCR2B.

Immunoprecipitation of HA-CCR2B using 12CA5 bound to protein A/G beads resulted in major contamination of the immunoprecipitated HA-CCR2B with immunoglobulin, which is observed by western blot as multiple contaminating bands (Figure 5.13). Cross-linking the antibody to AminoLink® Plus Coupling Resin or covalently binding it to BioMag® Amine particles resulted in much cleaner immunoprecipitated HA-CCR2B (Figure 5.13).

Figure 5.13 Immunoprecipitation of HA-CCR2B using 12CA5 and different bead supports.
Pre-cleared HEK or HEK HA-CCR2B cell lysate from four 70 % confluent plates was incubated with 16 μg 12CA5 or IgG2b pre-bound to protein A/G agarose (12CA5) or 16 μg 12CA5 covalently coupled to AminoLink Plus Coupling Resin (cross-linked 12CA5), for 2 hours at 4 °C. (B) Pre-cleared HEK HA-CCR2B cell lysate from four 70 % confluent plates was incubated with 10 μg 12CA5 covalently coupled to BioMag Amine for 2 hours at 4 °C. After washing, all beads were boiled in reducing loading buffer for 5 minutes to elute any bound proteins and this was analysed by SDS-PAGE followed by western blotting using α-HA (HA.11).
Several different ratios of 12CA5 antibody to cells were tested to find the best ratio to use to enable immunoprecipitation of the majority of the HA-CCR2B in a 2 hour incubation. Using a ratio of 200 μg 12CA5 antibody per 10 cm plate (70 % confluent) of transfected HEK293 cells was found to result in immunoprecipitation of approximately 97 % of the ‘normal’ HA-CCR2B band (Figure 5.14). Immunoprecipitation using 10 μg 12CA5 was sufficient to visualise the immunoprecipitated HA-CCR2B by western blot. However, identification of novel interacting proteins by MS following co-immunoprecipitation ideally requires sufficient protein for visualisation on a SDS-PAGE gel by Coomassie staining. No HA-CCR2B was observed by SDS-PAGE following immunoprecipitation using up to 100 μg 12CA5 suggesting that further scale up would be necessary. Based on the western blot results and the knowledge that western blotting is more than 100 fold more sensitive than Coomassie staining (Gillespie and Hudspeth, 1991), it was estimated that at least 1 mg 12CA5 antibody would be required to immunoprecipitate enough receptor to visualise on a Coomassie stained gel.

Figure 5.14 Immunoprecipitation of HA-CCR2B from HEK HA-CCR2B cell lysates. HEK HA-CCR2B cell lysate from half of a 70 % confluent plate was incubated with 100 μg 12CA5 covalently coupled to AminoLink Plus Coupling Resin for 2 hours at 4 °C. Equal fractions of the cell lysate before and after this immunoprecipitation were analysed by SDS-PAGE followed by western blotting using α-HA (HA.11). The green arrow indicates the “normal” molecular weight form of HA-CCR2B that is immunoprecipitated.

Following the identification of conditions for successful immunoprecipitation of HA-CCR2B, I encountered the unexpected technical problem of being unable to efficiently elute the immunoprecipitated receptor and interacting proteins. This elution step is necessary to enable reuse of the 12CA5 linked beads due to the large amount of 12CA5 antibody and AminoLink® Plus Coupling Resin required per immunoprecipitation experiment and to allow downstream MS analysis that is not dependent on SDS-PAGE. However, the standard Pierce elution buffer gave low recovery of the immunoprecipitated HA-CCR2B in the eluate (Figure 5.15A). A range of other elution conditions based on different principles, including altered pH and salt concentration, were tested but none gave an efficient elution (Figure 5.15B). Boiling the beads after
elution confirmed that only a small proportion of the immunoprecipitated bound receptor is actually eluted, the rest remains bound to the 12CA5 linked beads.

**Figure 5.15 Recovery of immunoprecipitated HA-CCR2B following elution.**

(A) HEK HA-CCR2B cell lysate from half of a 70 % confluent plate was incubated with 100 µg 12CA5 covalently coupled to AminoLink Plus Coupling Resin for 2 hours at 4 °C. After washing, bound proteins were eluted using Pierce elution buffer. Equal fractions of the cell lysate before and after this immunoprecipitation and of the eluate were analysed by SDS-PAGE followed by western blotting using α-HA (HA.11). The green arrow indicates the “normal” molecular weight form of HA-CCR2B that is immunoprecipitated. (B) HEK HA-CCR2B cell lysate from half of a 70 % confluent plate was incubated with 100 µg 12CA5 covalently coupled to AminoLink Plus Coupling Resin for 2 hours at 4 °C. After washing, the resin was split into eight portions and each was used for elution with either none or one of a range of elution buffers as indicated. The beads were then boiled in non-reducing buffer and analysed alongside the eluates as for (A).

The inefficient elution resulted in a low concentration eluate, which required concentration to enable analysis by SDS-PAGE or mass spectrometry. Several mass spectrometry compatible concentration methods were tested including the VivaSpin concentrator, using a dialysis cassette in conjunction with Aquacide and TCA precipitation. The Vivaspin and dialysis cassette concentration methods were both unsuccessful in retaining HA-CCR2B in the sample, however TCA precipitation resulted in some recovery of HA-CCR2B.
5.4 Discussion

5.4.1 GST pull-down: successful outcomes

Reasonable yields of the GST-CCR2Bct fusion protein were obtained and used for a set of initial GST pull down experiments using THP-1 cells under resting conditions. These experiments identified two potential CCR2B interacting partners: the structural protein β-tubulin and the nuclear import protein importin 7. Importin 7 was previously identified as a possible CCR2B interacting partner by co-immunoprecipitation, although this potential interaction was not further characterised (Favre et al., 2008).

β-tubulin is a structural protein that is present in large amounts in the cell and so may be hypothesised to bind non-specifically to GST-CCR2Bct. However, actin, another major structural protein was not identified and the β-tubulin-containing band was consistently present in all of the pull down samples whilst not being observed with either of the two controls. β-tubulin has been shown to be a non-GPCR substrate of GRK2 (Pitcher et al., 1998), which is also responsible for the agonist-induced phosphorylation of CCR2 (Aragay et al., 1998). As GPCR substrates have been shown to activate GRK2 upon binding (Kim et al., 1993), it is possible that β-tubulin may interact with CCR2 via GRK2. However, the identification of tubulin as a common protein in the sepharose beadome (Trinkle-Mulcahy et al., 2008) suggests that, despite not being obviously present in the controls, it is likely that β-tubulin binds non-specifically to the beads used for the pull down.

Importin 7 is a member of the importin β family, which are involved in translocation of proteins across the nuclear membrane. Therefore, its interaction with CCR2B might suggest the possibility of CCR2B localising to the nucleus. The idea of a chemokine receptors trafficking to the nucleus is controversial and currently under debate. Agonist binding of a GPCR at the cell surface leads to various intracellular signaling cascades and can result in transcriptional regulation within the nucleus. Over the last decade there have however emerged several reports of ligand–bound GPCRs trafficking to the nucleus where they can initiate different and sometimes opposite functional responses to the signaling from cell surface receptors (Goetzl, 2007). CXCR4 is commonly expressed by tumour cells where it has been observed to localise to the nucleus in addition to the cytoplasmic and some limited plasma membrane expression (Speetjens et al., 2009;
Wang et al., 2005). However, it is important to bear in mind that most studies showing nuclear localisation of CXCR4 were carried out on fixed cells (Na et al., 2008; Speetjens et al., 2009; Wang et al., 2009; Wang et al., 2010a; Wang et al., 2005; Woo et al., 2008; Yao et al., 2011). In some circumstances, fixation processes can cause the collapse of intracellular membranes and organelles (Hoetelmans et al., 2001; LaJeunesse et al., 2004), thus rendering it difficult to distinguish between ER and nuclear membranes. It has been suggested that under certain conditions CXCR4 can traffic to the nucleus following long-term agonist stimulation (Wang et al., 2009). As this study did not use cell surface labelled receptors, it cannot be determined that the CXCR4 observed in the nucleus was actually derived from the cell surface pool of receptor. Therefore the possibility of receptor transport from the ER to the nucleus during biosynthesis remains to be explored. A single study reported for CCR2 shows nuclear expression in transfected HEK and HeLa cells that is increased upon agonist stimulation (Favre et al., 2008).

The role played by nuclearly localised chemokine receptors is currently unknown. For CXCR4 it is believed to be different to that played by the cytoplasmic receptor and has been frequently linked to poor prognosis for cancer patients (Na et al., 2008; Speetjens et al., 2009; Wang et al., 2009; Wang et al., 2010b; Woo et al., 2008; Xiang et al., 2009; Yao et al., 2011). It has recently been suggested that nuclear CXCR4 may promote tumour metastasis, as its presence has been associated with metastasis of multiple cancers including renal cell carcinoma (Wang et al., 2009), gall bladder cancer (Yao et al., 2011), non-small cell lung cancer (Na et al., 2008), hepatocellular carcinoma (Xiang et al., 2009) and breast cancer (Woo et al., 2008). For nuclear CCR2 no role has yet been proposed. Nuclearly localised metabotropic glutamate 5 receptor was suggested to signal via a G protein-dependent mechanism (Kumar et al., 2008). However, as β-arrestin 1 has been recently shown to traffic to the nucleus (Hoeppner et al., 2012), it is possible that any nuclear chemokine receptor signalling may alternatively utilise the β-arrestin-dependent signalling pathways.

How chemokine receptors or other GPCRs could enter the nucleus is not yet established. Conventional nuclear import of large proteins involves binding of a classical nuclear localisation signal (NLS) in the protein that is being imported to the adaptor protein importin α, which then interacts with importin β to enable interaction with the nuclear
pore complex and translocation into the nucleus. More recently it has been shown that some proteins can bind directly to importin β1 itself or to one of the 18 other (Chook and Suel, 2011) other members of the importin β family. These interactions are not dependent on the presence of classical NLSs, rather on a diverse and still expanding selection of non-classical NLSs.

Analysis of the CCR2B primary amino acid sequence shows a couple of putative NLSs. A classical monopartite NLS (KRHR) is present in the third intracellular loop but if it acts as an actual NLS has not yet been investigated. However, the same sequence is present in the third intracellular loop of the better characterised chemokine receptor CCR5, for which no evidence of nuclear localisation has been reported. Additionally, as the third intracellular loop of CCR2 is quite short and is also a site of G protein (Arai and Charo, 1996) and probably β-arrestin binding (Cheng et al., 2000; Zheng et al., 2010), it is unlikely to be readily accessible for interactions with the nuclear import machinery. Accessibility is a key feature of an NLS (Xu et al., 2010). Indeed, despite the presence of a functional NLS in the relatively large third intracellular loops of the α1A and B adrenergic receptors (Wright et al., 2012), in general when present, putative classical NLSs are more commonly found in the eighth helix located between the seventh transmembrane domain and the cytoplasmic tail (Lee et al., 2004).

In contrast to the consensus sequences that form classical NLSs, the non-classical NLSs are less well defined and highly variant, although NLSs for transportin-1 and importin 7 have been suggested and the CCR2B sequence was screened for these. A phosphorylated S/T-P-S/T motif has been shown to be responsible for interaction of several proteins, including ERK2 (Chuderland et al., 2008) and early growth response 1 (Chen et al., 2011), with importin 7 leading to nuclear import. A version of this motif (TPS) is present in the CCR2B cytoplasmic tail and the serine/threonine residues are known to be phosphorylated in response to CCL2 stimulation. However, this short sequence is also present in several other typical chemokine receptors (CCR1/3/4/9/10 and CXCR7), which have not been shown to localise to the nucleus, suggesting that it alone is not sufficient for nuclear localisation. A search for the R/K/H-X(2,5)-P-Y consensus motif that has been reported to make up part of the PY-NLS responsible for binding to transportin-1 (Lee et al., 2006), showed no hits for CCR2B. An earlier report showing interaction of CCR2B with transportin-1 by CO-IP suggested that the
interaction was not direct and was instead mediated via a complex possibly involving TCP-1γ (Favre et al., 2008). The identification of both importin 7 and transportin-1 as proteins that interact with CCR2B is not inconsistent, as some proteins that are imported via the non-classical nuclear import pathways have been shown to be able to utilise various members of the importin β family (Chook and Suel, 2011; Jakel and Gorlich, 1998).

As GPCRs contain seven transmembrane domains, they are unlikely to be transported into the nucleus as for soluble cytoplasmic proteins, rather their insertion in the nuclear envelope is the more likely scenario. This raises the question of if the conventional nuclear translocation machinery can be adapted in an undetermined way to also play this role, or if it happens via a novel mechanism such as fusion of vesicle membranes with the nuclear membrane or lateral diffusion through the ER membrane.

The suggested interactions of CCR2 with several nuclear import proteins does not necessarily imply that the receptor actually undergoes nuclear localisation. Some nuclear transport proteins are known to moonlight and there thus remains the possibility that importin 7 may interact with CCR2 as part of a different function. For example, FROUNT is a member of the Nup107-160 complex where it plays a role in nuclear pore complex assembly, however, it also interacts with ligand-bound CCR2 and CCR5 at the plasma membrane and links them to the PI(3)K-Rac-lamellipodium protrusion cascade (Loiodice et al., 2004; Terashima et al., 2005; Toda et al., 2009).

5.4.2 GST pull-down: limitations

The main limitation for the GST pull-down was the inability to produce and purify sufficient GST-CCR5ct fusion proteins due to degradation and insolubility problems. Coupled with the lack of functional CCR5 in THP-1 cells, this meant that the second objective of comparing interacting proteins for these two receptors following agonist or LTA stimulation could not be carried out. The reason for the difference in stability of the two fusion proteins is currently unclear. When endogenously expressed, palmitoylation of the cysteines in the CCR5 cytoplasmic tail is thought to enable interaction with the plasma membrane creating an extra pseudo forth intracellular loop (Blanpain et al., 2001; Kraft et al., 2001; Percherancier et al., 2001) and this more rigid conformation may control the normal accessibility of parts of the tail protecting them...
against degradation. When expressed as part of a GST fusion protein in *E.coli*, the CCR5 cytoplasmic tail would not retain this same conformation and so may be more accessible to proteases. In contrast, there are no palmitoylated cysteines in the CCR2B cytoplasmic tail and so its conformation may be less affected by expression as a GST fusion protein.

The identification of importin 7, which had been previously corroborated as an interacting partner for CCR2B (Favre et al., 2008), validated the use of this GST pull-down technique. However, my study failed to identify the other known binding partners of CCR2B: FROUNT (Terashima et al., 2005), filamin A (Minsaas et al., 2010), other nuclear import proteins including in particular TRN-1, TCP-1γ (Favre et al., 2008), GRK2 or β-arrestin (Aragay et al., 1998). For GRK2 and β-arrestin this is likely to be simply explained by the use of non-stimulated cells as these proteins have been reported to interact with CCR2 following CCL2 stimulation (Aragay et al., 1998). An important difference between the GST pull-down and other methods such as CO-IP (Favre et al., 2008), previously used to identify CCR2B interacting partners, is that the GST pull-down only uses the cytoplasmic tail of CCR2B. Reports for GPCRs, including chemokine receptors, have identified other receptor domains as playing important roles in specific protein interactions. A key example is the DRY motif, which is highly conserved in second intracellular loops of almost all typical chemokine receptors, including CCR2, and is required for interaction with a heterotrimeric G protein for signalling (Mellado et al., 1998). Other intracellular loop residues, especially those at the N and C-terminal ends of the third intracellular loop are also important for G protein binding to GPCRs (Wess, 1997). Additionally, residues in the second intracellular loop encompassing the DRY motif (Huttenrauch et al., 2002; Marion et al., 2006) and in the third intracellular loop (Cheng et al., 2000; Zheng et al., 2010) of GPCRs including chemokine receptors, in addition to phosphorylated serines/thereonines in their cytoplasmic tail, can all contribute to β-arrestin binding. Precisely how CCR2B interacts with the nuclear import proteins and TCP-1γ is not yet clear and so the cytoplasmic tail alone may not be sufficient for these interactions. However, as FROUNT (Terashima et al., 2005) and filaminA (Minsaas et al., 2010) were first identified in a yeast-2-hybrid screens using the CCR2B cytoplasmic tail, they would be expected to also be identified in the GST pull-down experiment and this was not the case. This could be due to these
proteins either not being present at all or only in undetectable amounts/concentrations in the samples analysed by MS. There are several potential reasons why this might be.

The design of the GST pull-down experiment MS analysis was partially determined by the in-house facilities available at the start of the project, which have since improved in terms of sample type that can be analysed and capacity for identifying low abundance proteins. The conditions dictated that MS analysis could only be carried out on bands excised from a gel where they must be visible using a low sensitivity Coomassie stain.

The bands analysed were chosen based on both their presence in the GST pull down coupled with absence in controls and their reproducibility between experiments. It is possible therefore, that known CCR2 interacting partners were actually pulled down but were not analysed as they were not present in the bands chosen for analysis. This could be because their concentration was insufficient to be revealed by the low sensitivity Coomassie stain and so no band was observed. Or known CCR2 interacting partners could localise to a band that was not analysed as it was also present in the controls due to the presence of another protein that interacts non-specifically with GST or the support bead matrix. Alternatively, the known CCR2 interacting partners could be present at low concentrations in a band that was analysed and so were not identified due to masking by more abundant proteins. GST contamination due to smearing throughout the gel was a problem for the analysis as all bands were identified as containing GST and for most bands this was the major protein identified.

5.4.3 CO-IP: outcomes and limitations

The first approach used in this study to try to immunoprecipitate endogenous CCR2 and CCR5 from monocytic cell lines using a variety of anti-CCR2 and anti-CCR5 antibodies was unsuccessful under the conditions tested. This may be due to the lack of good CCR2 antibodies commercially available, and the level of epitope recognised by the anti-CCR5 antibody, MC5, being very low on THP-1 cells. However, the recent discovery that the CCR5 present on THP-1 cells is not functional (described in Chapter 3), meant that using this cell line for comparing the proteins co-immunoprecipitated with CCR2 and CCR5 would not be useful for addressing the aims of this study. In contrast, suitable conditions to immunoprecipitate HA-CCR5 and the non-modified form of HA-CCR2B from the HEK HA-CCR2B/5 cell lines using the anti-HA antibody 12CA5 were
successfully identified. Unfortunately, several limitations to the planned co-immunoprecipitation experiments were found. The main problems were that the immunoprecipitated HA-CCR2B was unexpectedly difficult to elute from the antibody-bound AminoLink® Plus Coupling Resin, and more importantly that no conditions were found to efficiently immunoprecipitate the glycosylated form of the receptor.

CCR2B and CCR5 appear to express some of the innate stickiness associated with 7TM receptors. The only condition found to efficiently elute HA-CCR2B from the antibody-bound AminoLink® Plus Coupling Resin was boiling in SDS-PAGE sample buffer. Sample concentration techniques involving membranes led to low retention of HA-CCR2B, presumably due to interaction with the membranes.

Studies in Chapter 4 have shown that the glycosylated form of HA-CCR2B is present at the cell surface, whereas the non-modified form is believed to be in the biosynthetic pathway. In addition, it is the glycosylated form of HA-CCR2B that responds to agonist stimulation. Therefore, it is the glycosylated form of HA-CCR2B that is of interest for identifying proteins interacting with the receptor during desensitisation. Precisely why this form of the receptor is not immunoprecipitated is currently unclear, however there are several possible explanations, which are discussed here.

The non-modified and glycosylated forms of HA-CCR2B are differentially localised within the cell. The lipid composition and thus the susceptibility to detergents differs between cellular membranes suggesting that the different HA-CCR2B forms may be solubilised to varying extents during the lysis conditions used for the immunoprecipitation assay. However, a major difference in solubility is unlikely to be the explanation for why the non-modified form is immunoprecipitated whilst the glycosylated form is not, as they were both present at similar concentrations in the soluble fraction of the cell lysate used for the immunoprecipitation assay.

The glycosylation modification itself could be responsible for the lack of immunoprecipitation of the glycosylated HA-CCR2B. The N-glycosylation site is believed to be on the N-terminus of CCR2B and it is likely that the O-linked glycosylation also takes place here. It is possible that the glycosylated N-terminus of the receptor is more likely to fold back on itself and interact with another part of the
receptor making the N-terminal triple HA-tag inaccessible to the 12CA5 antibody due to steric hinderance or masking.

5.4.4 Recommendations for future studies to identify binding partners for CCR2B

This chapter has shown that techniques that work for confirming protein-protein interactions do not necessarily work well for identifying unknown interactions without extensive adaptation. Various modifications that may improve future proteomics approaches to identifying interacting partners of CCR2B are discussed here.

The small number of interacting proteins identified through the GST pull-down assay suggests that cells endogenously expressing CCR2 may not yield sufficient material for identifying interacting partners. This illustrates the requirement to enrich the concentration of proteins of interest in the starting material. One approach to this is to use transfected cells expressing higher levels of CCR2. However, as described in Chapter 3, whilst the HEK HA-CCR2B cells over-expressed the HA-CCR2B receptor, the cell surface receptor level was similar to that observed in monocytic cells, with the excess receptors confined to the biosynthetic pathway. For CO-IP experiments, an alternative but more complex approach would be to immunoprecipitate HA-CCR2B directly from the cell surface, by pre-binding the antibody prior to cell lysis, or from a membrane fraction following sub-cellular fractionation.

As fractionation could lead to a loss of transient interactions, crosslinking prior to cell lysis may be required. Traditional crosslinking methods have various limitations, and often the most suitable crosslinker needs to be determined empirically (Kaake et al., 2010). Chemical crosslinking methods are not all protein specific, such as the case for formaldehyde, or may be dependent on larger specific distances between the interacting proteins, as for other bifunctional chemical crosslinkers with spacer arms (Sutherland et al., 2008). Site specific photochemical crosslinking is dependent on the incorporation of a photoactivatable amino acid into a peptide or protein, which can be carried out by chemical synthesis (Kauer et al., 1986), in vitro synthesis (Cornish et al., 1994), or in vivo synthesis in E.coli (Chin et al., 2002) or mammalian cells (Hino et al., 2005) by addition of a mutated cDNA and a specific orthogonal aminoacyl tRNA synthetase/tRNA pair. This therefore limits the number of proteins and sites that can be
used for photochemical crosslinking and thus the identification of proteins involved in complexes. An alternative photocrosslinking method has been developed, which involves growing cells in a medium containing modified photoactivatable leucine and methionine, which can be incorporated by the endogenous protein translation machinery thus enabling labelling of the majority of cellular proteins (Suchanek et al., 2005). CCR2 and CCR5 both contain leucine in their cytoplasmic tail and CCR5 has leucine and methionine residues in its cytoplasmic loops. Hence this crosslinking technique would be suitable for investigating proteins that interact directly or indirectly with the two receptors.

Due to improvements in MS sensitivity and resolution, there has been a general shift away from identifying proteins using individual bands or spots excised from a gel, towards utilising whole affinity purification, such as CO-IP or pull-down, samples for gel-free MS analysis (Gingras et al., 2007; Goudreault et al., 2009; Kaake et al., 2010; Raman et al., 2009). This should theoretically increase many fold the number of proteins identified from one sample. Carrying out MS analysis of whole samples instead of individual bands should therefore increase the likelihood of finding known interacting partners that were missed using the GST pull-down gel-based MS analysis in this study.

However due to the increase in sensitivity of MS, the identification of large numbers of non-specific binding proteins would be a greater issue when analysing affinity purification samples directly (Kaake et al., 2010). Various quantitative proteomics methods are available to reduce the problem of false positives caused by non-specific binding proteins whilst minimising the loss of real but weak interactions (Trinkle-Mulcahy, 2012). These methods work by differentially labelling specific and control samples used for affinity purification, either chemically or metabolically, and then determining the ratio of a candidate interacting protein indentified in both samples. Non-specific binding proteins should be present equally in both samples giving a 1:1 ratio, whereas those that bind specifically should show a greater presence in the specific sample. SILAC is a popular metabolic labelling method as it uses incorporation of isotopic versions of essential amino acids (lysine and/or arginine) in vivo and so labels all proteins and has little impact on their functions (Ong et al., 2002; Trinkle-Mulcahy, 2012). The use of SILAC should enable the identification of some interactions that were
missed in this study due to their presence in control and specific GST pull-down sample lanes.
6 General discussion

One of the major factors controlling the activation status of chemokine receptors is the regulation of their cell surface expression. The three main trafficking steps regulating cell surface receptor expression, namely biosynthesis, internalisation and degradation or recycling, have been considered for CCR2B in this thesis. This work presents an analysis of the intracellular trafficking of CCR2B using a HEK HA-CCR2B cell line created for this project. Certain aspects of the trafficking (endocytosis, recycling) are also supported by experiments using the monocytic cell line THP-1. The CCR2B post-endocytic trafficking pathway is compared to that of the related chemokine receptor CCR5, in part through my study of HEK HA-CCR5 cells and also other cell types, including monocytes, used in published work. Novel work showing some of the limitations of using monocytic cell lines for the study of functional CCR5 is also presented.

6.1 Biosynthesis

Chapter 4 presents the first evidence that in addition to undergoing N-linked glycosylation (this study and Preobrazhensky et al., 2000), CCR2 can also be O-glycosylated. The predominant localisation of glycosylated CCR2 in the plasma membrane and the reduction in cell surface expression of the receptor when glycosylation is inhibited, suggest that this post-translational modification enhances trafficking of the newly synthesised CCR2 to the plasma membrane. The involvement of glycosylation in GPCR trafficking to the cell surface is thought to vary between receptors (Dong et al., 2007; Duvernay et al., 2005). Predominantly N-, but also O-, linked glycosylation has been shown to enhance the cell surface expression of some GPCRs and for certain receptors it is essential, whilst for others it has no effect. How precisely glycosylation regulates cell surface expression has not yet been fully explained although for several receptors it has been suggested to act by aiding the trafficking of newly synthesised GPCRs to the cell surface (Angelotti et al., 2010; Ge et al., 2009), or by protecting existing cell surface GPCRs from proteolytic attack (Ludwig et al., 2000). Glycosylation can provide binding sites for certain chaperone proteins, which may be important for CCR2B trafficking to the plasma membrane. Lectin chaperones, such as calnexin and calreticulin, bind glycans and are involved in promoting correct protein
folding. For the $\alpha_{2c}$-AR receptor it has been suggested that glycosylation improves the cell surface receptor expression by increasing the efficiency of protein folding (Angelotti et al., 2010). Another receptor, the $\mu$-opioid receptor (MOR), is dependent on N-glycosylation for interaction with Ribophorin I (RPNI), which regulates its cell surface expression (Ge et al., 2009). The molecular mechanism is currently unknown but it has been suggested that in addition to its role in protein N-glycosylation as part of the oligosaccharide transferase complex (OST), RPNI may act in conjunction with BiP as a chaperone playing a role in ER quality control thus regulating MOR translocation out of the ER (Ge et al., 2009). In contrast, it has been reported that N-glycosylation of CXCR2 at two different sites is not required for the trafficking of the receptor to the cell surface, but is required for maintenance of its cell surface expression on human neutrophils (Ludwig et al., 2000). The addition of glycan chains was suggested to protect CXCR2 from proteolytic attack. Unlike CCR2, despite also being localised to the cell surface, CCR5 is not reported to be N-glycosylated but does undergo direct O-linked glycosylation (Bannert et al., 2001), thus reinforcing the variability in the role of glycosylation.

Glycosylation is not the only post-translational modification involved in trafficking of newly synthesised chemokine receptors to the plasma membrane. Palmitoylation is important for cell surface expression of CCR5 and this modification is believed to act by protecting against CCR5 degradation (Blanpain et al., 2001; Percherancier et al., 2001). Despite the presence of suitable cysteines in its cytoplasmic tail, the CCR2B receptor is not thought to undergo palmitoylation and thus this post-translational modification is unlikely to play a role in CCR2B cell surface expression. This demonstrates that even with closely related chemokine receptors, cell surface expression pathways dependent on post-translational modifications described for one receptor are not necessarily generic.

### 6.2 Internalisation

Chapters 3 and 4 confirmed the agonist-induced internalisation of CCR2 using THP-1 and HEK HA-CCR2B cell lines. The results of inhibition and colocalisation studies shown in this thesis support a role of clathrin-mediated endocytosis in agonist-mediated internalisation. Based on the lack of complete inhibition of internalisation by clathrin-
mediated endocytosis inhibitors and conflicting results when modulating the membrane lipid composition, a role for clathrin-independent lipid-dependent endocytosis pathways cannot be ruled out but probably do not play the predominant role in HEK HA-CCR2B cells. This work is in agreement with previous groups that reported the use of both pathways for CCR2 and suggested that there may be a level of cell type specificity (Andjelkovic et al., 2002; Garcia Lopez et al., 2009; Ge and Pachter, 2004). However, caution must be exerted in the interpretation of lipid modulation studies as for some receptors changing the lipid composition can have an indirect effect on receptor internalisation by changing receptor conformation and thus affecting ligand binding. Binding studies using radiolabelled chemokine have been used to show that this is the case for CCR5 (Nguyen and Taub, 2002; Nguyen and Taub, 2003a, b; Signoret et al., 2005), and would be required before any final conclusions could be drawn for CCR2.

If indeed CCR2 can use multiple endocytic pathways, how the choice of which pathway to use is regulated remains to be explored. It is becoming apparent that chemokine receptor internalisation is a complex process that can be modulated by multiple factors such as cell type and ligand used for stimulation. As the plasma membrane lipid composition differs between cell types, the influence of lipid on the internalisation of CCR2 could also be expected to differ. Caveolae are described as a subdomain of lipid rafts enriched in sphingolipids, cholesterol and caveolin proteins, and endocytosis via this route is sensitive to cholesterol depletion (Le Roy and Wrana, 2005). On the other hand, cholesterol depletion in general does not significantly inhibit clathrin-mediated endocytosis (Doherty and McMahon, 2009). In addition, different cellular protein content may lead to cell type variation in the availability of key endocytic pathway proteins. For example, β-arrestin expression has been shown to vary between different cell types at both the mRNA and protein levels (Komori et al., 1998; Menard et al., 1997; Parruti et al., 1993). Therefore, as clathrin-mediated endocytosis is dependent on interaction of the GPCR with β-arrestin (Luttrell and Lefkowitz, 2002), the proportion of CCR2B endocytosis using this pathway may be cell type-dependent. Indeed for β2-AR, a GPCR whose internalisation is predominantly clathrin-dependent (Goodman et al., 1996; Moore et al., 1995), a positive correlation between the cellular β-arrestin expression and the level of receptor internalisation has been demonstrated (Menard et al., 1997). Thus it could be hypothesised that the choice of endocytic route used may be in part dictated by availability of endocytic pathway proteins in the cell type studied.
In contrast, differences in the internalisation response of a specific chemokine receptor when stimulated with different ligands but using the same cell type, have also been reported for CCR4 (Mariani et al., 2004), CCR7 (Byers et al., 2008) and CXCR2 (Feniger-Barish et al., 2000). Indeed recent work for CCR2 has reported the existence of a ligand-dependent bias in the extent and rate of receptor internalisation and suggested that this bias was due to the stabilisation of different CCR2 homodimer conformations and differential β-arrestin recruitment (Berchiche et al., 2011). It would be interesting to determine if the observed positive correlation between β-arrestin recruitment and CCR2 internalisation supports uniquely a clathrin-mediated endocytic pathway, or if there are additional ligand-specific contributions of clathrin-independent pathways.

Whilst agonist-induced CCR2 internalisation appears to occur independent of cell type, there seem to be differences in internalisation resulting from receptor cross-talk from TLR2. In contrast to monocytes, LTA stimulation does not lead to efficient rapid CCR2 down-modulation/internalisation in HEK TLR2 HA-CCR2B cells. However, dose-dependent down-modulation of CCR2B was observed at relatively high concentrations of a different TLR2 ligand, Pam3CSK4, with slightly higher down-modulation observed in cells expressing the TLR1/2 dimer compared to the TLR2/6 dimer. The reason for this ligand-specific difference in the cross-talk response with CCR2B remains unclear. TLR2 can homo- and hetero-dimerise, and has been shown to partner with TLR1 (Wyllie et al., 2000), TLR6 (Takeuchi et al., 2001) and TLR10 (Hasan et al., 2005) resulting in different ligand specificity. TLR1/2 and TLR2/6 complexes are believed to generally bind triacylated (e.g. Pam3CSK4) and diacylated (e.g. LTA) lipoproteins and glycolipids respectively (Takeuchi and Akira, 2010). TLR1 and TLR6 are both expressed in monocytes (Chang et al., 2007) and it is not known which TLR2 heterodimer is responsible for the LTA-induced chemokine receptor down-modulation (personal communication from Nathalie Signoret, University of York, UK). The HEK TLR2 HA-CCR2B cell lines used in the present study expressed mouse TLR2 in combination with either mouse TLR1 or mouse TLR6, however HEK293 cells have also been shown to express endogenous human TLR1 and TLR6 (Kurt-Jones et al., 2004). As Pam3CSK4 has been reported to be able to induce responses in HEK293 cells only transfected with human TLR2 (Mandell et al., 2004), there remains the possibility that in my study the mouse TLR2 may partner with endogenous human TLR1 explaining the observation of Pam3CSK4 induced HA-CCR2B down-modulation regardless of which
other mouse TLR is expressed. The Pam3CSK4-induced HA-CCR2B down-modulation observed in my study was only observed at levels of Pam3CSK4 much higher than normally required to induce cytokine production (Mandell et al., 2004), unlike the LTA concentration required to down-modulate CCR2 in monocytes (Fox et al., 2011). As high concentrations of LTA gave no response, it is possible that mouse TLR1 is better suited than mouse TLR6 for acting in combination with TLR2 for transducing signals, thus enabling a response to very high Pam3CSK4 concentrations. These results suggest that the TLR2 is functional and that a TLR2-CCR2 cross-talk pathway is present to some extent but that it may be ligand-dependent and less efficient than in monocytes. On monocytes, LTA cross-talk induced CCR5 internalisation utilises the same machinery involved in agonist-induced internalisation, but following a much slower kinetic (Fox et al., 2011). Although not tested, this may also be the case for CCR2. The pathways downstream of TLR2 that lead to the recruitment of this machinery remain to be elucidated and may be in part cell type-specific.

The apparent importance of cell type specificity for the LTA-dependent TLR2 CCR2/5 cross-talk pathway but not for agonist-induced desensitisation illustrates the huge complexity of the processes that regulate chemokine receptor activity. There is thought to be a single agonist-induced homologous desensitisation pathway that is dependent on receptor cytoplasmic tail phosphorylation by a member of the GRK family. In contrast, multiple cross-talk pathways have been partially described, that utilise GRKs or second messenger kinases, with no single consensus pathway. In some cases, such as for the LTA induced cross-desensitisation of CCR2 and CCR5 described by our group, the cross-talk pathway is thought to utilise the homologous desensitisation machinery. However, there must still be a unique cross-talk pathway that feeds into the homologous desensitisation pathway, and proteins involved in this first step could be expressed in a cell type-specific manner.

6.3 Degradation

My study (Chapter 4) provides the first biochemical evidence that at least some CCR2 undergoes lysosomal degradation in response to agonist stimulation. This is supported by colocalisation immunofluorescence studies. Colocalisation of internalised HA-CCR2B with a marker of the degradative pathway, coupled with the fact that only
glycosylated HA-CCR2B is degraded, suggest that it is cell-surface derived HA-CCR2B that is internalised and undergoes degradation following agonist stimulation. N- or O-linked glycosylation has been shown to be required for efficient ligand binding to some chemokine receptors including CCR5 (Bannert et al., 2001), CCR8 (Gutierrez et al., 2004) and CXCR4 (Wang et al., 2004b). Therefore either of the CCR2 glycosylation modifications may be important for agonist-induced internalisation and subsequent intracellular trafficking leading to receptor degradation. These studies were carried out using HEK HA-CCR2B cells. Unfortunately, the commercial CCR2 antibodies currently available are not sufficient to confirm if endogenously expressed CCR2B in monocytic cell lines or monocytes is also degraded, or if this is a cell type-specific process. In contrast, CCR5 has been shown not to undergo enhanced degradation in response to agonist stimulation, and instead internalised receptors have been shown to be recycled back to the cell surface (Signoret et al., 2004; Signoret et al., 2000).

Agonist-induced receptor degradation and recycling provide temporally different levels of receptor desensitisation, with degradation leading to more long term inhibition of future signalling than the transient desensitisation observed for receptors that recycle. This thesis provides evidence that internalised CCR2B undergoes both degradative and recycling fates, as opposed to the single recycling pathway followed by internalised CCR5. Coupled with the fact that CCR2, but not CCR5, is down-regulated on non-activated leukocytes at the gene level in response to TLR2 stimulation with bacterial lipoprotein (McKimmie et al., 2009), this raises the question of why CCR2 requires more long-term desensitisation than CCR5? Although their overall functions in the immune system are complementary, the two receptors do differ in their precise roles in leukocyte recruitment. Monocytes and macrophages exhibit complementary cellular expression profiles for CCR2 and CCR5 and these receptors show reciprocal functions. CCR2 is expressed on human peripheral blood monocytes but not macrophages (Fantuzzi et al., 1999), whereas CCR5 shows a low level of expression on human monocytes, which is increased upon differentiation into macrophages (Kaufmann et al., 2001). Thus it is not surprising that CCR2 plays a predominant role in the initial recruitment of monocytes to tissues, where following differentiation into macrophages, CCR5 plays an important role in maintaining and retaining them (Zhao, 2010).
Unless degradation is the default pathway following internalisation, there must be a motif or modification of the receptor that is responsible for its sorting into this pathway. Ubiquitination has been shown to be important for degradation of CXCR4 (Marchese and Benovic, 2001) and some other GPCRs (Marchese et al., 2008). At the same time, this modification plays no role in the degradation of CXCR2 (Baugher and Richmond, 2008) or CXCR3 (Meiser et al., 2008) and no ubiquitination of CCR2B was observed in my study. The CCR2B cytoplasmic tail contains a putative tyrosine based motif (YLSV) of the type that can play a role in lysosomal targeting (Bonifacino and Traub, 2003). However, the localisation of the motif away from the C-terminal and the lack of the common preceding glycine residue (Bonifacino and Traub, 2003), coupled with the presence of a very similar motif (YLLV) at the same location in CCR5 suggest that this motif is unlikely to play a function role in lysosomal sorting of CCR2B. In addition, the CCR2B tail does not contain a functional acidic dileucine motif, which is the other motif commonly associated with lysosomal sorting (Bonifacino and Traub, 2003). This suggests that there may be at least one other less common motif or modification responsible for sorting of chemokine receptors into the degradation pathway and this may not be generic to all degraded chemokine receptors.

6.4 Recycling

I have shown that following internalisation of CCR2 from the plasma membrane, the cell surface receptor levels are partially recovered within a 60 minute period in both HEK HA-CCR2B and THP-1 cells. The results of experiments shown in Chapter 4 suggest that not all internalised CCR2 is degraded and instead a portion is recycled back to the cell surface. This data supports previous suggestions of varying levels of CCR2 cell surface recovery in astrocytes (Andjelkovic et al., 2002) and THP-1 cells (Wang et al., 1993a). The level of recycling observed in HEK HA-CCR2B cells is quite low suggesting that, at least in these cells, this is a minor pathway following internalisation. The level of recovery was considerably higher in THP-1 cells, suggesting that endogenous CCR2 also has the capability to recycle. Although it has been reported that CCR2B is the dominant isoform of CCR2 in THP-1 cells, the possibility of the post-internalisation cell surface recovery being enhanced by the presence of a low level of CCR2A cannot be ruled out, as the R&D α-CCR2 antibody used recognises both isoforms. Partially contrasting results regarding the main cellular localisation of CCR2A
have been reported (Tanaka et al., 2002; Wong et al., 1997). In stably transfected cells, N-terminal FLAG-tagged CCR2A has been shown by immunofluorescence on intact and permabilised cells to be predominantly located intracellularly unlike the plasma membrane localised CCR2B, although a small amount of CCR2A cell surface expression was observed by flow cytometry and ELISA (Wong et al., 1997). The CCR2A intracellular localisation was suggested to be dependent on an unidentified cytoplasmic retention signal in the C-terminal tail of CCR2A (Wong et al., 1997). Predominantly intracellular localisation of CCR2A has also been shown in transfected COS-7 cells by flow cytometry on intact and permabilised cells (Tanaka et al., 2002). In contrast, in THP-1 cells and monocytes, the ratio of intracellular and cell surface CCR2A has been shown, by a chemiluminescence immunoassay using total or plasma membrane fractions and by flow cytometry, to reflect that of CCR2B, although at an almost 10 fold lower expression level (Tanaka et al., 2002). However, the antibodies used by Tanaka et al. (Tanaka et al., 2002) for this work are not available commercially to confirm the relative CCR2A and CCR2B levels and localisations on our THP-1 cells.

The recycling pathway back to the cell surface utilised by internalised CCR2 is currently unclear. The partial colocalisation of internalised CCR2 observed with Rab4 and transferrin in the present study could represent the potential presence of the small proportion of CCR2B that is recycled in recycling endosomes. However, experiments using over-expression of Rab4 suggested that as for CCR5 (personal communication from Nathalie Signoret, University of York, UK), but unlike for some other receptors, CCR2B recycling is not Rab4-dependent. Based on this information and the disperse punctuate pattern of intracellular HA-CCR2B containing vesicles, it may be more likely that the colocalisation happens in the sorting endosome and that recycling takes place from here via a Rab4-independent pathway (Stenmark, 2009).

The molecular mechanisms responsible for sorting of chemokine receptors into the recycling pathway have not been established in all cases. For CCR5 and CXCR2, the presence of a PDZ ligand binding motif has been suggested to facilitate interactions with proteins of the sorting machinery. However, this motif is not present in CCR2B, again showing the lack of a conserved mechanism.
6.5 Nuclear localisation

Proteomics experiments in the present thesis suggested that CCR2B may interact with importin 7, implying the potential for nuclear localisation of CCR2B. This is in agreement with a previous study showing increased nuclear localisation of CCR2 upon agonist-stimulation, which is reportedly mediated by a related protein TRN-1 (Favre et al., 2008). Nuclear localisation of HA-CCR2B was not directly investigated by colocalisation experiments using nuclear markers in the present thesis. However, it is interesting to note that no localisation of internalised HA-CCR2B at the nucleus, as marked by DAPI staining, was observed in any of the immunofluorescence endocytosis experiments carried out using agonist-stimulated HEK293 transfectants. Although nuclear localisation has been reported for CXCR4 in tumour cells (Wang et al., 2005), it is not currently widely accepted as a general feature of the chemokine receptor response to stimulation.

The number of reports of nuclear localisation of various transmembrane receptors including GPCRs is increasing at a rapid rate (Boivin et al., 2008; Gobeil et al., 2006; Goetzl, 2007; Pickard et al., 2007; Planque, 2006; Wang and Hung, 2012; Wright et al., 2012). However, there is a distinct lack of knowledge of how this nuclear localisation could physically come about and what trafficking pathways and mechanisms are involved. As GPCRs contain seven transmembrane domains, they are unlikely to be transported actually into the nucleus as for soluble cytoplasmic proteins, rather their insertion in the nuclear envelope is the more likely scenario. Although for D-Frizzled 2 it was reported that a C-terminal fragment of the receptor is cleaved off and actually enters the nucleus (Mathew et al., 2005). Insertion of a transmembrane protein into the nuclear envelope is a very different process to the conventional translocation of a soluble cytosolic protein through the nuclear pore complex into the nucleus. Some studies have identified nuclear localisation sequences in the GPCRs (Lee et al., 2004; Wang et al., 2010a; Wright et al., 2012) but so far there has been little focus on the actual relocation and insertion processes.

What triggers the receptor relocation to the nucleus is unknown for some GPCRs, whereas for others the trigger has been suggested to be agonist stimulation (Favre et al., 2008; Mathew et al., 2005; Wang et al., 2009). Therefore it is possible that GPCRs
could traffic to the nucleus from either the biosynthetic pathway or from endocytic vesicles derived from the plasma membrane. In both cases, the first step would be insertion of the GPCR into the outer nuclear membrane. For GPCRs direct from the biosynthetic pathways this step could be simply envisaged to be lateral diffusion through the rough endoplasmic reticulum that is continuous with the outer nuclear membrane. For proteins trafficking from the plasma membrane it could be dependent on fusion of endocytic vesicle membranes with the outer nuclear membrane. The second step involving delivery from the outer to the inner nuclear membrane is more complex. Two different pathways have been proposed for the delivery of integral inner nuclear membrane proteins including those with multiple transmembrane domains, namely passive diffusion through the pore membrane surrounding nuclear pore complexes or utilisation of part of the nuclear pore complex itself (Lusk et al., 2007). Presumably, GPCRs hijack one of these pathways although this remains to be explored.

If nuclear signalling of CCR2 were confirmed to exist, this would offer more opportunities for therapeutically targeting this receptor but at the same time increase the complexity of doing so. The pharmaceutical industry is under pressure from the increasing requirement for the development of new assays to identify and test potential therapeutics in light of the emerging unexpected complexity of chemokine receptor regulation.

6.6 Factors responsible for trafficking

The work presented here, showing that agonist stimulation leads to internalisation of CCR2B followed by both degradation and recycling, raises several questions regarding CCR2B regulation. Firstly, what features, motifs or post-translational modifications of CCR2B are responsible for its utilisation of these intracellular signalling pathways? Secondly, what circumstances or conditions control/modulate which pathway CCR2B enters? Thirdly, is one pathway the default pathway?

Regulation appears to be a complex process that is dependent on a combination of multiple factors. Certain motifs or post-translational modifications of GPCRs have been associated with their intracellular trafficking. Whilst some such as cytoplasmic tail phosphorylation are believed to act universally, it is not yet clear how generic or
receptor specific some others actually are. The sorting step where receptors are sent towards degradative or recycling fates appears to be a key point where the regulatory mechanisms identified so far differ considerably between chemokine receptors. Unlike CCR5, CCR2B recycling does not appear to be dependent on interaction with PDZ containing proteins as no PDZ ligand motif is present in its cytoplasmic tail. Additionally, unlike CXCR4, but similar to CXCR2 and CXCR3, CCR2B recycling does not appear to be dependent on the post-translational ubiquitination modification. The protein-protein interactions governing this important step remain to be identified and based on the array of different protein-chemokine receptor interactions that have been described so far (Table 1.4), these may involve proteins with a currently unknown role in intracellular trafficking.

Several other chemokine receptors have been reported to be both degraded and recycled, with conditions modulating the pathway followed suggested for some only. For CXCR4, the dominant fate is thought to be dependent on the cellular background in which the receptor is expressed. An identical GFP-CXCR4 construct expressed in HeLa, UP37 and CEM cells resulted in quite varied but inefficient levels of recycling after agonist-induced internalisation (Tarasova et al., 1998) and the authors suggested that the main fate of internalised receptors was probably degradation. In contrast, in human hematopoietic CD34+ cells, CXCR4 was found to colocalise with markers of the recycling but not degradative pathways (Zhang et al., 2004). The HA-CCCR2B expressed in HEK293 cells appears to follow both pathways but degradation appears to be the dominant fate. It would be interesting to investigate if the ratio of degradation to recycling varies dependent on cell type as observed for CXCR4 in certain cells (Tarasova et al., 1998), however this would be time consuming with the tools currently available. Alternatively, the fate of CXCR2 has been suggested to be modulated by the duration of agonist stimulation, with short stimulation (up to 1 hour) resulting in recycling and longer stimulation (4 hours) leading to entry into the degradative pathway (Fan et al., 2003). This possibility would be simple to test using the HEK HA-CCR2B cells. As different ligands can determine the efficiency of CCR2B internalisation (Berchiche et al., 2011), the possibility of them also impacting on the downstream sorting process would be worth further investigation.
Many of these factors including cell type-, agonist- and post-translational modification-dependency highlight that how a chemokine receptor is presented to its immediate cellular environment can determine its internalisation and intracellular fate. Chemokine receptor presentation is determined by conformation, modifications, accessibility of motifs and oligomerisation state. As seven transmembrane receptors, chemokine receptors have potential for more conformational states than single transmembrane proteins. In addition, the multiple intra- and extra-cellular loops and N- and C-terminal tails provide many parts of the receptor that can be modified or influenced in some way.

The cellular background in which the receptor is expressed can impact on its conformation due to variability in the local membrane lipid composition or the availability of other membrane proteins that form oligomers with the chemokine receptor. Hetero-oligomerisation can change the behaviour of chemokine receptors, with reported examples of both negative and positive co-operativity for ligand binding, signalling and downstream functions including chemotaxis (Table 1.2). This co-operativity is thought to be mediated through allosteric changes in receptor conformation following ligand binding (Milligan and Smith, 2007; Salanga et al., 2009; Smith and Milligan, 2010).

Many cell surface GPCRs exist in an equilibrium between multiple inactive and active states with the equilibrium being shifted towards the former upon ligand binding and it is likely that the same scenario may exist for chemokine receptors (Thelen et al., 2010). Ligand binding has been shown to differentially influence cell surface chemokine receptor conformation. Indeed two CCR2 ligands, CCL2 and CCL11, have been suggested to induce different active conformations of CCR2 (Ogilvie et al., 2004). It is also believed that chemokine binding can stabilise certain chemokine receptor oligomers (Martinez Munoz et al., 2009). The conformation of a chemokine receptor influences how it is seen by other potentially interacting proteins in the cell.

Some motifs, such as the PDZ ligand motif, are present in multiple chemokine receptors (Marchese et al., 2008) but have only been shown to play a role in the regulation of certain receptors or under certain conditions (Baugher and Richmond, 2008; Delhaye et al., 2007). Other motifs, such as potential tyrosine motifs involved in lysosomal sorting, dileucine motifs, and putative nuclear localisation sequences, are present in multiple
GPCRs but do not always result in the associated action (Marchese et al., 2008; Meiser et al., 2008). In addition, GPCRs carry many putative sites for post-translational modifications, but these are not always utilised (Baugher and Richmond, 2008; Neel et al., 2005). Clearly accessibility of motifs is crucial to their potential roles and receptor conformation and oligmerisation could be envisaged to modulate the accessibility of specific motifs and receptor domains involved in protein-protein interactions that regulate the chemokine receptor.

As degradation appears to be a more dominant fate for CCR2B, it is possible that this is the default pathway for the internalised receptor and that the recycled fraction of CCR2B is modified in an as yet undetermined way that enables it to follow the recycling pathway. The requirement of modification of CXCR4 by ubiquitination for degradation may argue against this suggestion, however there is the emerging possibility of receptor specific regulatory mechanisms.

6.7 Conclusions

In summary, the present thesis explored the regulation, trafficking and fate of CCR2B and identified the two different intracellular routes followed by the receptor in response to agonist treatment. This work and the methods that were developed provide a solid basis to enable further characterisation of the factors regulating the fates of this receptor. The differences identified with the post-endocytic trafficking of CCR5 support the concept of receptor specific behaviours. This idea highlights the difficulty of drawing conclusions about receptor behaviour based on analogy even to highly homologous receptors or based on the presence of primary sequence post-translational modification motifs. In addition to receptor specific behaviours, the differences in CCR2B recycling observed in the two cell types tested suggest that there may be a cell type dependency for certain aspects of the regulation. Added to previously proposed ideas of cell type-dependent modes of internalisation (for CCR2) and recycling (for CXCR4), my findings suggest a greater influence of the cellular environment when investigating chemokine receptors. These new insights into the receptor and cell type dependency of the endocytic regulation of agonist treated CC chemokine receptors show that what was previously thought to be a relatively conserved process is now becoming established as more complex and influenced by a wide range of factors.
The post-endocytic trafficking path followed by chemokine receptors after agonist-stimulated internalisation directly impacts on the type of receptor desensitisation that is observed. Recycling results in transient desensitisation, whereas degradation typically has a long-term negative impact on the level of active receptors at the cell surface. Regulation of receptor availability via these desensitisation processes is important for the fine-tuning of cellular responses and consequently chemokine receptor dysregulation has been implicated in a variety of autoimmune and allergic inflammatory diseases. Thus the ability to modulate the receptor behaviour is a key way of targeting these diseases. A comprehensive understanding of chemokine receptor specific behaviours in the relevant conditions is essential to help facilitate this approach. As has been demonstrated here, even receptors playing complementary biological roles, such as CCR2B and CCR5, can be regulated differently, and so may require different therapeutic strategies.

Drugs designed against chemokine receptors commonly target the activation state of the cell surface receptor or its internalisation. However, these drugs are only acting on a single step of the regulatory process. The existence of two different possible fates for CCR2B provides the potential opportunity for alternative therapeutic targeting of the receptor. If the receptor is typically recycled, inventions that tip the balance in favour of receptor degradation may be a useful way to desensitise CCR2B to prevent overstimulation by agonists. It would be interesting to investigate whether the ligand itself can influence the post-endocytic trafficking of CCR2B. If so, natural ligands may provide a useful basis for design of therapeutics to manipulate receptor trafficking. A precedent for this lies in the previous development of modified forms of CCL5 that appear to block the recycling receptor at different intracellular locations (Bennett et al., 2011). Modified forms of CCL2 are starting to be developed as potential therapeutics (Severin et al., 2012) but there remain several other ligands for CCR2B that could be exploited, possibly with different effects.

It would be important to determine if the potential interaction with importin 7 translates into a nuclear role for CCR2B and if so, what is the involvement of nuclear CCR2B signalling in different diseases? This is a relatively new consideration as until recently all GPCR signalling responses were thought to be principally due to plasma membrane localised receptors. If CCR2B had different functional roles depending on its
localisation, it would be necessary to target the relevant form of the receptor when treating a disease. Investigation into where any nuclearly localised receptors could come from would be essential to decide how best to target them. Drugs designed to target cell surface receptors would not impact effectively on nuclear receptors derived from the biosynthetic pathway but may impact on receptors trafficking from the plasma membrane. This question regarding the origin of nuclear receptors and their subcellular trafficking is currently being addressed for some other types of cell surface receptors including RTKs (Wang and Hung., 2012).

GPCRs, including CCR2B, can undergo various post-translational modifications and it is important to target the relevant form of the receptor for the function of interest. The roles of glycosylation in cell surface expression and agonist-induced degradation of CCR2B, highlight the importance of this modification for chemokine receptor regulation. Some previous studies have focussed on the “normal” molecular weight form of CCR2B (Favre et al., 2008; Minsaas et al., 2010), which appears at least in HEK HA-CCR2B cells to be the immature form of the receptor. My investigations question the degree of relevance of these studies to the regulation of the mature cell surface receptor.

The conclusions drawn for CCR2B regarding the importance of studying disease relevant cell types are also applicable to CCR5 and are likely to extrapolate to other chemokine receptors. THP-1 cells have been widely used to study CCR5 since they were identified as expressing a form of the receptor that facilitates HIV entry (Cassol et al., 2006). There is now a growing appreciation of the fact that chemokine receptors can have multiple varying roles that may be dependent on different signalling pathways. It is thus possible that the same receptor expressed in different cellular environments may only be functional for a subset of these roles. Indeed results presented in this thesis suggest that this appears to be the case for the form of CCR5 expressed on THP-1 cells. This knowledge is of vital importance as it shows that THP-1 cells are not a suitable model for studying the role of CCR5 in diseases involving signalling of this receptor.

Although not focussed directly on drug development, this study made several novel observations regarding the factors influencing chemokine receptor regulation that may impact on future therapeutic development by highlighting a more relevant direction for the focus of the related basic research.
6.8 Future experimental directions

Future experimental work would be required to answer the many questions raised in this thesis. Based on the important involvement of monocytes in many auto-immune and allergic inflammatory diseases, the next logical step would be to determine if the fates observed for CCR2 in the HEK HA-CCR2B and THP-1 cell lines reflect what actually occurs in monocytes. Due to the lack of widespread knowledge of the cell type influence on chemokine receptor trafficking at the time, this avenue of investigation was not initially pursued for CCR2 as the small size of monocytes makes it difficult to effectively employ colocalisation techniques. However, following the identification of the post-endocytic pathways followed by CCR2B presented here, subsequent work could be carried out using the alternative techniques that have been developed in this project.

What precisely is responsible for the choice of fate undergone by CCR2B in a particular situation is currently unknown, although several possibilities have been addressed and/or highlighted during this project. Determining the ratio of recycling to degradation typically experienced by CCR2 in monocytes would enable subsequent investigation of any factors influencing the receptor fate. The first one to examine would be the influence of ligand, both identity and duration of stimulation, and could be directly tested using the experiments designed during this project.

Further dissection of the factors influencing CCR2B fate would require identification of the protein-protein interactions experienced by this receptor using improved approaches as discussed in Chapter 5. This work would be important for identifying if and/or how CCR2B regulation could be targeted via manipulation of individual steps of the intracellular trafficking pathway.

The essential next step to facilitate much of this work would be the development of a better anti-CCR2 antibody suited to various techniques, especially western blotting. This would enable experiments designed and used with HEK HA-CCR2B cells in this project to be extended to the endogenous receptor in work that would have more direct future application.
Appendix

Mechanisms regulating chemokine receptor activity

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Summary

Co-ordinated movement and controlled positioning of leucocytes is key to the development, maintenance and proper functioning of the immune system. Chemokines and their receptors play an essential role in these events by mediating directed cell migration, often referred to as chemotaxis. The chemotactic property of these molecules is also thought to contribute to an array of pathologies where inappropriate recruitment of specific chemokine receptor-expressing leucocytes is observed, including cancer and inflammatory diseases. As a result, chemokine receptors have become major targets for therapeutic intervention, and during the past 15 years much research has been devoted to understanding the regulation of their biological activity. From these studies, processes which govern the availability of functional chemokine receptors at the cell surface have emerged as playing a central role. In this review, we summarize and discuss current knowledge on the molecular mechanisms contributing to the regulation of chemokine receptor surface expression, from gene transcription and protein degradation to post-translational modifications, multimerization, intracellular transport and cross-talk.

Keywords: chemokine receptors; chemokines; regulation; immunity and infection

Chemokine receptor function and regulation

Chemokine receptors belong to the G protein-coupled receptor (GPCR) superfamily and are divided into four classes, named according to the type of chemokine (CC, CXC, CX3C or XC) with which they interact.1 Since the cloning of the interleukin-8 (CXCL8) receptor,2 a total of 10 CC, seven CXC, one CX3C and one XC receptors have been identified.1,3 There is apparent redundancy in the system, as many chemokines bind multiple receptors of one class and more than one receptor can interact with each chemokine. However, some groups have found different receptor signalling and trafficking responses to individual chemokines, suggesting that this redundancy may not be as widespread as thought previously.3,5

Chemokine receptors have a wide range of biological functions and can be grouped as constitutive or inflammatory receptors depending on whether they play a role predominantly in development and homeostasis, or in host response to inflammation and infection.6 They control the trafficking and positioning of leucocytes throughout the body by inducing directed cell movement towards the source of chemokine gradients (chemotaxis). In particular, inflammatory chemokine receptors have a significant role in host defence due to their ability to trigger leucocyte mobilization in response to chemokines secreted at sites of injury. Many chemokine receptors have been associated with various pathologies, including human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS), cancer and inflammatory diseases. However, with the exception of HIV/AIDS, for which it is established that CXCR4 and CCR5 act as co-receptors for virus entry,7–10 the molecular mechanisms by which chemokine receptors contribute to diseases are poorly understood. Work has been carried out in developing drugs targeting at least 10 of the known chemokine receptors. Although antagonists for several receptors are in clinical trials,11–13 the only drug licensed to date is a CCR5 antagonist (Maraviroc) used in HIV therapy.14 As CCR5 antagonism has failed to show clinical benefit with rheumatoid arthritis, it has been suggested that multiple chemokine receptor blockade may be more effective.14,15 Consequently, much effort is currently put towards developing promiscuous antagonists to tackle the problem of redundancy/compensation,12,13 but a greater understanding of the mechanisms regulating chemokine receptor
activity might also be required for the development of more efficient drugs.

The ability of cells to respond to chemokines can be modulated by mechanisms affecting either the chemokine or its receptor. Control can be exerted on the chemokine receptors to modulate the cellular levels of receptor molecules, or the presentation of functionally active receptors at the cell surface. Regulation of protein expression can be targeted at the level of gene regulation, mRNA and protein synthesis. However, these processes are too slow to be solely responsible for the changes required by individual cells to fine-tune their response according to the specific composition of the local environment. Therefore, tight control of the presence of functional chemokine receptors at the cell surface is essential, and can be achieved by affecting the activation state, signalling ability and/or cellular localization of the receptor. This rapid control can be mediated in response to ligand binding but also as a consequence of cross-talk from other receptors.

A considerable amount of our knowledge regarding chemokine receptor biology comes from concepts uncovered for other GPCRs. However, a few chemokine receptors such as CXCR1, CXCR2, CXCR4, CCR2 and CCR5 have received much attention in the last two decades, leading to the discovery that as part of the desensitization process, chemokine-stimulated receptors are removed from the plasma membrane by endocytosis and transported within the cell. Although the trafficking trend appears conserved between chemokine receptors, the mechanisms involved vary and thus cannot be considered generic. Understanding these mechanisms at the molecular and cellular levels could lead to new approaches to target chemokine receptors for disease therapy. In this review we summarize current knowledge about the various molecular mechanisms regulating the presence of functional chemokine receptors at the surface of cells.

Regulation of protein expression

Long-term regulation of chemokine receptors is achieved by controlling the cellular levels of receptor molecules through changes in gene expression, mRNA stability and protein degradation. This can lead to both up- and down-regulation of a specific receptor, as reported for CXCR4. With regard to leukocytes, the expression of chemokine receptors is tightly regulated on the different subtypes and changes through the processes of cell differentiation, activation and polarization. This regulation is particularly important for inducible chemokine receptors such as CCR2 and CCR5 helping to recruit blood neutrophils, monocytes and activated T cells to sites of infection. Host-pathogen interactions can also regulate chemokine receptor expression. For example, it was shown that bacterial lipopolysaccharide (LPS) interfered with CCL2-mediated recruitment of blood neutrophils and monocytes in vivo by down-regulating CCR2 expression. LPS was found to act in vitro by affecting CCR2 mRNA stability, as did the inflammatory cytokines interleukin-1 (IL-1), tumour necrosis factor (TNF-2) and interferon-γ (IFN-γ), but with no major effect on CCR3 transcripts. In contrast, reactive oxygen intermediates produced by phagocytes for killing pathogens increased CCR2, CCR5 and CXCR4 mRNA expression and opposed the down-regulation induced by LPS. Interestingly, chemokine receptor switch and modulation of mRNA expression has also been reported with Mycobacterium tuberculosis antigens and proposed to be part of a normal programme of cell co-ordination needed to contain infection. Enhancing protein degradation independently of, or in combination with, a transcriptional control is also an efficient way to down-regulate chemokine receptor expression, as described for CXCR1 and CXCR2 on activated neutrophils or CCR2 during monocyte differentiation. Significantly, changes in the regulation of chemokine receptor expression can contribute to pathological conditions such as Alzheimer’s disease, where there is evidence for binding of the amyloid β protein to the receptor for advanced glycation end-products (RAGE) up-regulating CCR5 expression on brain endothelial cells causing T cell infiltration in the brain.

Control of chemokine receptor functional activity

To be functionally active, cell surface chemokine receptors have to be coupled to a heterotrimetric G protein, presented in a conformation compatible with agonist binding, and ready to transmit intracellular signals. Other GPCRs are thought to reside in the plasma membrane in equilibrium between active and inactive states, depending on complex allosteric interactions and conformational changes affected by ligands as well as cell-specific parameters. This is still relatively uncharted territory for chemokine receptors but, as will be discussed in detail later, experimental findings suggest that they may be subject to similar regulation. There is evidence for conformational heterogeneity in cell surface CXCR4 and CCR5 receptor populations sometimes related, but not always, to post-translational modifications of the proteins. Indeed, sulphation and glycosylation have both been shown to influence ligand binding and signalling by CXCR4 and CCR5. The membrane environment is another factor influencing the activation state of CXCR4 and CCR5, which require cholesterol and lipid rafts for chemokine binding and signalling. However, if these parameters are important to maintain receptor integrity, whether or not they are accounting for their regulation remains unknown. One feature confirmed to impact on the functional regulation of many chemokine receptors is multimerization.
Table 1. Identified chemokine receptor homomers

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Formation</th>
<th>Methods</th>
<th>Cells</th>
<th>Endogenous</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR2</td>
<td>Constitutive, Inducible</td>
<td>BRET, IP</td>
<td>HEK-293</td>
<td>MM-1</td>
<td>133,134</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HEK-293</td>
<td></td>
<td>48,135</td>
</tr>
<tr>
<td>CCR5</td>
<td>Constitutive, Inducible</td>
<td>IP, Y2H, FLIM, BRET, FRET</td>
<td>HeLa, HEK-293, RBLs</td>
<td>57,76,78,133,136</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HEK-293</td>
<td>L1.2</td>
<td>136–138</td>
</tr>
<tr>
<td>CXCRI</td>
<td>Constitutive</td>
<td>Co-IP, FRET, BRET</td>
<td>HEK-293</td>
<td></td>
<td>56</td>
</tr>
<tr>
<td>CXCRII</td>
<td>Constitutive</td>
<td>IP, FRET, BRET, WB</td>
<td>HEK-293</td>
<td>Neurons</td>
<td>56,139</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Constitutive</td>
<td>IP, FRET, BRET</td>
<td>HEK-293, HEK-teA201</td>
<td></td>
<td>49,65,134,140</td>
</tr>
<tr>
<td></td>
<td>Inducible</td>
<td>IP, FRET, BRET</td>
<td>MOLT4</td>
<td></td>
<td>47</td>
</tr>
<tr>
<td>DARC</td>
<td>Constitutive</td>
<td>BRET</td>
<td>HEK-293</td>
<td></td>
<td>141</td>
</tr>
</tbody>
</table>

BRET: bioluminescence resonance energy transfer; CO-IP: co-immunoprecipitation; DARC: duffy antigen receptor for chemokines; FLIM: fluorescence lifetime imaging; FRET: fluorescence resonance energy transfer; IP: immunoprecipitation; WB: Western blot; Y2H: yeast-2 hybrid.

**Receptor multimerization**

It is now accepted that GPCRs not only operate as single entities (monomers), but can also function as multimers regulated by allosteric mechanisms.36-46 Chemokine receptors have been shown to form homomers as well as heteromers with other chemokine receptors, GPCRs or distinct types of cell surface receptors (Tables 1 and 2). Techniques used to ascertain receptor-receptor interactions and demonstrate the presence of multimers in living cells include co-immunoprecipitation and fluorescence or bioluminescence resonance energy transfer (FRET or BRET; Tables 1 and 2). Note that many of the studies describing chemokine receptor multimers have been carried out on transfected cells where at least one of the interacting partners is over-expressed, and features of endogenous receptor complexes as well as their biological significance in vivo remain largely to be explored.

Early work has indicated that chemokine receptor dimerization was ligand-induced, as described for CXCR4 homodimers and CXCR4/CCR5 or CCR2/CCR5 heterodimers.47-51 However, the current view is that chemokine receptor dimers are constitutively formed (Tables 1 and 2), and ligand binding stabilizes or reorganizes pre-existing complexes.52-54 CXCR1 and CXCR2 exemplify this: a recent study revealed that CXCL8 binding stabilizes homodimers but alters heterodimers.55 In fact, dimers are thought to assemble during biosynthesis prior to arriving at the cell surface, as shown for CXCR1/CXCR2 heterodimers56 or for CCR5 homomers.57 Other factors, such as the type of molecules complexed with the chemokine receptor or the cellular background, could affect where and how dimers form. For example, CXCR4 and the T cell receptor (TCR) only dimerize at the surface of T cells following CXCL12 stimulation,50 while CXCR4 interacts with the tetraspanin CD63 in the biosynthetic pathway of B cells.58-61 For CCR5, there are reports of constitutive intracellular interactions with CD4 in a monocytic cell-line61 and stable cell surface CCR5/CD4 heteromers complexed with or without CXCR4 on transfected cells or blood-derived dendritic cells.62-64 Another study described co-localized but independent monomeric CCR5 and CD4 molecules interacting upon binding of HIV-gp120 at the surface of transfected cells.65-67 Pathogen-induced interaction has also been established for CXCR4 and the Toll-like receptor 2 (TLR-2).68

Importantly, multimerization impacts on the cell’s biological response to chemokine exposure. Cross-talk within homomers or heteromers enables regulation of chemokine receptors in response to stimuli other than their own ligands. This process, called receptor or ligand-binding co-operativity, is known to occur within all types of GPCR dimers.69 Positive binding co-operativity has been shown for the constitutive CXCR4/CXCR7 dimer in which CXCR7, a chemokine receptor unable to trigger G protein signalling,70 enhances CXCR4-mediated signals following CXCL12 stimulation.71 Positive co-operativity has also been described for the CXCR2/α-opioid receptor (DOR) heterodimer, but in that case it is antagonism of CXCR2 that enhances the DOR response to ligand.72 Nevertheless, dimers of chemokine receptors have been shown more often to exhibit negative binding co-oper-
### Table 2. Identified chemokine receptor heteromers and their functional outcomes

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Formation</th>
<th>Methods</th>
<th>Cells</th>
<th>Endogenous</th>
<th>Co-operativity (assays)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemokine receptors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCR1/CXCR2</td>
<td>Constitutive</td>
<td>Co-IP, FRET</td>
<td>HEK-293</td>
<td>No</td>
<td></td>
<td>55,56</td>
</tr>
<tr>
<td>CXCR4/CXCR7</td>
<td>Constitutive</td>
<td>Co-IP, FRET</td>
<td>HEK-293</td>
<td>IM-9</td>
<td>Positive (Ca²⁺ flux)</td>
<td>71</td>
</tr>
<tr>
<td>CXCR4/CCR2</td>
<td>Constitutive</td>
<td>BRET</td>
<td>CHO-K1 HEK-293</td>
<td>Negative (binding, chemotaxis)</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>CXCR4/CCR3</td>
<td>Constitutive</td>
<td>Co-IP</td>
<td>NIH 3T3</td>
<td>Positive (chemotaxis)</td>
<td>63,130</td>
<td></td>
</tr>
<tr>
<td>CXCR4/CCR5/CCR3</td>
<td>Constitutive</td>
<td>BRET</td>
<td>HEK-293</td>
<td>Negative (binding, chemotaxis)</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>CCR2/CCR5</td>
<td>Inducible</td>
<td>Co-IP</td>
<td>HEK-293</td>
<td>Positive (Ca²⁺ flux)</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Constitutive</td>
<td>Co-IP, BRET</td>
<td>CHO-K1 HEK-293</td>
<td>CD4⁺ T cells</td>
<td>Negative (binding)</td>
<td>133</td>
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<tr>
<td>DARC/CCR5</td>
<td>Constitutive</td>
<td>Co-IP, BRET</td>
<td>HEK-293</td>
<td>Negative (chemotaxis)</td>
<td>141</td>
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<td>GPCRs</td>
<td></td>
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<tr>
<td>CCR5/C5αR</td>
<td>Constitutive</td>
<td>Co-IP, BRET</td>
<td>RBLs HEK-293</td>
<td>Negative (co-internalization)</td>
<td>76</td>
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<tr>
<td>CXCR2/DOP</td>
<td>Constitutive</td>
<td>Co-IP, FRET</td>
<td>HEK-293</td>
<td>Positive (G protein activation)</td>
<td>72</td>
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<tr>
<td>CXCR4/DOP</td>
<td>Constitutive</td>
<td>Co-IP, FRET</td>
<td>HEK-293</td>
<td>Negative (chemotaxis, adhesion, Ca²⁺ flux)</td>
<td>142</td>
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</tr>
<tr>
<td>CCR3/opioid receptors</td>
<td>Constitutive</td>
<td>Co-IP</td>
<td>CHO CEMx174</td>
<td>Negative (chemotaxis)</td>
<td>132,143</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>CXCR2/AMPA GluR1</td>
<td>Constitutive</td>
<td>Co-IP</td>
<td>HEK-293</td>
<td>Neurons</td>
<td>Negative (chemotaxis)</td>
<td>144</td>
</tr>
<tr>
<td>CXCR4/CD4</td>
<td>Inducible (HIV)</td>
<td>Co-IP</td>
<td>Jurkat T</td>
<td>PBMCs</td>
<td>N.D.</td>
<td>145,146</td>
</tr>
<tr>
<td>CXCR4/TCR</td>
<td>Inducible</td>
<td>Co-IP, FRET</td>
<td>PBMCs</td>
<td>Positive (Ca²⁺ flux)</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>CXCR4/IGF-I R1</td>
<td>Constitutive</td>
<td>Co-IP</td>
<td>MCF-7</td>
<td>MDA-MB-231</td>
<td>Positive (chemotaxis)</td>
<td>147</td>
</tr>
<tr>
<td>CXCR4/CD63/CCR5/CD4</td>
<td>Inducible</td>
<td>Co-IP, FRET</td>
<td>HEK-293</td>
<td>N.D.</td>
<td>60</td>
<td></td>
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<tr>
<td></td>
<td>Inducible</td>
<td>BRET, Co-IP</td>
<td>CHO K1 HEK-293</td>
<td>N.D.</td>
<td>61,64,148</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inducible (HIV)</td>
<td>FRET</td>
<td>HEK-293</td>
<td>DCs</td>
<td>N.D.</td>
<td>66</td>
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</table>

AMPA GluR1: a-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid glutamate receptor 1; BRET: bioluminescence resonance energy transfer; C5αR: complement component 5α receptor; Co-IP: co-immunoprecipitation; DARC: duffy antigen receptor for chemokines; DCs: dendritic cells; DOP: δ-opioid receptor; FRET: fluorescence resonance energy transfer; IGF-R1: insulin-like growth factor-1 receptor; N.D.: not determined; PBMCs: peripheral blood mononuclear cells.

Activity, where binding of an agonist to one receptor inhibits ligand binding to the other. Antagonist binding to one chemokine receptor has also been shown to cross-inhibit the other chemokine receptor in the pair, both in vitro and in vivo. Although a few publications have shown that binding co-operativity within a dimer can involve co-internalization of receptors, it is not considered to be the rule. As for other GPCRs, it is thought that both negative and positive co-operativity are mediated through allosteric changes in receptor conformation following ligand binding. A 'cigar bundle' model has been proposed recently for chemokine receptors.
Chemokine receptor desensitization

Chemokine receptors are coupled to heterotrimeric G proteins and undergo conformational changes following ligand binding. The G protein dissociates into guanosine triphosphate (GTP)-bound Gα and the Gβγ complex, which activate second messengers and stimulate effector proteins leading to intracellular signalling. It has emerged that GPCRs can also elicit G protein-independent signals through interaction with the scaffolding proteins β-arrestins, linking activated receptors to various signalling pathways that act independently of, in synergy with, or in opposition to, G protein-mediated signals. However, β-arrestins are best known for their pivotal role in the regulation of GPCR signals via the process of desensitization, a feedback mechanism protecting cells from overstimulation. In this section we consider what is called homologous desensitization, only affecting agonist-activated receptors (Fig. 1). Briefly, following agonist binding, signalling receptors become rapidly phosphorylated on their cytoplasmic tail, usually by one member of the G protein receptor kinase (GRK) family, which uncouples the G protein from the receptor and prevents further activation. Phosphorylated receptors interact with one of the β-arrestins acting as a scaffold targeting receptors for internalization, leading to a permanent or transient loss of cell surface receptors due to degradation or subsequent recycling of internalized molecules, respectively. The ability of a chemokine receptor to interact with β-arrestins can influence its fate in multiple ways. First, the strength and stability of receptor/β-arrestin interactions seem critical in determining whether or not an agonist-activated chemokine receptor is internalized, as described for CCR5. Secondly, the affinity of these interactions can influence the destiny of receptors once internalized. Indeed, GPCRs that rapidly recycle (Class A) preferentially bind β-arrestin 2 with low affinity and dissociate from it upon internalization, whereas those that slowly recycle or are degraded (Class B) bind both β-arrestins with high affinity and remain β-arrestin-bound inside the cell. To date, only class B chemokine receptors have been described, with evidence for β-arrestins binding to agonist-treated CXCRR4, CCR2 and CCR5 in internal compartments (Fig. 2).

Chemokine receptors can be internalized via clathrin- or caveolin-dependent endocytosis, although other independent pathways have also been reported. Interestingly, CCR2 and CCR5 have been shown to follow both clathrin-dependent and caveolin-mediated pathways and the route of endocytosis could be cell-type dependent. The intracellular path followed by a chemokine receptor determines the fate of this receptor, i.e., being sent for degradation (down-regulation) or being sequestered intracellularly before returning to the cell surface (resensitization). Receptors can follow one path exclusively, such as.

Figure 1. Agonist-dependent (a) and independent (b, heterologous) chemokine receptor desensitization. (a) Following agonist binding and G protein mediated signalling, the chemokine receptor cytoplasmic tail is rapidly phosphorylated, usually by a G protein receptor kinase (GRK); this uncouples the G protein, which dissociates into guanosine triphosphate (GTP)-bound Gα and the Gβγ complex, and enables interaction with a β-arrestin, which acts as a scaffold targeting the receptor for internalization. Once internalized, the receptor follows recycling or degradation pathways. (b) Receptor X mediates cross-phosphorylation of the chemokine receptor, which may involve protein kinase C (PKC), leading to inhibition of chemokine-induced signalling and in some cases internalization of the receptor.
Figure 2. Intracellular transport of β-arrestin-bound CCR5 receptors following CCL5-treatment. Isolated human blood monocytes were treated with 100 nM CCL5 for the indicated time-period. Cells were fixed and permeabilized before labelling for CCR5 (red) and β-arrestins (green), as described previously. Scale bar 5 μm.

CCR5 or CXCR3 sent for recycling or degradation, respectively. Alternatively, they can enter either pathway depending on the cell-type and duration of ligand treatment, as reported for CXCR2 and CXCR4. Note that the agonist itself can impact upon the fate of a receptor. For instance, with CCR5, any agonist-stimulated receptors seem to follow the recycling route but the distribution of receptors along the pathway could be agonist-specific (Fig. 3). Following internalization, CCR5 receptors treated with the natural chemokine CCL5 regulated upon activation normal T cell expressed and secreted (RANTES) are located in recycling endosomes (RE) before re-accumulating in the plasma membrane. In contrast, they keep cycling back from the cell surface to the RE after exposure to the chemically modified amino-oxepane (AOP)-RANTES become trapped in the trans-Golgi network (TGN) after passage through RE with Nα-(n-nanonoyl)-des-Ser1-[γ-thioprolin2, 1-z-cyclohexyl-glycine3] PSC-RANTES and appear to bypass the RE to accumulate in the TGN with methionine MET-RANTES.

Sorting of internalized chemokine receptors to the recycling or degradative pathways requires complex interactions with the machinery mediating movement of molecules between intracellular compartments. Endocytic adaptors recognize specific determinants in the cytoplasmic domains of the receptors, mainly small sorting motifs and post-translational modifications. Two of these determinants, the PDZ ligand motif and ubiquitination, have received much interest recently, and were shown to support recycling or degradation of chemokine receptors, respectively. At least 12 chemokine receptors have been identified as containing potential PDZ ligand motifs in their extreme C-terminal cytoplasmic tail. The PDZ ligand motifs are presumed to interact with PDZ domain containing proteins of the sorting machinery, but only a few of these interactions have been unveiled. CCR5 post-endocytic sorting to the recycling pathway is dependent on its PDZ ligand motif, which has been shown to interact with a protein implicated in receptor recycling called EBP50/NHERF-1. For CXCR2 that can be both recycled following short ligand exposure and degraded following more prolonged ligand treatment, the PDZ ligand motif serves to delay degradation by preventing lysosomal sorting, due probably to interaction with an as yet unknown PDZ-containing protein. Ubiquitination has emerged as an important modification for sending the chemokine receptor CXCR4 and other GPCRs to degradation. For CXCR4, CXCL12 stimulation leads to ubiquitination of cell surface receptors as well as ubiquitin-dependent endocytosis and trafficking of ubiquitinated CXCR4 to lysosomes. However, ubiquitination does not seem to be required for the degradation of all chemokine receptors.

Cross-talk and heterologous regulation

In addition to co-operativity within chemokine receptor multimers, various examples for regulation by indirect
cross-talk with other receptors, without evidence of physical interactions but occurring through interconnectivity of cellular signalling networks, have been described.\(^{55}\) Note that such regulation can be bidirectional, although here we consider only cases of cross-talk towards chemokine receptors. The cross-talk can be targeted at the receptor itself, the heterotrimeric G protein it is coupled to or downstream signalling components, resulting in trans-inhibition or activation of chemokine receptor activity.

Trans-inhibition results from a negative pathway of cross-talk leading to desensitization of chemokine receptors or the down-regulation of their expression, as discussed in an earlier section. Here we are considering agonist-independent (heterologous) desensitization involving inactivation and/or down-modulation of cell surface chemokine receptors. As for the other mechanisms of regulation presented in this review, the pathways of heterologous desensitization are undoubtedly receptor- and cell-type dependent. Heterologous desensitization often implies rapid signalling inactivation of surface chemokine receptors, inhibiting chemokine-induced intracellular calcium mobilization. It happens whether the cross-talk comes from another chemokine receptor such as for CXCR1 and CXCR2 with CCR5 in transfected cells,\(^{109}\) or CXCR4 with CCR5 in human pre-B and T cells,\(^{111,112}\) another GPCR as for CXCR1 with the N-formyl peptide (FFP) and C5a receptors,\(^{113}\) or an unrelated surface receptor such as the TcR with CXCR4 in immortalized cell lines.\(^{114}\) In many reports, the inactivation has been linked to rapid cross-phosphorylation of the chemokine receptor, with some studies identifying protein kinase C (PKC) as the point of convergence between the different receptor pathways.\(^{110,113,115,116}\) Alternatively, receptor inactivation can result from indirect effects as reported for CXCR4 either in pre-B cells, where CD24 altered its distribution in membrane lipid rafts by changing cholesterol levels,\(^{117}\) or in leukaemia cells, where an oncoprotein has been shown to highjack kinases of the CXCR4-dependent calcium pathway.\(^{118}\) Signalling inactivation can be, but is not always, followed by the down-regulation of cell-surface chemokine receptors.\(^{116,119,120}\)

Conversely, heterologous down-modulation can occur without prior desensitization of chemokine-mediated signalling, as we uncovered with the cross-regulation of CC chemokine receptors 1, 2 and 5 by TLR-2 on human blood monocytes.\(^{86}\) In this instance, we found that activation of TLR-2 triggered relatively slow phosphorylation and removal of cell-surface CCR5 molecules by activating the machinery used to support chemokine-dependent endocytosis.\(^{86}\)

Cross-talk can also lead to trans-activation of chemokine receptors and a potentiation of their functional activity, but few studies have been able to identify the mechanisms involved.\(^{53}\) Potentiation of calcium signalling has been reported for CXCR2 upon co-stimulation of another GPCR, the PY2 nucleotide receptor, and suggested ligand-induced synergy between the two receptors.\(^{121}\) Activation of the neurokinin 1 receptor has also been shown to potentiate the effect of CXCL8 on human neutrophils and was proposed to have a priming effect on CXCR1 and CXCR2.\(^{122}\) The chemokinetic effect of cytokines is thought to prime cells to increase their migratory response to chemokines, as found with IL-5-enhancing eosinophil chemotaxis in response to CCL11.\(^{123}\) Furthermore, potentiation and synergy between different chemokine receptors has been involved in the migration of primary cells. For example, CXCL8 has been shown to increase monocyte migration towards CCL2 and CCL7,\(^{124}\) while CCL2 and CCL7 can stimulate neutrophil chemotaxis towards CXCL8.\(^{125}\) An intriguing finding came from the study of cross-talk between CCR1 and the high-affinity IgE receptor FcεRI in transfected cells, whereby engagement of FcεRI inhibited CCL3-mediated chemotaxis but engagement of both CCR1 and FcεRI had a synergistic effect on cell degranulation.\(^{126}\) This would suggest that receptor cross-talk can take place at multiple levels and could have a relatively complex bearing on cell response to chemokine stimulation.

The impact of receptor cross-talk on how immune cells adapt their behaviour to specific situations is undeniable. Combinations of chemokines, cytokines and growth factors act synergistically to amplify inflammatory responses, and this is thought to be due to integration of multiple signalling pathways.\(^{123,124}\) Cross-talk initiated from non-chemokine receptors is also emerging as an important and complex phenomenon used to enhance or modulate innate immune responses to pathogens. Synergy between CCR2 and FPR agonists has recently been shown to co-operate with TLR-4 for production of the inflammatory chemokine CXCL8 upon LPS stimulation, which in turn synergizes with CCL2 to mediate CXCR1/ CXCR2-dependent chemotaxis of human monocytic cells.\(^{127}\) In addition, heterologous desensitization between TLR-2 and the CC chemokine receptors 1, 2 and 5 or CXCR2 has been shown to take place in vivo, affecting the migration and homing of mouse monocytes and neutrophils.\(^{128,129}\) Furthermore, synergy and cross-talk may have therapeutic implications, as illustrated with some HIV-related studies. Synergy between CXCR4 and CCR5 was recently shown to enhance human monocyte and T cell chemotaxis and to completely block infection by a dual tropic HIV-1 strain,\(^{130}\) while cross-desensitization of CCR5 by the opioid receptor specifically decreased the susceptibility of peripheral blood mononuclear cells (PBMCs) and macrophages to HIV-1 R5 viruses.\(^{131}\) However, it remains to be ascertained whether these are pure cross-talk situations or involve receptor multimerization.\(^{63,132}\)
Conclusion

Advances in our understanding of chemokine receptor biology have highlighted the fact that a controlled regulation of their activity is probably more important than their activation per se, certainly in the context of the immune system for both homeostasis and inflammatory responses. It is becoming apparent that individual receptors are subject to different mechanisms of regulation depending upon the type of cells on which they are expressed, the cell differentiation and activation status, as well as the microenvironment. We have learnt that some of the molecular mechanisms involved in the regulation are shared among chemokine receptors while others are purely receptor-specific, with either transient or permanent consequences on cell responsiveness to chemokine stimulation. Overall, we can conclude that the complexity of the regulation process confers specificity to what is an apparently redundant chemokine/chemokine receptor system. Nevertheless, much more research is needed to appreciate the ins and outs of this regulation, evaluate the true relevance of individual mechanisms in vivo and establish how the chemokine system integrates with the rest of the immunoregulatory machinery.

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We thank Professor Paul Kaye from the CII for constructive discussions and critically reading the manuscript. The Signoret laboratory was supported by grants from the Biotechnology and Biological Sciences Research Council and The Royal Society (London, UK). L.D.B. is the recipient of a BBSRC studentship.

Disclosures

The authors declare having no conflicts of interest.

References


## Definitions

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<thead>
<tr>
<th>Symbol</th>
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<td>µg</td>
<td>Microgram</td>
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<tr>
<td>AC</td>
<td>Adenylate cyclase</td>
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<td>AMPA GluR1</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid Glutamate receptor 1</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>Aminooxypentane-regulated on activation, normal T cell expressed and secreted</td>
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<td>Binding immunoglobulin protein</td>
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<td>BRET</td>
<td>Bioluminescence resonance energy transfer</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>C5aR</td>
<td>Complement 5 a receptor</td>
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<td>Clathrin coated pit</td>
</tr>
<tr>
<td>CCV</td>
<td>Clathrin coated vesicle</td>
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<tr>
<td>CD4</td>
<td>Cluster of differentiation 4</td>
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<td>cDNA</td>
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<td>CREB</td>
<td>cAMP response element-binding</td>
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<td>DAPI</td>
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<td>Dulbecco's Modified Eagle Medium</td>
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<td>Deutsche Sammlung von mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures)</td>
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<td>ERC</td>
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<td>Extracellular signal-regulated kinase</td>
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<td>FACS</td>
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<tr>
<td>g</td>
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<td>Laser scanning microscopy</td>
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<td>LY</td>
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<td>MCAF</td>
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<td>mRNA</td>
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<td>MS</td>
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<td>MVB</td>
<td>Multivesicular body</td>
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<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<tr>
<td>NHERF-1</td>
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<td>NK</td>
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<td>NLS</td>
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<td>nm</td>
<td>Nanometre</td>
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<tr>
<td>ns</td>
<td>Non significant</td>
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<tr>
<td>PBMCs</td>
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<tr>
<td>PBS</td>
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<td>PE</td>
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<tr>
<td>PFA</td>
<td>Para-formaldehyde</td>
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<td>PSC-RANTES</td>
<td>N(^\alpha)-(n-nonanoyl)-des-Ser(^1)-[1-thioproline(^2),1-(\alpha)-cyclohexylglycine(^3)] RANTES</td>
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<td>PY2R</td>
<td>Bis (2-ethylpyridine) amine receptor</td>
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<td>RANTES</td>
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<td>Sorting endosome</td>
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<td>SILAC</td>
<td>Stable isotopes labelling by amino acids in cell culture</td>
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<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
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<td>Trans Golgi network</td>
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<tr>
<td>TIR</td>
<td>Toll-interleukin 1 receptor</td>
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<td>TIRAP</td>
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<td>Yeast two hybrid</td>
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<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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AtT20, BV2, and N18 cell lines as revealed by microarray analysis. BMC Genomics 12, 14.


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