

# Design of modulatory peptides against chemokines



Benoît Darlot

Lincoln College  
University of Oxford

Co-Supervised by  
Professor Akane Kawamura  
Professor Shoumo Bhattacharya  
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# Declaration

I declare that this thesis is entirely my own work except figures:

**Figure 2.2** P672/CCL8 interface characterised using HDxMS – Data obtained by Dr. James Eaton and Dr. Lucia Geis-Asteggianti

**Figure 2.3** Chimeric Evasin-1 design and analysis – Data obtained by Dr. James Eaton

**Figure 2.4** Biophysical analysis of BK1.1 binding to CCL8 – Data obtained by Dr. James Eaton

**Figure 2.5** FP assays confirming BK1.1 multiple chemokine binding property – Data obtained by Dr. James Eaton

**Figure 2.14** Cell based dose-response experiments assessing BK1.3 potency – data from panel **C** and **D** obtained by Dr. James Eaton in an assay developed by Mr. Graham Davies

**Figure 2.15** *In vivo* local anti-inflammatory activity of BK1.3 – Data obtained by Dr. Gopala Yakala and Dr. Kalimuthu Karuppanan

**Figure 2.16** *In vivo* distal anti-inflammatory activity of BK1.3 – Data obtained by Dr. Gopala Yakala and Dr. Kalimuthu Karuppanan

**Figure 3.4** Cell based dose-response experiments assessing BK1.2 potency – data from panel **C** and **D** obtained by Dr. James Eaton in an assay developed by Mr. Graham Davies

I confirm that appropriate credit has been given within the thesis where reference has been made to the work of others.

# Dedication

This thesis is dedicated to my grandparents who unfortunately will not have the opportunity to read it. Liliane and Henri Champion as well as Jeanne and Robert Darlot taught me perseverance and dedication, qualities without which I would not have been able to conduct this work. In particular, Jeanne and Robert left us in the last year of my Doctorate, living abroad for my studies, I have not had the chance to spend much time with them or be present for Robert's funeral. I profoundly hope that the dedication, time and effort I put into this work will make them proud. They will all forever be remembered. I deposit their name here, so their memory survive time, close to a part of myself in the archives of the Bodleian Library.

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Firstly, I would like to thank Professor Akane Kawamura and Professor Shoumo Bhattacharya for their kind supervision for the last three years and a half. Their guidance, support and advice was invaluable and made my DPhil experience one I will always remember and cherish.

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Finally, I would also like thank my family, back in France, for their unwavering encouragements along those years. It seems very distant, but when I decided, young, that I wanted to become a chemist, they were the one, sometime authoritatively, sometime supportively, to push me to achieve my ambitions. Through a pandemic, distant, their trust in my success and their support helped me continue to go forward in a world frozen still.

# Abstract

Chemotactic cytokines or chemokines are small signalling proteins responsible for the recruitment of leukocytes to sites of inflammation. Although, chemokines are critical to orchestrate effective immune response, many have been associated to several inflammatory disorders. Despite being recognised as therapeutic target, no efficient treatments targeting the chemokine network for inflammatory diseases are available to date. This is attributed with the complexity of the many and varied chemokine interactions with themselves, their receptors and glycosaminoglycans on extracellular matrix. Thus, the pharmaceutical industry dogma of “one molecule, one target” is rendered ineffective against this network. Evasins are small tick saliva proteins that can bind and neutralise several chemokines, therefore allowing the tick to feed on their host for days without sparking an immune response. The potential of Evasins as effective anti-inflammatory agents was proven in many pre-clinical disease models. The interest in Evasins led to the discovery of a novel Evasin, P672, that can bind a wide range of CC chemokines. This work describes the discovery of peptides inspired by the chemokine binding site of P672. These peptides were shown to neutralise several chemokines and exhibited *in vivo* anti-inflammatory activity. A novel generation of P672-inspired peptides were designed and exhibited multiple chemokine-binding properties with increased human plasma resistance. Finally, P672/chemokine interfaces were mapped using peptide display techniques informing on P672 binding sites and its mode of action to neutralise chemokines. This work demonstrates the exciting potential of utilising Evasin/chemokine interaction studies with informed mode of action as starting points for anti-inflammatory peptide development.

# Publication

The results from Chapter 2 and Chapter 3 are the basis of this publication:

**B. Darlot**, J. R. O. Eaton, L. Geis-Asteggianti, G. K. Yakala, K. Karuppanan, G. Davies, C. V. Robinson, A. Kawamura, S. Bhattacharya. “Engineered anti-inflammatory peptides inspired by mapping an Evasin–chemokine interaction”, *J. Biol. Chem.* **2020**, 295, 10926 – 10939.

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# List of abbreviations

|             |                                                          |
|-------------|----------------------------------------------------------|
| ACKR        | atypical chemokine receptor                              |
| ADME        | absorption distribution metabolism and excretion         |
| AIDS        | acquired immune deficiency syndrome                      |
| AlphaScreen | amplified luminescent proximity homogeneous assay screen |
| AS          | AlphaScreen                                              |
| ATP         | adenosine triphosphate                                   |
| BLI         | biolayer interferometry                                  |
| BSA         | bovine serum albumin                                     |
| cAMP        | cyclic adenosine monophosphate                           |
| CCRL1       | CC chemokine receptor like 1                             |
| cDNA        | cyclic or complementary deoxyribonucleic acid            |
| CKBP        | chemokine binding protein                                |
| CKR         | chemokine receptor                                       |
| CRS         | chemokine receptor site                                  |
| cryo-EM     | cryogenic electron microscopy                            |
| DAMP        | damage associated molecular pattern                      |
| DARC        | Duffy antigen receptor for chemokine                     |
| DCM         | dichloromethane                                          |
| DIC         | N,N'-diisopropylcarbodiimide                             |
| DIPEA       | N,N-Diisopropylethylamine                                |
| DMF         | dimethylformamide                                        |
| DMSO        | dimethyl sulfoxide                                       |
| DNA         | deoxyribonucleic acid                                    |
| DNase       | desoxyribonuclease                                       |
| DTT         | dithiothreitol                                           |
| ELISA       | enzyme-linked immunosorbent assay                        |
| FACS        | fluorescence-activated cell sorting                      |
| FBS         | foetal bovine serum                                      |
| FDA         | Food and Drug Administration                             |

|        |                                                    |
|--------|----------------------------------------------------|
| FITC   | fluorescein isothiocyanate                         |
| fMLP   | <i>N</i> -formylmethionine-leucyl-phenylalanine    |
| Fmoc   | fluorenylmethoxycarbonyl protecting group          |
| FP     | fluorescence polarisation                          |
| GAG    | glycosaminoglycan                                  |
| GPCR   | G protein-coupled receptor                         |
| HDxMS  | hydrogen-Deuterium exchange mass spectrometry      |
| HEK293 | Human embryonic kidney 293 cell                    |
| HEPES  | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HIV    | human immunodeficiency virus                       |
| HpARI  | <i>H. polygyrus</i> Alarmin Release Inhibitor      |
| HPLC   | high-performance liquid chromatography             |
| HRP    | horseradish peroxidase                             |
| ICAM   | intercellular adhesion molecule                    |
| IgG    | immunoglobulin G                                   |
| IL     | interleukin                                        |
| IMAC   | immobilised metal affinity chromatography          |
| IPTG   | isopropyl $\beta$ -d-1-thiogalactopyranoside       |
| ITC    | isothermal titration calorimetry                   |
| IVTT   | in vitro transcription translation                 |
| JAM    | junctional adhesion molecule                       |
| LCMS   | liquid chromatography coupled to mass spectrometry |
| LDL    | low-density lipoprotein                            |
| LFA-1  | lymphocyte function-associated antigen-1           |
| Mac-1  | macrophage-1 antigen                               |
| MALDI  | matrix-assisted laser desorption/ionization        |
| mRNA   | messenger ribonucleic acid                         |
| MS     | mass spectrometry                                  |
| MS/MS  | mass spectrometry coupled to mass spectrometry     |
| NET    | neutrophil extracellular traps                     |
| NGS    | next-generation sequencing                         |

|                  |                                                                 |
|------------------|-----------------------------------------------------------------|
| NK               | natural killer cell                                             |
| NLR              | Nodd-like receptor                                              |
| NMI              | N-methylimidazole                                               |
| NMP              | N-methylpyrrolidone                                             |
| NMR              | nuclear magnetic resonance                                      |
| nP               | neopentyl                                                       |
| PAMP             | pathogen associated molecular pattern                           |
| PBS              | phosphate buffer saline                                         |
| PECAM            | platelet endothelial cell adhesion molecule                     |
| PPI              | protein-protein interaction                                     |
| PCR              | polymerase chain reaction                                       |
| qPCR             | quantitative polymerase chain reaction                          |
| ROS              | reactive oxygen species                                         |
| RT               | room temperature                                                |
| SAR              | structure-activity relationship                                 |
| SDS-PAGE         | sodium dodecyl sulfate polyacrylamide-based gel electrophoresis |
| SEM              | standard error from the mean                                    |
| SGE              | salivary gland extract                                          |
| SPR              | surface plasmon resonance                                       |
| sY               | sulfotyrosine                                                   |
| TAA              | tumour associated antigen                                       |
| TAM              | tumour associated macrophage                                    |
| TFA              | trifluoroacetic acid                                            |
| T <sub>H</sub> 1 | T helper cell type 1                                            |
| TIPS             | triisopropyl silane                                             |
| TLR              | Toll-like receptor                                              |
| TNF              | tumour necrosis factor                                          |
| TOF              | time of flight                                                  |
| tRNA             | transfer ribonucleic acid                                       |
| TSP              | trimethylsilylpropanoic acid                                    |
| VLA4             | very late antigen-4                                             |

# COVID-19 pandemic impact

In October 2019, our lab moved to Newcastle University, and I transferred there as a visiting student. Unfortunately, there was significant delays in the lab refurbishment at Newcastle University, and it was late February 2020 when I was able to access any lab facilities. I started back in the labs following the completion of the studies described in the manuscript<sup>1</sup> and its submission.

After the move, I had planned to develop the work presented here in Chapter 4, involving not only screening and data analysis but also peptide synthesis and hit validation using the AlphaScreen assay I optimised in our lab. However, on the 18<sup>th</sup> of March 2020, all facilities were shut down at Newcastle University, ahead of the national lockdown, announced few days later. This prevented me from producing any experimental work until a phased reopening of laboratories in mid-June 2020. During this period, I focussed on the writing of the introductory Chapter 1.

Return in the lab was phased and involved part-time working, slowing down my progress. This was the basis of my funded two terms extension, which allowed me to produce enough results to present in Chapter 4.

Although I am confident about the body of experimental work presented in this thesis, I would like to take this occasion to stress the impact the SARS-COV-2 virus pandemic had on the amount of experimental data presented but also its toll on my mental wellbeing during the last year of my Doctorate studies.

I am humbled to present this work, although the current pandemic had an impact on it, it taught me to be resilient and to adapt under pressure. I hope it will still be appreciated and fairly examined.

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# Chapter 1 Introduction

## 1.1 Inflammation in the context of disease

Derived from the Latin *inflammare*, literally meaning “to set on fire”, inflammation is usually seen as a detrimental process associated with pathogenicity. Aulus Cornelius Celsus (29 B.C. - 37 A.C.), Roman encyclopaedist, was the first one to describe inflammation in his only surviving piece, *De Medicina* in book III: “*Notae vero inflammationis sunt quatuor: rubor et tumour cum calore et dolore*” (now the inflammation signs are four: redness, swelling, heat and pain).<sup>2</sup> Rudolf Virchow (1821 - 1902), German pathologist, centuries later, added “*functio laesa*” (function loss) to the list of inflammation markers.<sup>3</sup> To date, this list of markers are still used by clinicians to diagnose inflammation, but since Celsus and Virchow, the field of immunobiology has expanded. Inflammation is now acknowledged as an exceedingly complex but equally fascinating process.

### 1.1.1 An overview of the inflammation process

Inflammation has been extensively studied in the context of infection but it is also involved in several other pathological and physiological processes.<sup>4</sup> It consists of the recruitment of blood elements (plasma proteins and leukocytes) to the site of infection or tissue injury.<sup>5,6</sup> In a typical inflammatory response, resident leukocytes mainly sense pathogens and host cellular damage through the recognition of molecular patterns; either Pathogen-Associated Molecular Patterns (PAMPs) or Damage-Associated Molecular Patterns (DAMPs)<sup>7</sup> which are recognised through molecular pattern receptors such as Toll-Like Receptors (TLRs)<sup>8</sup> and NOD-Like Receptors (NLRs).<sup>9</sup> PAMPs include: pathogen membrane components like lipopolysaccharides, pathogen proteins like flagellin or foreign

genetic material like non-methylated DNA. DAMPs can be much more varied including free chromatin, ATP, and uric acid crystals for example.<sup>8,9</sup>

Once the initial recognition is achieved, the resident leukocytes secrete a large variety of inflammatory mediators (Table 1.1) including cytokines, vasoactive amines, products of proteolytic cascades, eicosanoids, and chemokines (which are the focus of this work) (Figure 1.1).<sup>5</sup> The initial effect is to activate the endothelium of the neighbouring vasculature in order to increase its permeability, thus leading to a local inflammatory exudate containing plasma protein (mostly opsonins like IgG, component proteins and mannose lectin to amplify the response against the homeostasis threat).<sup>10</sup> Additionally, through selectins/integrins and chemokine/chemokine receptor interactions, the activated endothelium permits the selective recruitment of neutrophils in the exudate, keeping the erythrocytes in the blood circulation (Figure 1.1).<sup>10,11</sup> These specific interactions and the mechanism of leukocyte migration will be discussed more extensively later in this chapter.

When neutrophils reach the afflicted tissue, they react either with the pathogen itself or with cytokines secreted by neighbouring cells and then activate to attack the invading agent (Figure 1.1). To do so, they have three distinct mechanisms: phagocytosis, Neutrophil Extracellular Traps (NETs) or degranulation.<sup>11</sup> Whilst the two former mechanisms are targeted towards the pathogens or damaged cells, the latter is less selective. The release from granules of agents like proteinase 3, elastase, cathepsin G, reactive oxygen species (ROS) and reactive nitrogen species leads to unavoidable collateral damage to host tissue.<sup>12,13</sup>

| MEDIATOR                       | SOURCE                                         | ACTIONS                                                                                                |
|--------------------------------|------------------------------------------------|--------------------------------------------------------------------------------------------------------|
| Histamine                      | Mast cells<br>Basophils<br>Platelets           | Vasodilatation<br>Increased vascular permeability<br>Endothelial activation                            |
| Prostaglandins                 | Mast cells<br>Leukocytes                       | Vasodilatation<br>Pain<br>Fever                                                                        |
| Leukotrienes                   | Mast cells<br>Leukocytes                       | Increased vascular permeability<br>Chemotaxis<br>Leukocyte adhesion and activation                     |
| Cytokines<br>(TNF, IL-1, IL-6) | Macrophages<br>Endothelial cells<br>Mast cells | Locally: endothelial activation<br>Systemically: fever, hypotension                                    |
| Chemokines                     | Leukocytes<br>Activated macrophages            | Chemotaxis<br>Leukocyte activation                                                                     |
| Platelet-activating<br>factor  | Leukocytes<br>Mast cells                       | Vasodilatation<br>Increased vascular permeability<br>Leukocyte adhesion<br>Chemotaxis<br>Degranulation |
| Complement                     | Plasma (liver)                                 | Leukocyte chemotaxis and activation<br>Direct target killing                                           |
| Kinins                         | Plasma (liver)                                 | Increased vascular permeability<br>Smooth muscle contraction<br>Vasodilatation<br>Pain                 |

**Table 1.1** Principal mediators of inflammation: their sources and general actions  
*Adapted from reference<sup>5</sup>*

If the neutrophil action permits the elimination of the offending agent, the inflammatory response is considered successful and is followed by a phase of resolution and initiation of tissue repair that is mostly orchestrated by macrophages, either tissue-resident or recruited (Figure 1.1).<sup>14</sup> The switch in lipid mediators released is paramount to a clear resolution of inflammation, from the pro-inflammatory prostaglandins and leukotrienes<sup>15</sup> to anti-inflammatory lipoxins.<sup>16</sup> Lipoxins shutdown neutrophil recruitment and promote macrophage infiltration into the tissue to clear dead cells and initiate tissue repair.<sup>14</sup> Resolvins and protectins<sup>17</sup>, which are other anti-inflammatory lipid

mediators, and macrophage secreted growth factors<sup>14</sup> (especially transforming growth factor- $\beta$ )<sup>18</sup> also play a pivotal role in inflammation resolution.

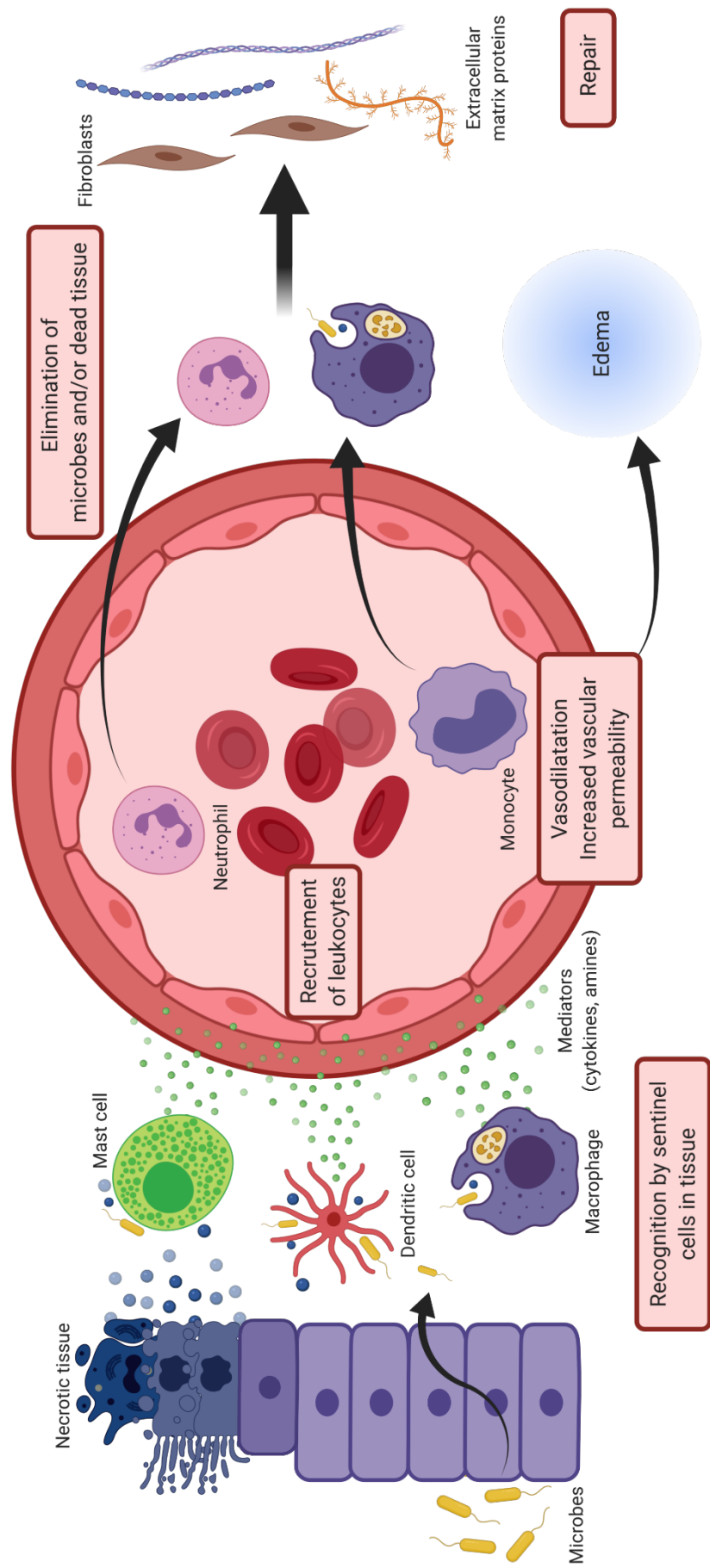
If neutrophils are not able to clear the offending agent, inflammation continues while changing its characteristics. Neutrophils are then replaced by macrophages and T cells (particularly in the case of infection), both of which work in a cooperative effort to fight the resisting agent. If this effort still fails, a state of chronic inflammation follows<sup>5</sup>, which can involve formation of granulomas<sup>19</sup> and tertiary lymphoid tissues.<sup>20</sup> The chronic inflammation state can also result from other causes like an autoimmune response (due to self-antigen persistence) or non-degradable foreign bodies.<sup>5</sup>

### 1.1.2 Inflammation in disease

Most occurrences of inflammation are protective, relatively short lived and therefore benign. Yet this relies on the fine balance and timing of release of pro- and anti-inflammatory cytokines. Unfortunately, a slight imbalance can lead to a range of pathologies linked to inflammatory response, of which some examples are given below.

#### 1.1.2.1 Myocarditis

Myocarditis is generally defined as an inflammatory disease of the heart muscle tissue. It is characterised by the infiltration of mononuclear leukocytes to the myocardium.<sup>21</sup> It can be triggered by a range of pathogens like viruses, bacteria, fungi and protozoa but also from other stimuli like toxins and hypersensitive reactions.<sup>22</sup>



**Figure 1.1** Sequence of events in the inflammation response  
*Illustration created with BioRender.com*

The leukocyte infiltrate during the inflammatory reaction, while clearing the offending agent, produces collateral heart tissue damage, which can vary from quite mild to severe depending on the magnitude of the inflammation response. Most cases of myocarditis are mild but in the most severe cases, damages can cause dilated cardiomyopathy and heart failure.<sup>22</sup>

#### 1.1.2.2 Atherosclerosis

Atherosclerosis is described as the build-up of fatty plaques in arterial walls and has been extensively studied since the 1980s.<sup>23</sup> Lesions are mostly seen at specific sites that possess hemodynamic and mechanical aggravating factors.<sup>24</sup> Low density lipoproteins (LDL) starts accumulating in the artery wall and once secluded from the circulating anti-oxidants, start to oxidise. Oxidised LDL in turn trigger an inflammatory response, recruiting monocytes to the arterial wall, which activate and differentiate in macrophages. Macrophages attempt to scavenge the excess oxidised LDL by phagocytosis leading to a cytosolic build-up of cholesterol.<sup>25</sup> Such high cholesterol content cytosols have a “foamy” aspect, which gave the term “foam cell” formation. These cholesterol-loaded macrophages then accumulate in the artery wall, forming fatty streaks in the plaque. Plaques can grow to a stage of artery occlusion or may rupture leading to thrombosis causing stroke or myocardial infarction.

#### 1.1.2.3 Cancer

Cancer is a disease in which there is abnormal and uncontrolled cell proliferation, which ultimately invade nearby tissues.<sup>26</sup> In some cases, metastasis can occur when cancerous cells spread to other parts of the body through the blood stream or lymphatic system.<sup>26</sup> Unfortunately, cancer comes in many different forms depending on multiple

factors, rendering it a challenge to treat effectively. Recently there has been an increasing focus on cancer immunology and immunotherapy, looking at immune cell recruitment in the tumour microenvironment and their effects on tumour growth, metastasis formation, angiogenesis and ultimately patient prognosis and outcome.<sup>27,28</sup> In fact, the pro-inflammatory characteristic of cancerous tumours is clear<sup>29</sup>, as tumour associated macrophages (TAMs) are the major components of cancer microenvironment and they are associated with poor patient prognosis in certain cancers, for instance breast cancer.<sup>30</sup> Other immune cells such as CD8<sup>+</sup> T cells, T helper 1 cells (T<sub>H</sub>1) and natural killer cells (NK), recruited to tumours through tumour-associated antigens (TAAs), can directly engage with the tumour to drive antitumour immunity, thus limiting metastasis and providing better survival in patients with ovarian and colon cancer.<sup>31,32</sup>

## 1.2 Leukocyte recruitment mechanism

Although there may be slight differences between leukocyte subpopulations in the molecular details of the different steps leading to their recruitment to inflamed tissues, they seem to follow a very similar and highly regulated process: slow rolling mediated by selectin interactions, chemokine activation, integrin-mediated arrest and finally trans-endothelial migration.<sup>33</sup>

### 1.2.1 Leukocyte slow rolling

At the onset of inflammation, the rapid release of vasoactive amines, like histamine, provoke vasodilatation of post capillary venules reducing the hemodynamic flow and allowing leukocytes to interact more closely with the vessel wall.<sup>34</sup> In that context leukocytes can start rolling, mediated by the interaction of integrins and selectins with their ligands (Figure 1.2).<sup>35</sup> However, these interactions are weak (high on-off rate)<sup>36</sup>, thus it is

through constant binding and release that leukocytes start slowing down and rolling on the epithelium.

Selectins are single chain transmembrane receptors of three different types: E-selectin, P-selectin and L-selectin.<sup>37</sup> P-selectin is displayed on endothelial membrane after activation by histamine or thrombin.<sup>38</sup> E-selectin expression is readily induced via stimulation of the endothelium by inflammatory cytokines like TNF- $\alpha$  and IL-1.<sup>38</sup> Therefore, P-selectin and E-selectin are only displayed in a time and location controlled manner, for an effective recruitment of leukocytes to the inflamed site. L-selectin is expressed on most leukocytes and plays a critical role in lymphocytes homing.<sup>39</sup>

Additionally, leukocytes express heterodimeric membrane receptors, called integrins, which are also able to modulate leukocyte rolling through interaction with endothelium ligands. Of the many integrins expressed on leukocytes very late antigen 4 (VLA4), lymphocyte function-associated antigen-1 (LFA-1) and macrophage antigen-1 (Mac-1) are amongst the best studied.<sup>40</sup> VLA4<sup>41</sup>, LFA-1 and Mac-1<sup>42</sup> have been shown to promote leukocyte rolling independently of E- and P-selectin.

### 1.2.2 Leukocyte chemokine activation

While rolling, leukocytes have prolonged exposure to Glycosaminoglycan(GAG)-displayed chemokines on inflamed endothelium surface.<sup>43</sup> Such activation through chemokines ligand/chemokine receptor interactions, in parallel with selectin mediated signalling, leads to activation of integrins into their high affinity conformation through so called inside-out activation (Figure 1.2).<sup>44</sup> For example chemokines such as CXCL1 and CXCL8 are activating LFA-1, binding to intracellular adhesion molecule-1 (ICAM-1) through

respective interactions with CXCR2 and CXCR1.<sup>45</sup> Many other arrest chemokines play similar roles in the process leading to firm adhesion of leukocytes.<sup>46</sup>

### 1.2.3 Leukocyte arrest

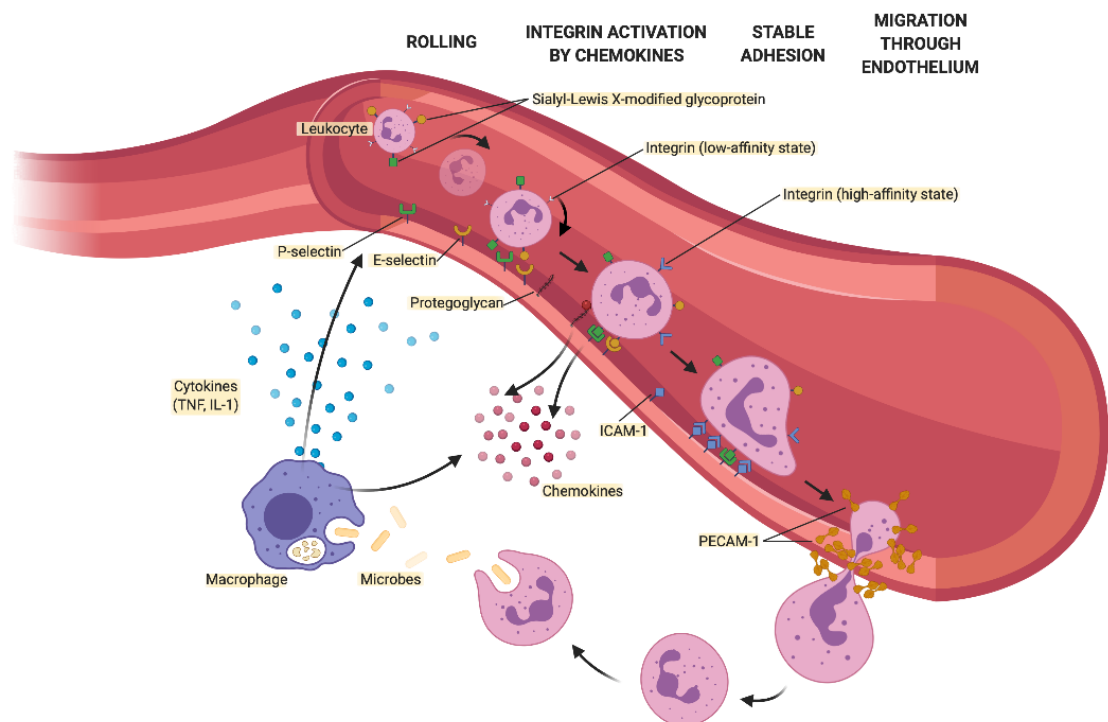
The high affinity activated integrins mediate leukocyte arrest and further signalling, so called outside-in integrin signalling, leading to cytoskeleton reorganisation and display of a higher density of integrins on leukocyte surface<sup>47,48</sup>, strengthening adhesion to the endothelium. Additionally, leukocytes proceed to a slow crawling to find the optimal transmigration location still mediated through clusters of integrins on their surface (Figure 1.2).<sup>49</sup>

### 1.2.4 Leukocyte trans-endothelial migration

Once firmly adhered on the vascular wall leukocytes can migrate through the endothelium via two different mechanisms: through endothelial junctions, a process called diapedesis, or through endothelial cells themselves, a transcellular mechanism which is much less common and will not be described in this section for more clarity (Figure 1.2).<sup>50</sup> Within the endothelial junction, adhesion molecules cooperate and guide leukocytes through. Wherein platelet/endothelial cell adhesion molecule-1 (PECAM1), ICAM-1 and ICAM-2, CD99, junction adhesion molecule-A (JAM-A), JAM-B and -C play an important role.<sup>51–53</sup> ICAM-1 and ICAM-2 are ligands of integrin LFA-1, while PECAM and CD99 form homodimers between endothelial cells and leukocytes. JAMs can interact either with themselves or with integrins. These different interactions continue to guide leukocytes through the endothelial junction.<sup>52,54,55</sup> Additionally, integrin/ICAM-1 interactions produce endothelial signalling that leads to myosin-light-chain kinase activation and ultimately to

cell contraction, increasing the permeability of the endothelium and favouring migration of leukocytes.<sup>51</sup>

Then leukocytes face the vascular basement membrane: a thick extracellular matrix rich in collagen, laminins and glycoproteins.<sup>56</sup> Therefore, during the migration process leukocytes start to secrete matrix proteases like matrix metalloproteases and gelatinases to facilitate navigation through the basement membrane.<sup>57,58</sup> Furthermore, as they reach the basement membrane, leukocytes can sense another set of chemoattractants as they get closer to the site of inflammation. Such chemoattractants includes mainly chemokine gradients displayed on GAGs but also complement components and some pathogen associated chemoattractants like bacterial *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), inducing further chemotaxis towards the site of inflammation.<sup>59</sup>



**Figure 1.2** Multistep process of leukocyte migration  
*Illustration created with BioRender.com and adapted from reference<sup>5</sup>*

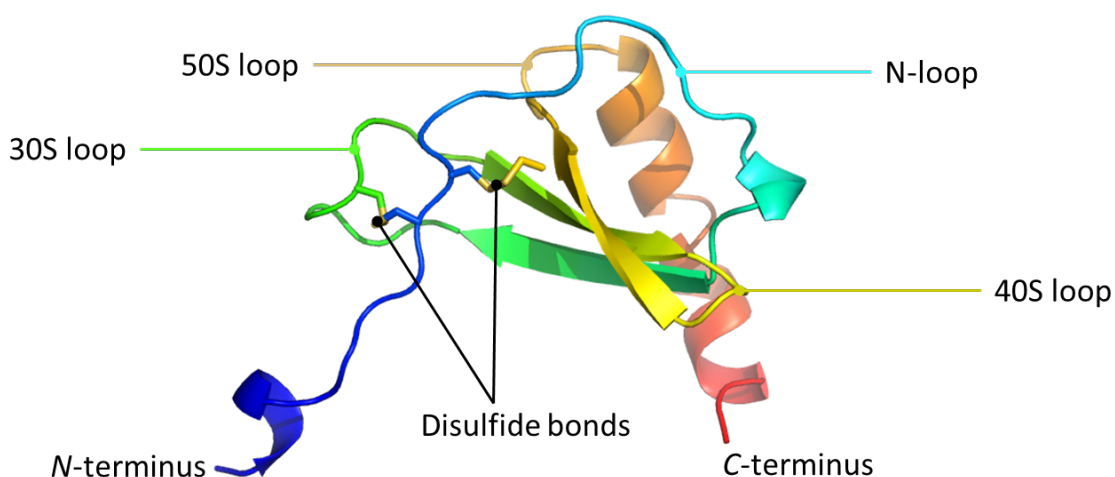
## 1.3 Chemokines

Chemotactic cytokines, or chemokines are small secreted proteins (8-12 kDa) primarily responsible for directional migration of leukocytes and critical for inflammation processes as discussed earlier.<sup>60</sup> Although each of the almost 50 different chemokines are unique in sequence, they have conserved *N*-terminal cysteine residues. These cysteines are involved in disulfide bonds with the third and fourth cysteines creating the conserved core scaffold of the chemokines (Figure 1.3).<sup>61</sup> They can therefore be classified into four distinct families depending on the spacing between the *N*-terminal cysteines: CCL, CXCL, CX<sub>3</sub>L, and XCL, in which X stands for any amino acid, and L stands for ligand.<sup>62</sup> Chemokine ligands signal through G-protein Coupled receptors (GPCRs), which stimulate cell migration. These receptors follow a similar nomenclature depending on the type of chemokine ligand they primarily interact with, leading to CCR, CXCR, CX<sub>3</sub>R or XCR classes of chemokine receptors.<sup>62</sup>

### 1.3.1 Chemokine ligands

#### 1.3.1.1 General structure

Although chemokines are all fairly distinct in sequence, their tertiary structure is highly conserved. They are composed of a flexible *N*-terminal tail followed by an *N*-terminal loop (N-loop), a short  $\beta$ -sheet composed of three anti-parallel  $\beta$ -strands and finally a C-terminal  $\alpha$ -helix (Figure 1.3).<sup>61</sup> These features are linked together with several loops, 30S and 40S loops respectively connecting the first and second and the second and third  $\beta$ -strands. The final loop connecting the  $\beta$ -sheet to the C-terminal  $\alpha$ -helix is known as the 50S loop. Disulfide bridges constrain the fold by stapling cysteine motif with the  $\beta$ -sheet motif.<sup>61</sup>



**Figure 1.3** Representative fold of chemokines  
*CCL8 structure solved by Dr. Max Renner (unpublished data). Created using PyMol*

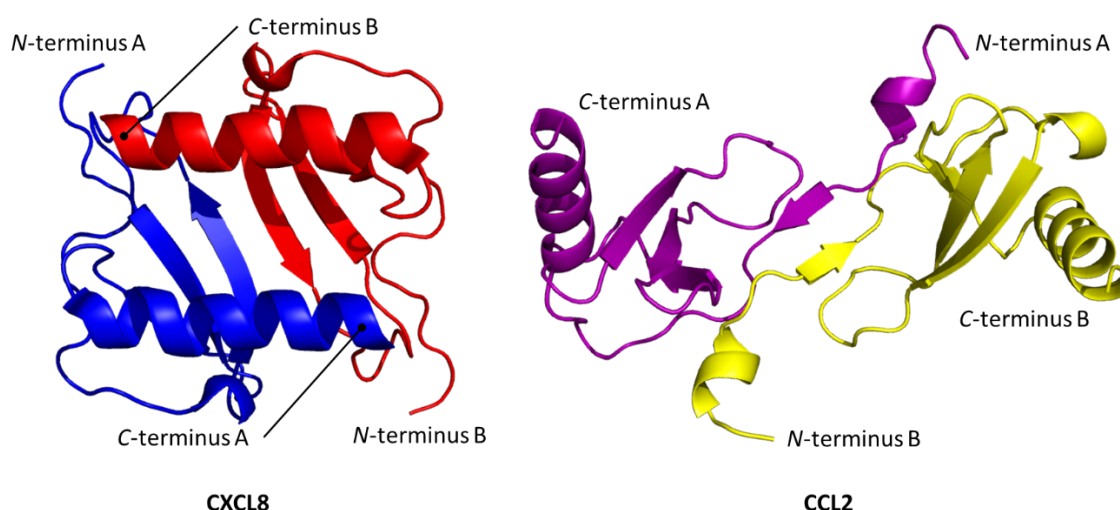
#### 1.3.1.2 Chemokine dimerization

One of the important features of chemokines is their propensity to oligomerise as shown in many crystal and NMR structures.<sup>61</sup> Chemokines dimerise in different modalities depending on their family. CXC chemokines, like CXCL8<sup>63</sup>, dimerise through their  $\beta$ -sheets forming an extended six  $\beta$ -stranded anti-parallel  $\beta$ -sheet with C-terminal  $\alpha$ -helix resting on the side of this  $\beta$ -sheet stabilising this interaction (Figure 1.4). CC chemokines, like CCL2<sup>64</sup>, dimerise through their N-terminal tail forming a small anti-parallel  $\beta$ -sheet. Higher order entities, especially tetramers have been observed in structural studies for CCL2<sup>64</sup> and CXCL10<sup>65</sup> showing quaternary structures composed of dimer of dimers displaying a mix of CC and CXC type dimer interaction. It is yet unclear if those tetramer structures have biological relevance.

Adding further complexity, due to their conserved structure and the relative high sequence identity, many chemokines are able to heterodimerise.<sup>66</sup> For example, CCR2 binding chemokines (CCL2, CCL8, CCL7, CCL13 and CCL11) have been shown to form CC

heterodimers.<sup>67</sup> Moreover, CC/CXC heterodimers were also reported in the case of the CCL5/CXCL4 interaction in atherosclerosis.<sup>68</sup>

Chemokine oligomers dissociate readily to their monomeric form and the equilibrium constant for such interaction is in the nanomolar range, although at a physiological level chemokine concentration is expected to be sub-nanomolar, making the dimeric form less likely.<sup>69</sup> However there is growing evidence that oligomeric states of chemokines is key for binding to GAGs in order to create the critical local gradient that drives leukocyte chemotaxis *in vivo*.<sup>70</sup>



**Figure 1.4** Structure of CXCL8 and CCL2 dimer  
CXCL8 (PDB 1IL8, solution NMR structure) and CCL2 (PDB 1DOK, X-ray diffraction structure) coloured depending on chain A and B. Created using PyMol.

### 1.3.1.3 Glycosaminoglycans binding relevance

Haptotaxis describes the migration of cells driven by surface immobilised reagents. Early experiments revealed that chemokines were able to trigger leukocyte migration by haptotaxis as well as by chemotaxis.<sup>71</sup> Following these results, it was shown that not only were chemokines able to oligomerise and interact with their receptors but that they were also able to bind to glycosaminoglycans (GAGs) on endothelial cell surface and heparin.<sup>72</sup>

Interactions with GAGs on the cell surface immobilise chemokines near their site of secretion to create a high local concentration that was shown to induce oligomerisation.<sup>72</sup>

It remains complex to characterise GAG binding sites on chemokine surface. Consistently it is found that cationic surfaces containing repeats of Lys, Arg or even His are often associated with GAG binding patches.<sup>73</sup> A review from Monneau et al. collected results from extended studies to map existing information on chemokine GAG binding sites.<sup>74</sup> This collection of data suggests that CC and CXC chemokines bind GAGs in different modes. CC chemokines show a major GAG binding region located on their 40S loop containing basic residues and some binding on their N-loop, whereas CXC chemokines seem to display the same regions and one additional GAG interacting patch located on their C-terminal  $\alpha$ -helix. However, CCL21<sup>75</sup> and CXCL9<sup>76</sup> are quite unique as they display a long C-terminal basic tail providing them with an anchor to bind to GAGs. Interestingly, the dimeric forms of chemokines displays larger GAGs binding surfaces, which strengthens the link between GAG binding and oligomerisation.<sup>77</sup>

### 1.3.2 Chemokine receptors

Chemokine receptors (CKRs) belong to the rhodopsin-like family of G-protein coupled receptors (GPCRs). They are composed of seven helical transmembrane domains, extended extracellular disordered N-terminal tail and the highly conserved E/DRY cytosolic motif.<sup>78</sup> There are about 20 known CKRs, and as they are selective to chemokine sub-families, they follow the same nomenclature (i.e. CCR, CXCR, CR and CX<sub>3</sub>R) depending on the specific family they are activated by.<sup>79</sup>

### 1.3.2.1 Chemokine ligand/receptor interaction

It is believed that the interaction between chemokine ligand and receptors operates via a two-step binding model. Early studies showed the importance of the *N*-terminal tail of CCL2 for receptor activation<sup>80,81</sup> but also the importance of the N-loop of CCL2 for receptor binding especially the highly conserved Tyr13 (or Phe13) and cationic residues on the N-loop.<sup>82</sup> These results pointed out that the chemokine N-loop is responsible for receptor binding and specificity while the *N*-terminal tail is responsible for the activation of the receptor, indicating a multi-step process.

Insights into CKRs binding came from a domain swap study between CCR2 and CCR3, and between CXCR1 and CXCR2 showing that the disordered *N*-terminal tail was critical to the binding of their respective ligands.<sup>83,84</sup> Additionally, a protein fusion of the *N*-terminus of CCR2 onto CD-8 to display it on cell surface was sufficient to record high affinity binding of CCL2.<sup>85</sup>

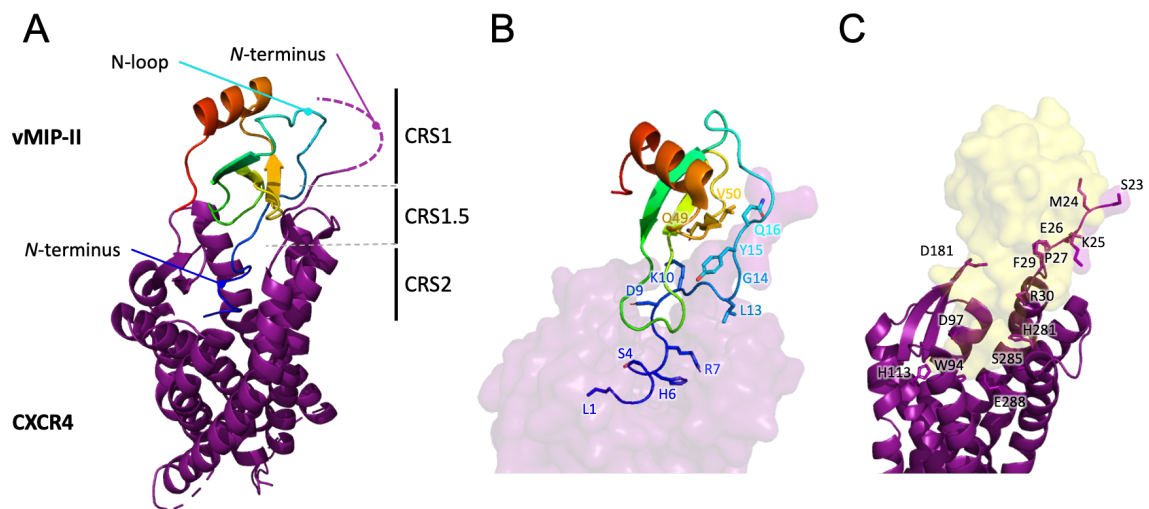
Furthermore, mutational studies of transmembrane domains of CCR5 and the *N*-terminal tails of its chemokine ligands revealed the importance of these regions in receptor activation.<sup>86</sup> These results led to the definition of the two-site binding model where CKRs are activated through chemokine recognition site 1 (CRS1) and chemokine recognition site 2 (CRS2) on the receptor.<sup>87</sup> CRS1 encompasses the *N*-terminal tail of the receptor interaction with the N-loop of the ligand and CRS2 the transmembrane extracellular loops from the receptor interacting with the *N*-terminal tail of the ligand. Across the combination of those two binding events, chemokines ligands can interact with their receptors and drive their function.

CXCR4 crystal structure in complex with the viral chemokine vMIP-II gave more certainty on this process (Figure 1.5).<sup>88</sup> This structure confirms the initial two-site binding

model and reveals a contact surface comprising the whole *N*-terminal tail of the receptor interacting with the N-loop of vMIP-II. Due to the lack of defined separation between the two interacting surfaces, CRS1 and CRS2, this study defined an intermediate interaction site: CRS1.5. According to this study the interaction is defined as: *N*-terminal tail of the receptor (CRS1) interacts with the N-loop of the ligand, the next residues of the receptor (CRS1.5) interact with the beginning of the *N*-terminal tail of the ligand, finally the rest of the *N*-terminal tail of the ligand interacts with the helical transmembrane domains of the receptor (CRS2) inferring conformational change and receptor activation. Using homology modelling this study also reveals the importance of the cationic residues at the *N*-terminus of CXC chemokines (highly conserved in CXC chemokines) and the cationic residues of the N-loop of CC chemokines (highly conserved in CC chemokines) for specificity of receptor binding.

Moreover, post-translational modification of chemokine receptors *N*-terminal tail, especially tyrosine sulfation, was shown to increase receptor binding to chemokine ligands by several folds.<sup>89</sup> The sulfotyrosine (sY) group on the *N*-terminal tail of the receptor interacts closely with residues located on the N-loop region of the ligand, facilitating the first binding event.<sup>90</sup> Additionally, it was shown, as for their ligands, that chemokine receptors have the ability to homo- and hetero-oligomerise in receptor rafts on leukocyte surface.<sup>91</sup> The effect of receptor oligomerisation on the overall chemokine signalling is currently unclear, but in a seminal study it was shown that cells co-expressing CCR2 and CCR5 responded cooperatively to their ligands.<sup>92</sup> It demonstrated that stimulation of a cocktail of CCL2 and CCL5 had a more than 10-fold higher response compared to the additive effect of the stimulation of each chemokine alone. This would indicate that

receptor oligomerisation can at least in some cases play a role in the regulation of chemokine signalling.



**Figure 1.5** X-ray structure of CXCR4/vMIP-II complex  
 (A) Structure of CXCR4 (cartoon, purple) and vMIP-II (cartoon, rainbow) in complex with important features annotated (PDB 4RWS). (B) Structure of CXCR4 (transparent surface, purple) and vMIP-II (cartoon, rainbow) in complex with vMIP-II residues involved in the interaction annotated. (C) Structure of CXCR4 (cartoon, purple) and vMIP-II (transparent surface, yellow) in complex with CXCR4 residues involved in the interaction annotated. Figure created using PyMol.

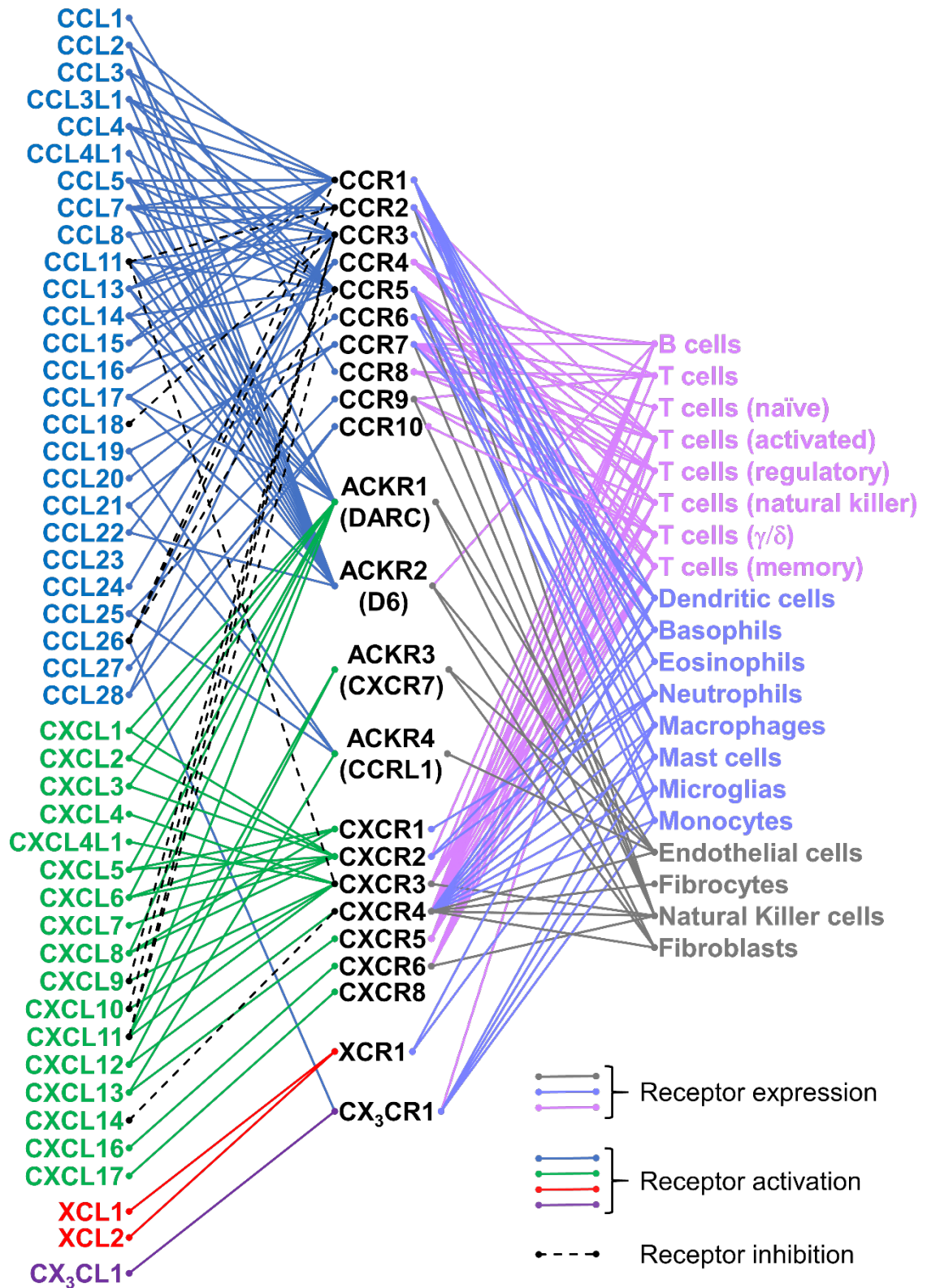
### 1.3.2.2 Atypical chemokine receptors

Atypical chemokine receptors are non-signalling receptors.<sup>93</sup> They are structurally close to the “typical” chemokine receptors with the exception that they are lacking the DRYLAIV cytosolic motif responsible for G-protein coupling. Therefore, they can be activated by binding chemokines but are unable to signal through G-protein activation, disabling their ability to drive cell migration, survival or other associated functions. There are four well identified Atypical Chemokine Receptors (ACKR).<sup>94</sup> The Duffy antigen receptor for chemokines (DARC or ACKR1), D6 or ACKR2, CXCR7 or ACKR3 and CC chemokine receptor like 1 (CCRL1 or ACKR4).

These receptors regulate chemokines through two main mechanisms. First, acting as decoy receptor that binds chemokine, then through  $\beta$ -arrestin signalling internalises and degrades the ligand, therefore controlling the level of chemokine present.<sup>95</sup> Second, supporting the delivery of chemokines to their optimal site of function.<sup>96,97</sup> This trafficking function provides adequate chemokine levels to specific locations to provide tailored tuning of the chemokine signalling network. In fact, deletion of these receptors in mice produces damaging phenotypes. For example, ACKR1 presents chemokines on endothelial cells to circulating leukocytes.<sup>96,98</sup> On erythrocytes, ACKR1 safeguards chemokine levels in blood which prevents chemokine receptors on leukocytes being desensitised.<sup>99–102</sup> These decoy receptors add to the subtlety of the chemokine signalling network with chemokine and receptor oligomerisation and GAG binding.

### 1.3.3 Complexity of the chemokine network

All previous factors depict a complex system of signalling with multiple layers of regulations. However, another unique property of the chemokine network is its incredible robustness.<sup>103,104</sup> This robustness comes from the fact that a single chemokine can bind multiple receptors and a single receptor can bind multiple ligands (Figure 1.6). This so called “one-to-many” binding modality drives the chemokine response. Although, all chemokine ligand/receptor interactions are not equal and *vice versa*. As an example, the receptor CCR7 interacts with two ligands CCL19 and CCL21, but only CCL19 induces its internalisation;<sup>105</sup> the ligand CXCL8 interacts with two receptors, CXCR1 and CXCR2, yet both are internalised at different levels.<sup>106</sup> These examples convey the complexity and subtlety of response for ligands binding to different receptors, and receptors being activated by different ligands. Moreover, single cells can express multiple receptors on their surface (Figure 1.6).<sup>108,109</sup>



**Figure 1.6** Complexity of the chemokine network  
*Human chemokines and receptors network, linked to receptor expression on human cells. Adapted from refence<sup>107</sup>*

Finally, chemokines can be expressed as parts of a “feedforward loop” where initial chemokine expression leads to further expression of different chemokines in the inflamed tissue.<sup>110</sup> Thus, with this complex and robust network, single chemokine or single receptor targeted therapeutics would become fairly inefficacious as the redundancy of the network is able to withstand such limited intervention.<sup>104,111</sup>

Complicating matters further, location and time of chemokine expression can also play a role in the complexity of this network.<sup>112</sup> Expression of chemokines and their receptors can change over time in the inflamed tissue and due to this “one-to-many” ligand/receptor interaction, it can modulate initiation of leukocyte migration. Therefore, a single target agent could be somehow effective at one point but lose its efficacy over time as the array of chemokines and receptors expressed changes.

## 1.4 Interfering with the chemokine network

The previous sections exemplified the importance of inflammation in various diseases states, the crucial role of the chemokine network in the onset and regulation of inflammation, and finally the great complexity and robustness of this network. Because of its central role in many pathologies, the chemokine network has been investigated as a therapeutic target over the last 30 years. Next, we will discuss how current clinical work and nature have found ways to interfere with the chemokine network.

### 1.4.1 Current Chemokine network modulators

The extreme complexity of the chemokine network has frustrated the therapeutic development of effective molecules.<sup>111</sup> Most of drug development to date has focussed on the chemokine receptors, as GPCRs are a target of choice for the pharmaceutical industry (Table 1.2).<sup>113</sup> The most successful molecules developed so far are Maraviroc and Plerixafor

(Figure 1.7) targeting CCR5<sup>114</sup> and CXCR4<sup>115</sup> respectively. CCR5 has been a target of choice as it was shown that patients with non-functional CCR5 gene mutation were resistant to HIV infection<sup>116</sup> and that CCR5 was a co-receptor for HIV cell entry.<sup>117</sup> These discoveries led to multiple molecules being developed against CCR5 for HIV and AIDS treatment, of which Maraviroc was the first to be approved by the FDA in 2007. Plerixafor was initially developed for its HIV resistance properties, but it was noted that patients were showing increased level of circulating haematopoietic stem cells.<sup>118</sup> It was later discovered that Plerixafor was inhibiting the action of CXCL12, ligand of CXCR4, responsible for homing of haematopoietic stem cells in the bone marrow.<sup>119</sup> Therefore, it was later approved by the FDA in 2008 for autologous transplantation in patients with Non-Hodgkins Lymphoma or multiple myeloma. Although, these molecules have safe pharmaceutical profiles and provide much needed medicines for important disorders, they do not produce their effect through reducing inflammation. One could therefore question why there are not more molecules in the clinic modulating inflammation by targeting the chemokine network, as chemokines are the main actors of leukocyte migration.

The answer lies in the robustness of the chemokine network, which grants it an incredible resistance to mono-target agents. Many molecules develop through preclinical studies and early clinical trials, only to find that there is no clinically relevant pharmacodynamic effect. For example, this was the case for one of the rare monoclonal antibodies (Carlumab) targeting a chemokine ligand. Carlumab (CNTO 888) was discovered to bind CCL2 with very high affinity ( $K_d = 22$  pM).<sup>120</sup> In several xenograft preclinical studies, it was shown that CCL2 neutralization using monoclonal antibodies was able to inhibit tumour growth, inflammation and angiogenesis.<sup>121–124</sup> Following these studies, Janssen Biotech decided to launch a first-in-human study to assess the safety profile of Carlumab in

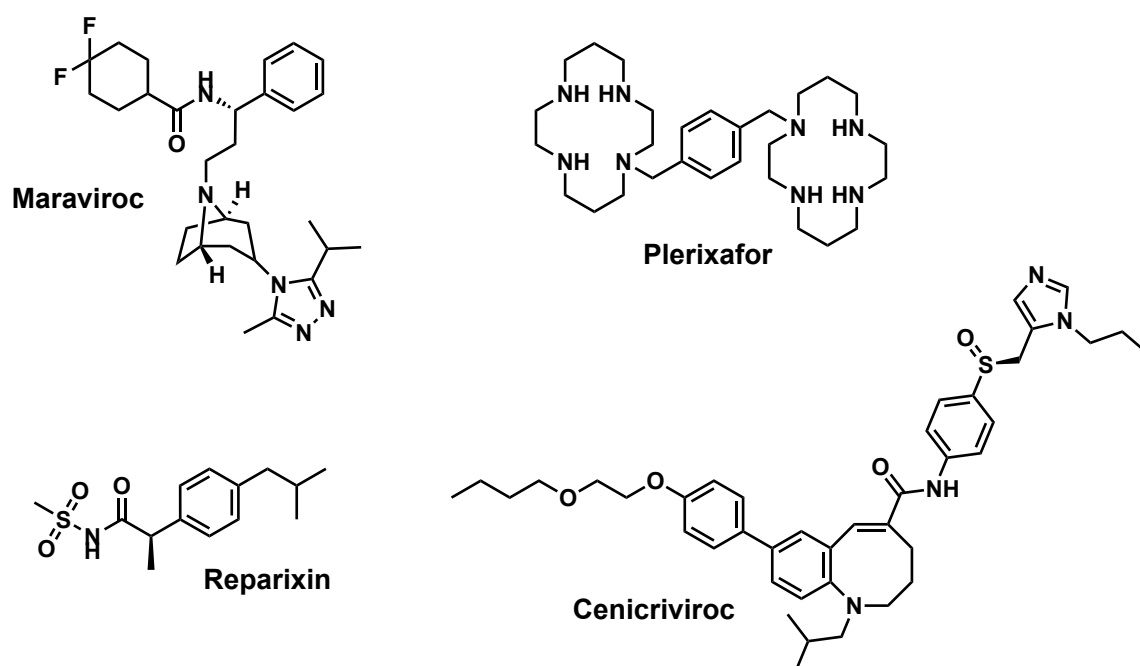
patients with solid tumour, which was found to be well tolerated up to doses of 15 mg/kg.<sup>125</sup> However, Janssen Biotech terminated the development of Carlumab as it did not show clinically significant efficacy in phase 2 on a larger sample of patients.<sup>126,127</sup> It was shown that the level of free CCL2 decreased drastically after the first injection of Carlumab indicating its engagement with the target, but free CCL2 concentration then increased again after a week post-injection. More worryingly, subsequent injections did not reduce free CCL2 concentration explaining the lack in efficacy observed and ultimately the missed primary end point of this study.<sup>127</sup> This is unfortunately the fate that befalls many promising chemokine ligand or receptor inhibitors. One exception is with Mogamulizumab (KW-0761), a monoclonal antibody against CCR4.<sup>128</sup> CCR4-positive tumour cells in Adult T-cell leukaemia-lymphoma patients indicate an unfavourable prognosis.<sup>129</sup> Therefore, investigation of antibody dependent cellular cytotoxicity activity of Mogamulizumab was performed and showed increased clinical benefit to standard of care Vorinostat (an histone deacetylase small molecule inhibitor).<sup>130</sup> Following these results, Mogamulizumab was approved by the FDA in 2018 for the treatment of relapsed cutaneous T-cell lymphoma.

Although, single target molecules have shown limited success in human clinical trials, there is some hope for different types of molecules with multiple targets. Cenicriviroc (Figure 1.7) is a novel small molecule developed by Takeda, able to bind and inhibit CCR2 and CCR5. As with many CCR5 inhibitors, it was first investigated as an anti-HIV medicine with good success.<sup>131</sup> More interestingly, it was shown preclinically<sup>132,133</sup> and clinically<sup>134–136</sup> that its unique blockade of both CCR5 and CCR2 was anti-inflammatory and antifibrotic. Thus, it is currently being investigated in phase II clinical trial for the treatment of fatty-liver diseases.<sup>137</sup>

| TARGET    | DRUG          | CONDITION                                           | TRIAL PHASE | TRIAL REFERENCE            |
|-----------|---------------|-----------------------------------------------------|-------------|----------------------------|
| CCL2      | Carlumab      | Solid tumor                                         | I (D)       | NCT00537368 <sup>138</sup> |
|           | Carlumab      | Metastatic prostate cancer                          | II (D)      | NCT00992186 <sup>127</sup> |
|           | Carlumab      | Idiopathic pulmonary fibrosis                       | II (S)      | NCT00786201 <sup>139</sup> |
| CCL20     | GSK3050002    | Colitis ulcerative                                  | I (C)       | NCT01984047 <sup>140</sup> |
| CCR1      | CP-481715     | Dermatitis (Allergic Contact)                       | I (D)       | NCT00141180 <sup>141</sup> |
| CCR2      | AZD2423       | Painful diabetic polyneuropathy                     | II (D)      | NCT01201317 <sup>142</sup> |
|           | CCX872        | Pancreatic cancer                                   | I (C)       | NCT02345408 <sup>143</sup> |
|           | PF-04136309   | Pancreatic cancer                                   | I (C)       | NCT01413022 <sup>144</sup> |
|           | PF-04136309   | Pancreatic ductal adenocarcinoma                    | II (S)      | NCT02732938 <sup>145</sup> |
| CCR3      | GW766944      | Asthma                                              | II (D)      | NCT01160224 <sup>146</sup> |
| CCR4      | Mogamulizumab | Adult T-cell Leukemia-lymphoma                      | II (C)      | NCT00920790 <sup>147</sup> |
|           | Mogamulizumab | Cutaneous T-cell lymphoma                           | III (A)     | NCT01728805 <sup>148</sup> |
| CCR5      | INCB009471    | HIV Infections                                      | II (O)      | NCT00393120                |
|           | Maraviroc     | Hematologic Malignancy                              | II (O)      | NCT01785810                |
|           | Maraviroc     | HIV Infections                                      | IV (A)      | NCT00884858 <sup>149</sup> |
|           | Maraviroc     | Metastatic colorectal cancer                        | I (C)       | NCT01736813 <sup>150</sup> |
|           | PF-00232798   | HIV Infections                                      | II (O)      | NCT00495677                |
|           | Vicriviroc    | Colorectal Neoplasms                                | II (O)      | NCT03631407                |
|           | Vicriviroc    | HIV infection                                       | III (D)     | NCT00474370 <sup>151</sup> |
| CXCR2     | AZD5069       | Pancreatic cancer                                   | II (O)      | NCT02583477                |
|           | Danirixin     | Chronic Obstructive Lung Disease                    | II (C)      | NCT03034967 <sup>152</sup> |
| CXCR4     | Balixafortide | HER2- metastatic breast cancer                      | I (C)       | NCT01837095 <sup>153</sup> |
|           | LY2510924     | Solid Tumour                                        | I (C)       | NCT02737072 <sup>154</sup> |
|           | PF-06747143   | Acute Myeloid Leukemia                              | I (C)       | NCT02954653 <sup>155</sup> |
|           | Plerixafor    | Multiple Myeloma and Lymphoma                       | III (A)     | NCT00838357 <sup>156</sup> |
|           | Plerixafor    | Recurrent Glioblastoma multiform                    | I/II (C)    | NCT01977677 <sup>157</sup> |
|           | Plerixafor    | Relapsed Acute Myeloid Leukemia                     | I/II (C)    | NCT00512252 <sup>158</sup> |
|           | Ulocuplumab   | Acute Myeloid Leukemia                              | I (C)       | NCT01120457 <sup>159</sup> |
|           | USL311        | Solid tumour & Glioblastoma multiform               | I/II (O)    | NCT02765165                |
| CCR2 & 5  | Cenicriviroc  | Non-alcoholic Fatty Liver Disease                   | II (C)      | NCT02330549 <sup>160</sup> |
|           | PF-04634817   | Diabetic Nephropathy                                | II (D)      | NCT01712061 <sup>161</sup> |
| CXCR1 & 2 | Reparixin     | Delayed Graft Function After Kidney Transplantation | II/III (O)  | NCT00248040                |
|           | Reparixin     | Islet Transplantation in Diabetes                   | III (D)     | NCT01817959 <sup>162</sup> |
|           | Reparixin     | Metastatic breast cancer                            | II (O)      | NCT02370238                |

**Table 1.2** Drugs in clinical development targeting the chemokine network.  
Information obtained in January 2021 on <http://www.clinicaltrials.gov> looking at intervention study containing the word “chemokine”. A: approved, C: completed, D: discontinued for lack of efficacy, O: ongoing, S: discontinued for safety reasons.

Reparixin (Figure 1.7) is a small molecule developed by Dompé Farmaceutici that can allosterically bind CXCR1 and CXCR2 and block CXCL8 signalling.<sup>163</sup> The CXCL8/CXCR1-2 axis is known for the chemotaxis of polynuclear leukocytes (mostly neutrophils).<sup>164</sup> CXCL8 was also shown to stimulate proliferation of cancer stem cells.<sup>165</sup> On these bases Reparixin was studied to prevent inflammation damage following transplantation with no success in phase III clinical trials, raising questions on the role of CXCR1 and CXCR2 in transplantation inflammation response.<sup>166</sup> However, its action on inhibiting CXCL8 signalling on cancer stem cells has shown some success in combination with chemotherapy to increase patient survival in metastatic breast cancer (NCT02370238). Some of these studies are still underway and might yield unsatisfactory results, but it is apparent that targeting a single ligand or receptor in the complex chemokine network has yet been ineffective, potentially also due to poor target selection for those indications.<sup>167</sup> Thus, there is a rationale behind inhibiting several chemokine ligands or receptors, and this is how Nature overcomes the robustness of the chemokine network.



**Figure 1.7** Chemical structure of several inhibitors of the chemokine network

### 1.4.2 Nature's approach

Many parasites and certain viruses have evolved to evade immune response in order to survive long enough in their host. This usually involves modulation or inhibition of pro-inflammatory cytokines. For example helminths are experts in the art of host immunity modulation.<sup>168</sup> *Heligmosomoides polygyrus* secretes the Alarmin release inhibitor (HpARI), which is able to block the activity of IL-33 in human and mouse, a cytokine responsible for further pro-inflammatory cytokines and chemokines release.<sup>169</sup> *Schistosoma mansoni* produces a chemokine binding protein (CKBP) able to neutralise CCL2, CCL3, CCL5, CXCL8, and CX<sub>3</sub>CL1 therefore inhibiting the recruitment of neutrophils and blocking acute host inflammation response.<sup>170</sup> *Necator americanus* secretes a range of metalloproteinases causing the proteolysis of CCL11 inducing the de-activation of dendritic cells.<sup>171</sup> Poxviruses have also developed several CKBPs able to bind multiple chemokine ligands, such as CCL2, CCL3, CCL5, CCL7, CCL11 and CCL23, blocking their signalling by prohibiting receptor binding.<sup>172,173</sup> Structures of poxvirus CKBP in complex with a range of chemokines showed that it was able to bind the N-loop of chemokines, thus inhibiting initial interaction with the first chemokine receptor binding site.<sup>174</sup>

Several of these natural proteins display an advantage on the previously described therapeutic agents as they can successfully inhibit multiple chemokines. These show great potential for the development of a novel generation of inflammation modulators derived from CKBPs to overcome the “one-to-many” chemokine mode of binding.

## 1.5 Evasins

### 1.5.1 Ticks and Evasins

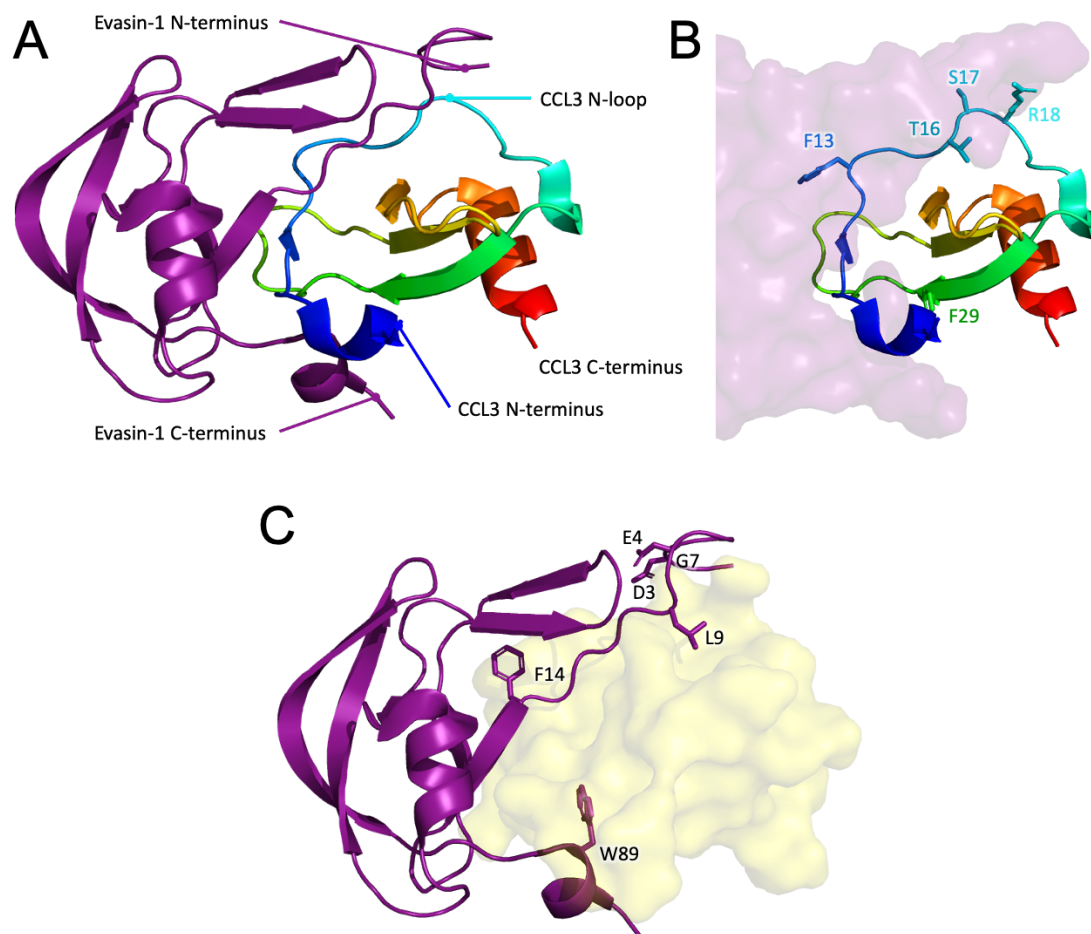
Ticks are parasites that feed on blood and therefore must remain attached to their host for several days. Biting their host, and the prolonged period of blood feeding is likely to produce a strong immune response. Therefore, they evolved to produce an array of anti-coagulant, immunomodulatory and anti-inflammatory factors to evade detection by their host while feeding.<sup>175–177</sup> Tick salivary gland extracts (SGEs) was shown to block the activity of pro-inflammatory cytokine TNF- $\alpha$ , suggesting some level of host immune response modulation.<sup>178</sup> Moreover, it was later showed, using radioligand binding, chemotaxis and ELISA assays, that SGE had anti CXCL8, CCL2, CCL3, CCL5 and CCL11 properties, indicating further immune response modulation through interference with the chemokine network.<sup>179–181</sup>

The agents behind the anti-chemokine properties of tick saliva were named “Evasins”.<sup>182</sup> The first Evasin ever described was Evasin-1, discovered using expression-cloning technology from *Rhipicephalus sanguineus* and <sup>125</sup>I-CCL3 crosslinking selection.<sup>183</sup> Evasin-1 was then produced in HEK293 mammalian cells and TN5 insect cells. It was discovered as a glycosylated protein and able to form a 1:1 complex with CCL3. Following surface plasmon resonance experiments, it was shown to not only bind CCL3 but also CCL4 and CCL18. Interestingly it was not able to bind any of the other chemokines tested including a large portion of the CC and CXC chemokine family as well as XCL1 and CX<sub>3</sub>CL1. This indicated that Evasin-1 was only able to bind selectively to a sub-set of CC chemokines. This study identified the first tick CKBP and hinted at the possible presence of many others.

Evasin-3 and 4 were later identified using similar techniques that were used for the discovery of Evasin-1. Using <sup>125</sup>I-CXCL8 as a bait identified Evasin-3 and using <sup>125</sup>I-CCL5 and <sup>125</sup>I-CCL11 identified Evasin-4.<sup>183</sup> Evasin-3 is unrelated to Evasin-1, only binds CXC chemokines and specifically CXCL1 and CXCL8. Evasin-4 was found to be related to Evasin-1, and like Evasin-1 only binds to CC chemokines but unlike Evasin-1, it can bind a much larger range including CCL1, CCL3, CCL4, CCL7, CCL8, CCL11, CCL13, CCL14, CCL15, CCL16, CCL17, CCL18, CCL19, CCL21, CCL22, CCL23, CCL24, CCL25 and CCL26.<sup>184</sup> These Evasins discoveries indicate *Rhipicephalus sanguineus* evolved to secrete these tick CKBPs. Additionally, there seem to be some level of specificity attached to a specific Evasin not only to a certain class of chemokines like Evasin-1 and 4 for CC chemokines and Evasin-3 for CXC chemokines, but also in the range of chemokines it can bind to.<sup>183,184</sup> A putative scenario is that ticks use an array of Evasins in order to block leukocyte infiltration at the bite site through chemokine network interference. Tick would have therefore evolved to overcome the complexity and robustness of the chemokine network by successfully neutralising several chemokines. The potential therapeutic benefit that could be provided by Evasins,<sup>185</sup> led to their evaluation in a number of pre-clinical studies including against lung fibrosis, post-infarction myocardial injury, colitis and myocardial reperfusion injury.<sup>186–189</sup> Their success to treat these phenotypes is probably due to their multiple chemokine binding properties and clearly shows the benefits of such therapeutic approach.

The growing interest in these new classes of CKBPs drove the desire to better understand their mode of action. These efforts led to the resolution of the crystal structure of Evasin-1 and CCL3 in complex (Figure 1.8).<sup>190</sup> The structure revealed that the *N*- and *C*-termini of Evasin-1 were engaging the *N*-terminus tail and loop of CCL3. This points out that Evasin-1 most certainly inhibits CCL3 signalling by blocking its binding to its receptors.

Further studies using phage display of Evasin-1 and Evasin-4 alanine mutants showed that Evasin-4 only uses its *N*-terminus to interact with CCL3, CCL5 and CCL8.<sup>190</sup> This work also revealed that Evasin-4 was also targeting the *N*-terminus of CCL5 in a similar way as Evasin-1, providing further evidence that Evasins are targeting the *N*-terminus of chemokines to inhibit receptor binding.



**Figure 1.8** X-ray structure of Evasin-1/CCL3 complex

(A) Structure of Evasin-1 (cartoon, purple) and CCL3 (cartoon, rainbow) in complex with important features annotated (PDB 3FPU). (B) Structure of Evasin-1 (transparent surface, purple) CCL3 (cartoon, rainbow) in complex with CCL3 residues involved in the interaction annotated. (C) Structure of Evasin-1 (cartoon, purple) and CCL3 (transparent surface, yellow) in complex with Evasin-1 residues involved in the interaction annotated. Figure created using PyMol.

### 1.5.2 Next generation Evasins

It was later described that the cDNA library constructed from *Rhipicephalus sanguineus* contained five more potential Evasins<sup>191</sup> and other cDNA libraries from different tick species were also identified to contain sequences of putative Evasins.<sup>192,193</sup> These led to the discovery of the next generation of Evasins with yeast surface display.<sup>194</sup> A library of more than 350 potential Evasins was identified using sequence homology to Evasin-1, 3 and 4 in psiBLAST. After inserting this library in a yeast surface display vector, Evasins were expressed on yeast surface to screen against several CC chemokine fluorescently labelled, using fluorescent assisted cell sorting (FACS) to assess binding clones. This study identified 26 Evasin sequences, of which only 10 were characterised after recombinant expression in HEK293 cells. They were then shown to bind and neutralise chemokines *in vitro* using biolayer interferometry (BLI) and Boyden chamber assays.<sup>194</sup> Another work using a library of over 250 potential Evasins sequences identified nine more Evasins. After *E. coli* expression and using a fluorescence polarisation assay and a cAMP cell reporter assay, these Evasins showed binding to and signal neutralisation of several CC chemokines.<sup>195</sup> Finally, a more recent study using yeast surface display identified 27 novel Evasins from a library of nearly 120 putative sequences based on Evasin-3.<sup>196</sup> These new Evasins were able to bind and neutralise several CXC chemokines using BLI and Boyden chamber assays like Evasin-3. This study revealed a knottin scaffold that is likely to play a role in CXC chemokine binding Evasins by presentation of different binding motifs, explaining in parts their promiscuous binding properties.

The discovery of many new Evasins, and their proven *in vivo* efficacy in disease models is opening a new avenue in contradiction to the pharmaceutical industry dogma:

“one target, one molecule”. Evasins promiscuous binding to chemokine could be the key to effectively addressing inflammation.<sup>197</sup>

### 1.5.3 A new CC chemokine binding Evasin: P672

One particular study of interest for this project reported the discovery of a novel Evasin named P672 from *Rhipicephalus sanguineus* using yeast surface display.<sup>198</sup> This 104 amino acid Evasin was discovered through a selection against CCL8. P672 is related to Evasin-1 and 4 and like them, binds multiple CC chemokines. This study showed that P672 was able to bind to CCL1, CCL2, CCL3, CCL3L1, CCL7, CCL8, CCL11, CCL13, CCL14, CCL15, CCL16, CCL18 and CCL23 by BLI with  $K_d$  ranging from 1 to 31 nM. It was also shown that this novel Evasin can successfully neutralise CCL2, CCL3, CCL3L1, CCL7 and CCL8 chemokine driven cell migration. Further experiments using intact mass spectrometry showed that CCL8 can dimerise and that P672 is able to disrupt this dimer. Finally, using homology modelling of P672 and CCL8 on the structure of the Evasin-1/CCL3 complex, it was hypothesised that the final 44 amino-acids at the *N*-terminus of P672 was responsible for its interaction with CCL8. To test this hypothesis, chimeric Evasin were recombinantly produced and purified using Evasin-1 as a scaffold and swapping different segment of its *N*-terminus with *N*-terminal regions of P672. Using BLI, it was shown that although Evasin-1 cannot bind CCL8, chimeras containing the 44-amino-acid-*N*-terminus of P672 were able to bind CCL8.<sup>198</sup>

These results revealed that Evasins could not only neutralise chemokine signalling by blocking ligand/receptor interaction, but also through oligomer disruption. Another key finding on this study was that the binding property of P672 is not on a diffuse surface but can be carried by a “shorter” *N*-terminal stretch. This could lead to the development of

smaller, yet potent promiscuous chemokine binders that would recapitulate the “one-to-many” binding mode of Evasins.

## 1.6 Aims of this work

The studies above outlined the many unique properties of Evasins and more particularly of the *N*-terminus of Evasin P672. The aim of the Chapter 2 of this work is to harness the unique properties of P672 Evasin by synthesising peptides that are able to bind and neutralise multiple chemokines. Binding patterns and anti-inflammatory activity will be assessed to deliver the first generation of Evasin-inspired promiscuous chemokine binding peptides. These will be tested in *in vivo* models to determine their effects on inflammation.

Biological therapeutics are usually associated with high manufacturing costs, parenteral delivery and potential immunogenicity, which deter development of many therapeutics. The opportunity to develop smaller entities, chemically synthesisable, could overcome a major hurdle for the wider development of CKBP derived medicine against inflammatory disorders. It is envisaged that pharmacokinetic and pharmacodynamic properties can further be optimised for the newly identified peptides and will be discussed in Chapter 3.

Finally, this work seeks to better understand Evasin/chemokine binding by the systematic study of their interaction through different high throughput techniques. Chapter 4 will provide deeper insight into the mechanism of action of Evasins, which is pivotal for the design of a new generation of effective anti-inflammatory medicines inspired by Nature’s own work.

# Chapter 2   Synthesis and characterisation of

## P672-derived chemokine-binding peptides

### 2.1 Introduction

Inflammation is linked to many diseases from myocarditis to atherosclerosis, cancer and even COVID-19-induced cytokine storm.<sup>22,25,29,199</sup> Chemokines are small chemotactic cytokines responsible for leukocyte migration in tissue and are classified in four families depending on the spacing of their cysteine residues on their *N*-terminus namely: CC, CXC, CX<sub>3</sub>C, XC.<sup>79</sup> They trigger cell migration through GPCRs binding, thus driving inflammation and tissue homeostasis.<sup>79</sup> Chemokines and their corresponding receptors are validated promising targets for pharmaceutical research but have mostly failed to deliver successful medicine in the clinic.<sup>111</sup> It is thought that this is mainly imputable to the incredible redundancy of the chemokine network, providing it with resilience.<sup>104</sup>

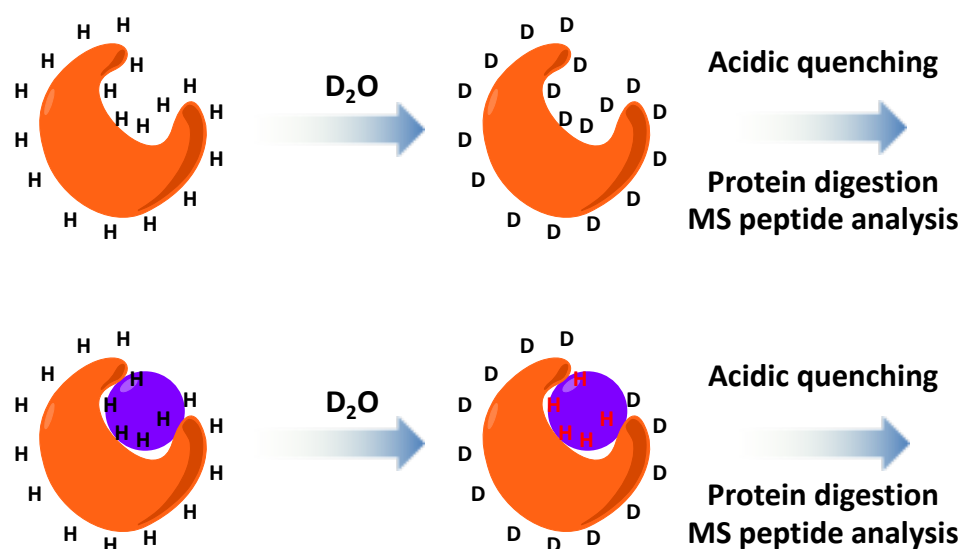
Natural convergent evolution in many pathogens such as helminths, viruses and ticks yielded structurally unrelated proteins that are able to bind multiple chemokines.<sup>182,200,201</sup> This implies that the ability to block several chemokines has an evolutionary advantage to disable the host's immune response. Such promiscuous chemokine-binding proteins found in ticks called Evasins, originally discovered in the Proudfoot laboratory, are of particular interest for therapeutic development targeting the chemokine network.<sup>185</sup> In fact, they showed great efficacy *in vivo* in preclinical models against myocardial ischemia, colitis, reperfusion injury, intestinal ischemia, lung inflammation, psoriasis, acute pancreatitis, and arthritis.<sup>185</sup> Over 40 Evasins were identified

and classified into two classes: class A, Evasins exclusively binding CC type chemokines and class B, Evasins exclusively binding CXC type chemokines.<sup>182</sup>

Unfortunately, the clinical translation of these tick saliva proteins as anti-inflammatory agents has been limited by the risk of foreign protein immunogenicity, parenteral delivery and high manufacturing cost.<sup>202,203</sup> However, the discovery of a novel Evasin P672<sup>198</sup> and the identification of a chemokine binding region within its *N*-terminus raised the opportunity to design peptides or peptidomimetics that could overcome some hurdles for the pharmaceutical development of Evasins.<sup>204</sup>

Previous work on the Evasin P672/CCL8 interface was performed by Dr. Lucia Geis-Asteggianti and Dr. James Eaton. These results are summarised in the next three sections (Sections 2.1.1, 2.1.2, and 2.1.3) and form the basis of the work described in this chapter.

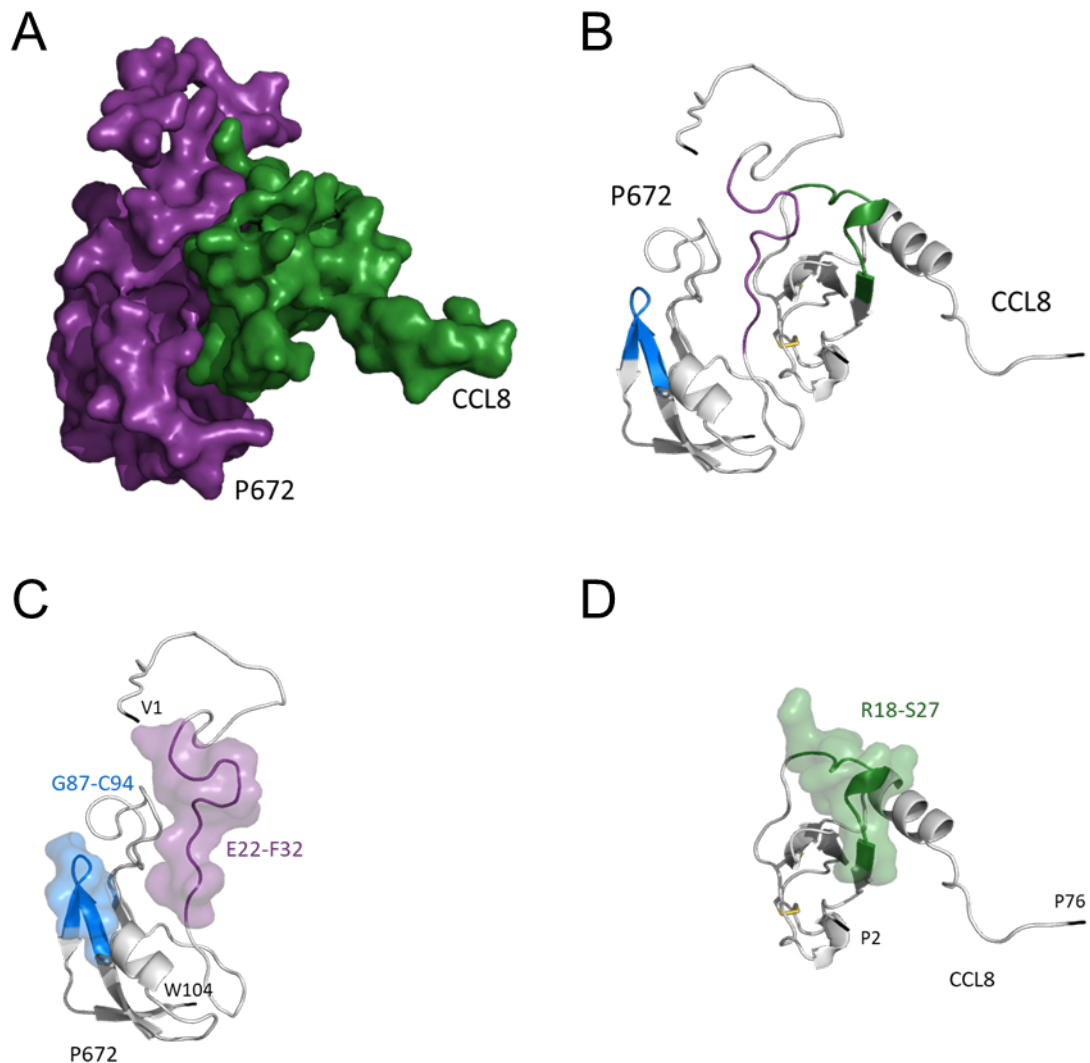
### 2.1.1 Identification of the Evasin P672/CCL8 interface



**Figure 2.1** Illustration summarising HDxMS experiment principle  
*Protein of interest alone and in complex are analysed in parallel. Decrease in uptake of deuterium from the free protein to the complexed protein usually indicates areas of interaction.*

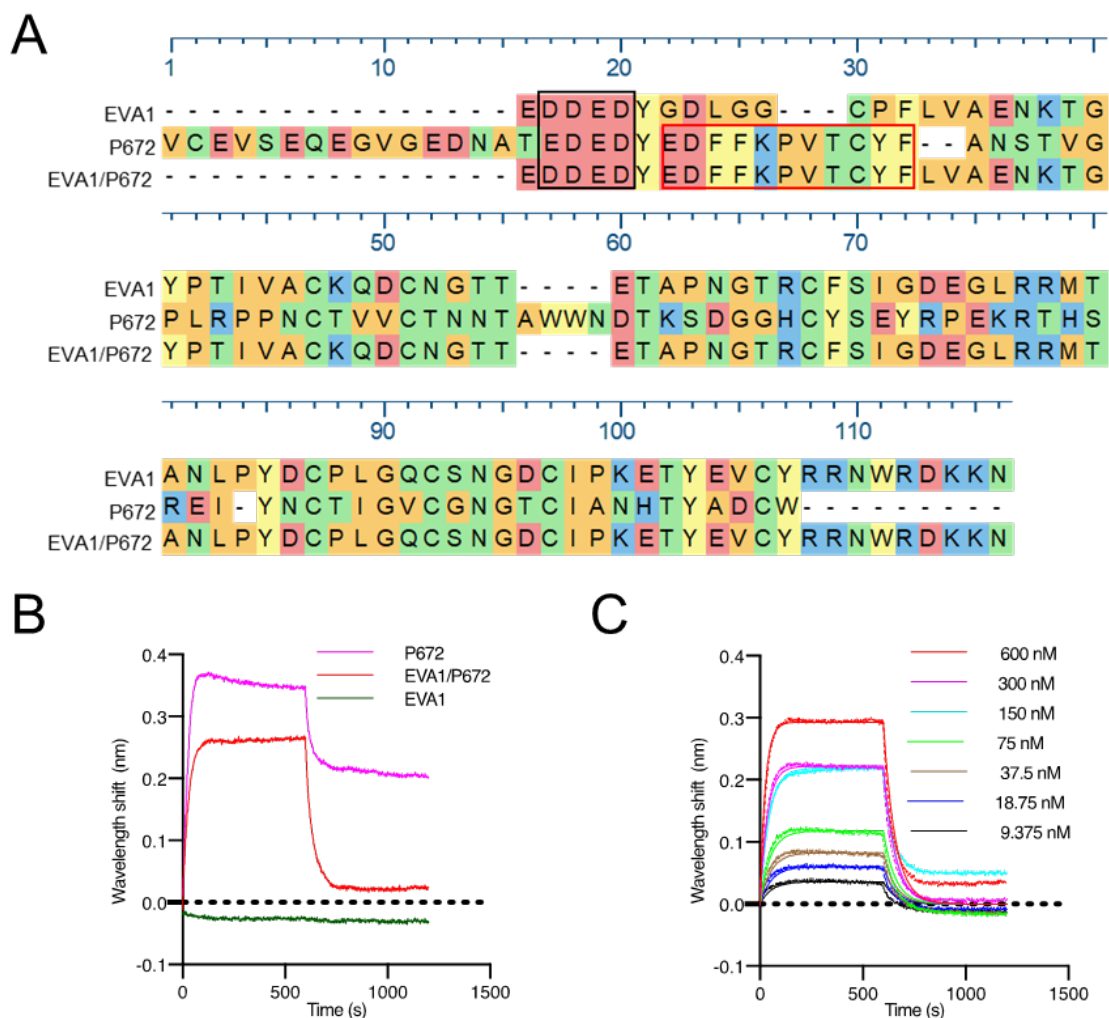
Peptide resolution hydrogen-deuterium exchange mass spectrometry (HDxMS) using deglycosylated P672 and commercial CCL8 enabled the initial characterisation of their protein-protein interaction. HDxMS is a technique measuring the rate of exchange of protein backbone hydrogens with deuterium present in excess in solvent ( $D_2O$ ) (Figure 2.1).<sup>205</sup> Differences in rate of uptake between free and complexed protein can provide information on protein-protein interfaces.<sup>206</sup> Residues that are involved in the binding interface, typically have a lower rate of deuterium exchange for backbone amide hydrogen atoms because they are engaged in interprotein hydrogen bonding rather than protein-solvent hydrogen bonding (Figure 2.1).<sup>207</sup> Free CCL8 and free P672 deuterium uptake were measured (satisfactory sequence coverage of P672: 100% and CCL8: 96.9%) and then compared to the P672/CCL8 complex deuterium uptake measurements at 5 sec, 30 sec, 5 min, and 60 min incubation times. These results were then mapped based on a previously published homology model of the P672/CCL8 complex<sup>198</sup> (Figure 2.2 A and B). Most of P672 and CCL8 showed no significant change in HDx rates. However, the region of CCL8 from Arg18 to Ser27 displayed significant decrease in HDx (% relative deuterium uptake (%D) from -6 to -18) (Figure 2.2 D). Additionally, a substantial region of P672, located on its *N*-terminal unstructured region from Glu22 to Phe32, showed significant decrease in HDx (%D up to -58%) (Figure 2.2 C). Most of these residues appeared to be protected at all times during the experiment, indicating that the interface between P672 and CCL8 lies in these residues. Furthermore, an increase in HDx was noticed on the C-terminal region of P672 (from Gly87 to Cys94, %D from 15% to 18%), indicating a higher exposure to solvent upon complexing with CCL8 pointing to a potential conformational change upon binding CCL8 (Figure 2.2 C). These results indicate that the interface is formed between P672 (Glu22-Phe32) and CCL8 (Arg18-Ser27). This region fits the predicted binding interface from the

homology model and the little change in deuterium uptake in those regions is in line with the tight-binding of P672 to CCL8 ( $K_d = 8.5 \text{ nM}$ ).<sup>198</sup>



**Figure 2.2** P672/CCL8 interface characterised using HDxMS  
**(A)** Surface representation of the homology model used to map HDxMS results, P672 (purple) and CCL8 (green). **(B)** Ribbon representation of P672/CCL8 complex with deuterium-exchange-protected region highlighted in P672 (purple) and CCL8 (green) as well as deuterium-exchange-exposed region highlighted in P672 (blue). **(C)** Ribbon representation of P672, deuterium-exchange-protected region (Glu22-Phe32) highlighted in purple and deuterium-exchange-exposed region (Gly87-Cys94) highlighted in blue with interface surface. **(D)** Ribbon representation of CCL8, deuterium-exchange-protected region (Arg18-Ser27) highlighted in green with surface interface. Figure adapted from reference<sup>1</sup>

To further characterise the function of P672 (Glu22-Phe32), this region was swapped with the corresponding section of Evasin-1 (Figure 2.3), a related Evasin that is unable to bind CCL8 while its parent Evasin-1 binds CCL8<sup>198</sup>, creating the chimeric Evasin: EVA1(P672<sub>22-32</sub>). Biolayer interferometry (BLI) revealed that the chimeric EVA1(P672<sub>22-32</sub>) was able to bind CCL8 while its parent Evasin-1 was not. Dose-response titration using BLI experiment showed that EVA1(P672<sub>22-32</sub>) bound CCL8 with a moderate affinity ( $K_d = 490$  nM) compared to P672 ( $K_d = 8.5$  nM).<sup>198</sup> These results confirmed that P672<sub>22-32</sub> alone was able to carry CCL8 binding properties.

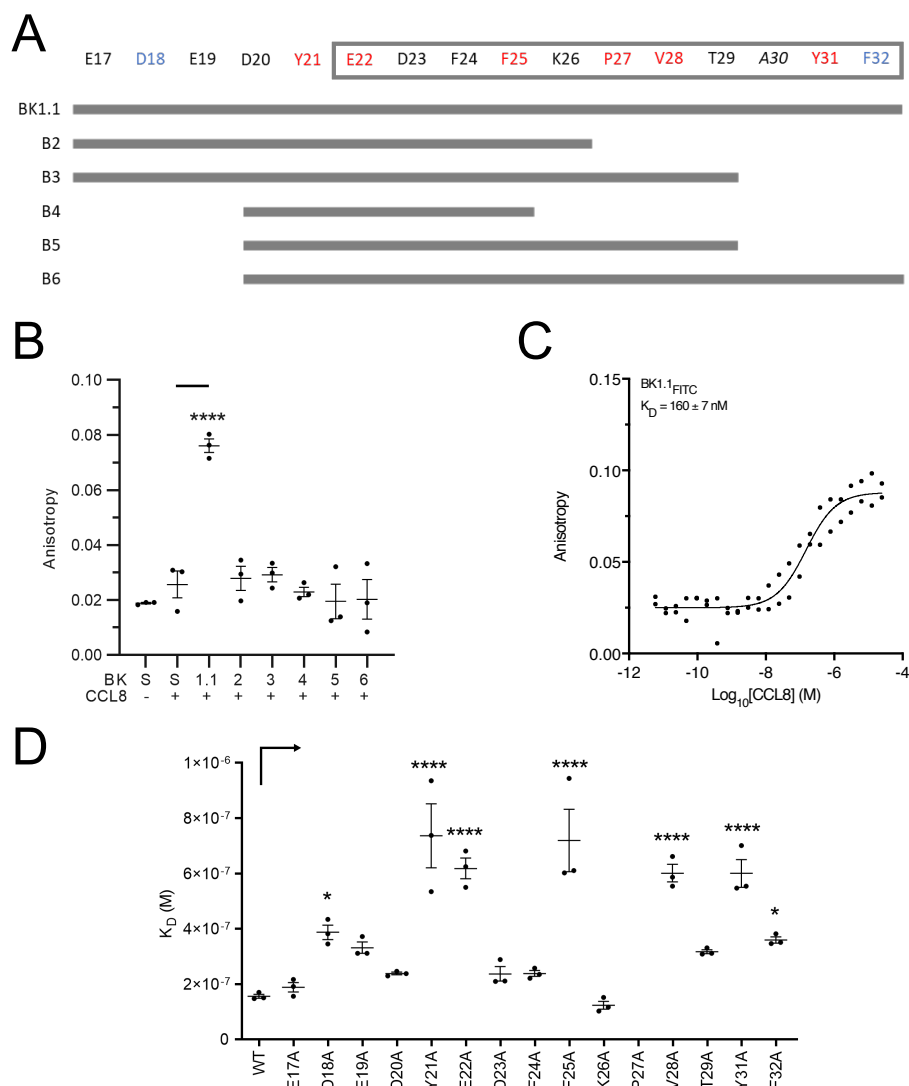


**Figure 2.3** Chimeric Evasin-1 design and analysis

**(A)** Alignment of Evasin-1 (EVA1), Evasin P672 (P672) and chimeric EVA1(P672<sub>E22-F32</sub>) (EVA1/P672) using the MUSCLE algorithm. P672 HDxMS protected region is in the red box and the conserved acidic patch on EVA1 and P672 is boxed in black. **(B)** BLI sensogram with P672, EVA1/P672 and EVA1 immobilised on sensor and exposed to 600 nM of CCL8. **(C)** BLI sensograms of EVA1/P672 immobilised on sensor and exposed to a range of concentration of CCL8. Showing collected data (dotted lines) and model (solid lines). Figure adapted from reference<sup>1</sup>

### 2.1.2 Development of CCL8 binding peptide BK1.1

Using the information provided by the HDxMS experiment, a range of *N*-terminally FITC labelled peptides, covering P672 protected region (Glu22-Phe32) and the acidic patch including Tyr21 (conserved in Evasin-1), were commercially sourced (Figure 2.3 A). To avoid potential dimerisation, all Cysteine residues were mutated to Alanine. These peptides were tested in a fluorescence polarisation assay against CCL8 and only the full contiguous peptide (Glu17-Phe32, C30A) showed significant binding (at 1  $\mu$ M) compared to the scrambled peptide control (FITC-EFTEVYEFDFKYDAPD, SCR<sub>FITC</sub>) (Figure 2.4 B). This peptide was termed BK1.1<sub>FITC</sub> and tested in fluorescence polarisation (FP) assay against CCL8 (Figure 2.4 C) and its affinity was found to be relatively high ( $K_d = 160 \pm 7$  nM, mean  $\pm$  SEM of three biological replicates). A library of BK1.1<sub>FITC</sub> Ala mutants, scanning every position, was tested using FP assay to look at the importance of each residue in its binding affinity to CCL8 (Figure 2.4 D). This experiment pointed to key residues contributing to CCL8 binding. The most significant differences were observed for aromatic residues Tyr and Phe (Tyr21, Tyr31, Phe25 and Phe32) as their Ala mutants showed reduced binding affinity of the whole peptide for CCL8. Asp18 and Glu22 mutants also showed reduced binding affinity to CCL8, indicating the importance of acidic residues at the *N*-terminus of the peptide, and supporting the tiling data that previously showed the importance of the Glu17-Tyr21 segment for CCL8 binding (Figure 2.4 B). Interestingly, P27A mutant abolished binding to CCL8, indicating strongly that it has a key importance for CCL8 binding.

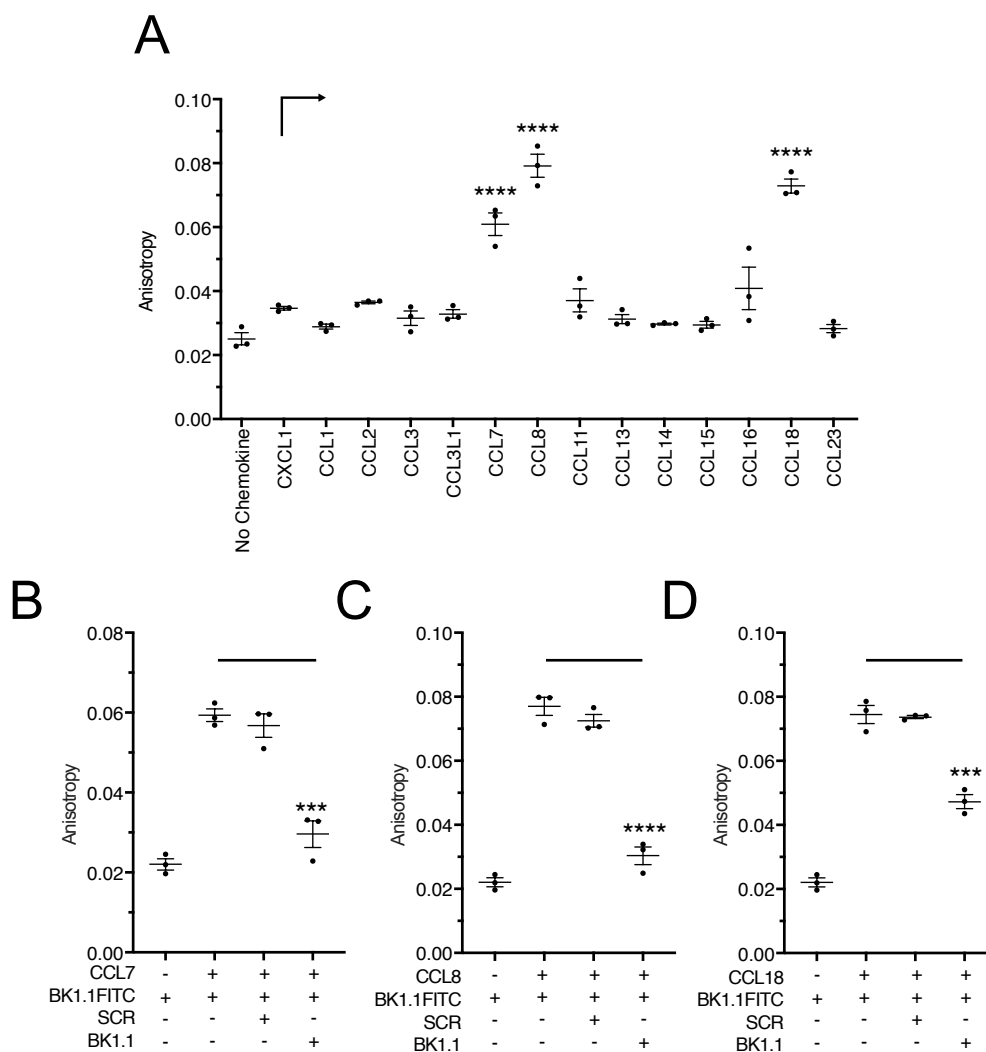


**Figure 2.4** Biophysical analysis of BK1.1 binding to CCL8

**(A)** Design of tiling peptides indicating in the grey box the HDxMS interface region, in red residues showing strong loss of activity and in blue residues showing moderate loss of activity according to the Ala-scan experiment (below). **(B)** Each FITC-labelled peptide (50 nM) incubated with CCL8 (1  $\mu$ M). A scrambled peptide was used as negative control (SCR<sub>FITC</sub> or S). Data presented as means  $\pm$  SEM of three biological replicates. **(C)** Fluorescent polarisation assay for binding affinity of BK1.1<sub>FITC</sub> (50 nM) to CCL8. Binding curve obtained using a one site binding model. **(D)** Fluorescent polarisation assay of alanine-scanning mutagenesis for BK1.1<sub>FITC</sub> on CCL8 binding.  $K_d$  values for each peptide are means  $\pm$  SEM of three biological replicates. The mutant P27A showed no detectable binding. Statistically significant differences (compared with SCR **(B)** or WT **(D)**) was calculated using a one-way ANOVA with Sidak's multiple comparisons test are indicated by asterisks: \*\*\*\*,  $p \leq 0.0001$ ; \*\*\*,  $p \leq 0.001$ ; \*\*,  $p \leq 0.01$ ; \*,  $p \leq 0.05$ . Figure adapted from reference<sup>1</sup>

### 2.1.3 Investigating promiscuous chemokine binding for BK1.1

As BK1.1<sub>FITC</sub> could bind to CCL8, it was also tested, using FP, against all 13 CC chemokines that P672 was previously shown to bind to<sup>198</sup> (Figure 2.5 A). This work revealed that BK1.1<sub>FITC</sub> (50 nM) was able to bind CCL7 and CCL18 (1  $\mu$ M) showing significant increase in anisotropy compared with the negative control CXCL1 (a chemokine that P672 does not bind to). Displacement of BK1.1<sub>FITC</sub> with unlabelled ligand confirmed its binding to CCL7, CCL8 and CCL18 (Figure 2.5 B to D). This data was compared to a scrambled version of BK1.1 (SCR) with the sequence EFTEVYEFDFKYDAPD.



**Figure 2.5** FP assays confirming BK1.1 multiple chemokine binding property  
**(A)** FP assay for the binding of BK1.1<sub>FITC</sub> (50 nM) to CC-chemokine panel (1  $\mu$ M). Data presented as means  $\pm$  SEM of three biological replicates. CC-chemokine binding compared with the negative control (CXCL1). **(B)–(D)** FP competition assay looking at BK1.1<sub>FITC</sub> (50 nM) binding to CCL7, CCL8, and CCL18 (1  $\mu$ M) with or without unlabelled BK1.1 (50  $\mu$ M). Data presented as means  $\pm$  SEM of three biological replicates. Statistically significant differences (compared with SCR) was calculated using a one-way ANOVA with Sidak's multiple comparisons test are indicated by asterisks: \*\*\*\*,  $p \leq 0.0001$ ; \*\*\*,  $p \leq 0.001$ ; \*\*,  $p \leq 0.01$ ; \*,  $p \leq 0.05$ . Figure adapted from reference<sup>1</sup>

## 2.2 Aims

The discovery of BK1.1, inspired by the HDxMS study of P672/CCL8 interface, provided an ideal starting point for further protein-protein interaction (PPI) analysis work and optimisation of chemokine binding peptides.

Aims of the work described in this chapter are to:

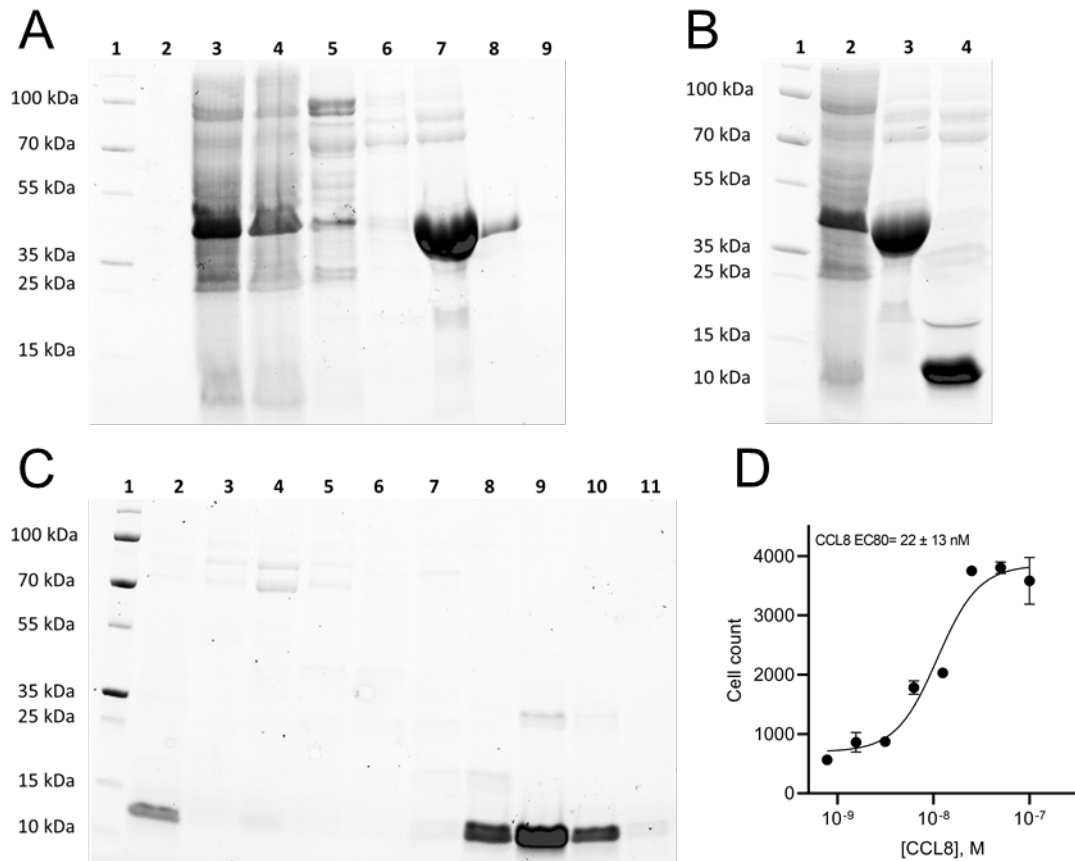
- a. Recombinantly express CCL8
- b. Design peptides from P672/CCL8 interface
- c. Evaluate the chemokine binding profile of such peptides *in vitro*
- d. Assess *in vivo* anti-inflammatory efficacy of generated peptides

## 2.3 Results

### 2.3.1 Expression of biologically functional CCL8

To allow the study of Evasin P672 interaction with CCL8, a large amount of biotinylated, recombinant CCL8 was required to enable assays involving immobilisation of the target on a solid support, such as AlphaScreen, biolayer interferometry (BLI), or surface plasmon resonance (SPR). Additionally, modified or tagged chemokines are expensive and difficult to source commercially. Therefore, CCL8 was expressed as a His-tagged SUMO fusion in a pNIC-BIO3 *E. coli* expression vector (GenBank: JN792439). This vector adds an AviTag on the C-terminus of the expressed protein, allowing for selective enzymatic biotinylation of a specific Lys residue on the AviTag *in vivo*. This can be achieved by co-expressing biotin ligase (BirA) in *E. coli*. The His-SUMO-CCL8 was expressed reasonably in the soluble fraction of Rosetta-Gammi 2 *E. coli*. His-SUMO tagged protein was purified using nickel affinity chromatography, followed by His-SUMO tag cleavage and gel filtration (Figure 2.6 A to C). This purification yielded 10.5 mg of biotinylated CCL8 from 12 L of culture, and the protein was concentrated and stored at 21 mg.mL<sup>-1</sup> (1.8 mM). The recombinantly produced biotinylated CCL8 was characterised using protein mass-spectroscopy (MS) and after deconvolution of the spectra two mass were observed 11526 *m/z* and 11509 *m/z*. The expected mass of the biotinylated construct is [M+H]<sup>+</sup>=11526 *m/z* (Figure 6.1). The peak at 11509 *m/z* has been attributed to a known dehydration event for CCL8, with pyroglutamine formation at the N-terminus occurring spontaneously and conferring increased chemotactic properties.<sup>208</sup> The purified biotinylated CCL8 was then tested on chemokine driven cell migration in a Boyden chamber assay using human monocytic THP-1 cells (Figure 2.6 D). Its EC<sub>80</sub> (22 ± 13 nM) was found to

be comparable to the commercial CCL8 (Peprotech:  $EC_{80} = 5.75$  nM, data not shown) tested in the same assay, indicating that the protein was folded and functional.



**Figure 2.6** Biotinylated CCL8 purification and characterisation

**(A)** SDS-PAGE gel stained using colloidal Coomassie blue showing: 1: Molecular weight ladder, 2: Empty, 3: Cell lysate, 4: Nickel affinity chromatography flow through, 5: Nickel affinity chromatography wash, and 6-9: Nickel affinity chromatography elution fractions (1 to 4). **(B)** SDS-PAGE gel stained using colloidal Coomassie blue showing: 1: Molecular weight ladder, 2: Sonication supernatant, 3: Nickel affinity chromatography elution and 4: Nickel affinity chromatography elution after over-night SUMO cleavage. **(C)** SDS-PAGE gel stained using colloidal Coomassie blue showing: 1: Molecular weight ladder, 2: Gel filtration input (Nickel affinity chromatography elution after over-night SUMO cleavage) and 3-1: Gel filtration elution fractions (1 to 9). **(D)** Human monocytic THP-1 cell migration in response to increasing concentrations of biotinylated CCL8. Three technical replicates with SEM are shown fitted to a four-parameters binding model. CCL8-AviTag: 11.5 kDa and His<sub>6</sub>-SUMO-CCL8-AviTag: 25.3 kDa.

### 2.3.2 Design of more potent CC chemokine binding peptides

To develop and optimise chemokine-binding peptides, it is important to have direct binding assays, providing proof of binding as well as binding constants for direct comparison of the derivatives. Ideally, these assays would have the capabilities to be used in high-throughput manner with low reagent requirements.

Fluorescence polarisation assays are very efficient in delivering binding affinity data, using reasonable amount of protein (typically low  $\mu\text{M}$  concentration) and peptides (typically 50-100 nM concentration), although they require fluorescently labelled peptides that are expensive to synthesise. Unlabelled peptides can then be tested to displace the binding probe, providing some means for structure-activity relationship (SAR) analysis. However, this is entirely dependent on displacing the probe, and additionally, the probe may not bind to multiple chemokines. The effect of the fluorophore on the binding of a certain peptide to a certain protein may also differ depending on the peptide. Our initial probe, BK1.1<sub>FITC</sub>, is a weak binder and only binds to a limited number of chemokines by FP, therefore its use was limited for extensive SAR analysis of new chemokine-binding peptides. Thus, FP assay was not used to assess peptide binding to multiple chemokines.

In order to screen multiple peptides for affinity against specific chemokines, we initially envisaged using BLI assay, where chemokines are immobilised to streptavidin sensors and peptide ligand binding is measured. The BLI technique was used effectively for screening Evasins, and has the advantage of using small amount of protein and peptides while producing very high quality binding data.<sup>198</sup> Unfortunately, despite extensive buffer and assay optimisation for BK1.1 binding to CCL8, robust peptide-binding signals were not observed on BLI, likely due to assay interference or peptide ligand size (data not shown,

Table 2.1). The signal that was observed was non-specific, which might indicate that the highly negatively charged peptides we developed are not suitable for use in BLI experiments. Therefore, BLI was not used further to analyse chemokine binding peptides.

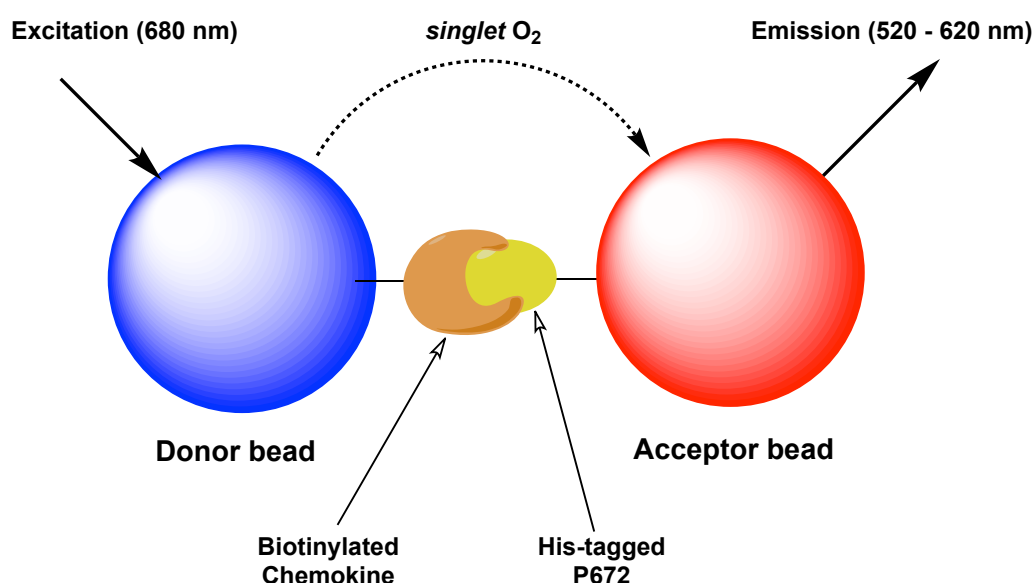
| Buffer system | [NaCl] (mM) | BSA (w/v) | Tween20 (v/v) | [Peptide]  |
|---------------|-------------|-----------|---------------|------------|
| PBS           | 137         | 0.1%      | 0.1%          | 10 $\mu$ M |
| PBS           | 137         | 0.1%      | 0.1%          | 100 nM     |
| PBS           | 137         | 0.1%      | X             | 100 nM     |
| PBS           | 137         | X         | 0.1%          | 100 nM     |
| HEPES 20 mM   | 150         | 0.1%      | 0.05%         | 10 $\mu$ M |
| HEPES 20 mM   | 150         | 0.1%      | 0.05%         | 1 $\mu$ M  |
| HEPES 20 mM   | 50          | X         | X             | 5 $\mu$ M  |
| HEPES 20 mM   | 50          | X         | X             | 1 $\mu$ M  |

**Table 2.1** BLI optimisation attempts summarised

*SuperStreptavidin sensors (ForteBio) were used for each experiment. Biotinylated CCL8 (in-house production) was charged on sensor until 2 nm before attempting recording sensogram of peptides binding to chemokine. Binding data recorded was non-specific for all these experimental conditions.*

AlphaScreen (AS) assay relies on the proximal interaction of an acceptor bead and a donor bead both of which are able to bind different interaction partners (Figure 2.7).<sup>209</sup> Light excitation at 680 nm of phthalocyanine (photosensitiser) in the donor bead converts ambient oxygen into singlet oxygen. If the acceptor bead is in close proximity, the singlet oxygen reacts with the thioxene derivative in the acceptor bead to generate a chemiluminescence at 370 nm. The energy transfer to the fluorescence acceptor within the same bead then shifts the emission, ultimately emitting light at 520-620 nm, which can be measured as a function of the binding affinity of the immobilised partners (Figure 2.7). Therefore, increase in signal indicates a larger portion of bound partners and a decrease in signal a smaller portion of bound partners. They are very sensitive assays requiring small amount of protein material (typically single digit nM protein concentration). As all peptides described herein are derived from Evasin P672 binding region to CCL8, the AS assay was set

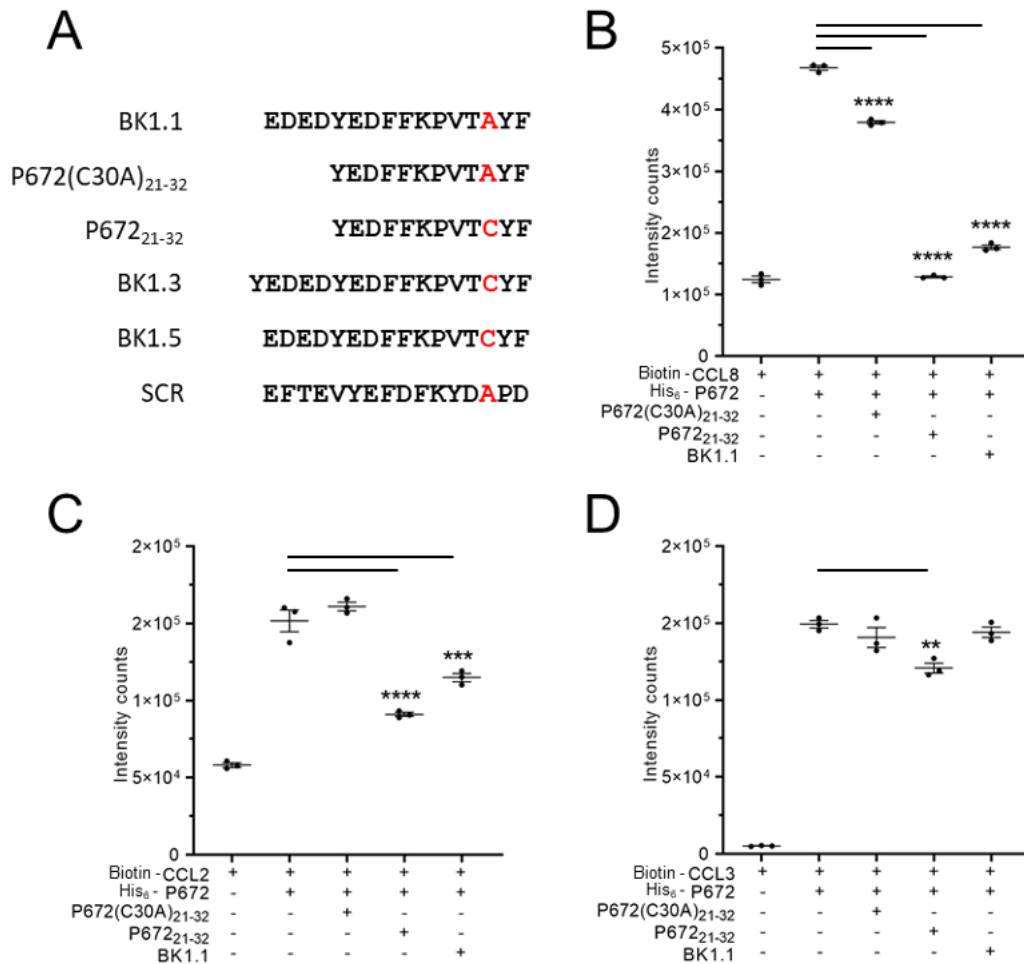
up to measure the displacement of the Evasin P672 and a specific chemokine interaction to allow comparative affinity determination of different P672-derived peptides against a specific chemokine. Therefore, for the purpose of this work, peptide affinity to chemokines were mostly assessed using AS displacement assay of P672/chemokine interaction using His<sub>6</sub>-P672 and biotinylated chemokines, using nickel donor and streptavidin acceptor bead pair.



**Figure 2.7** Illustration summarising AlphaScreen experiment principle

To understand the importance of the acidic patch *N*-terminal to Tyr21 and the effect of the C30A mutation in BK1.1, two more shorter peptides were designed only containing Tyr21-Phe32 with either Ala30 (P672(C30A)<sub>21-32</sub>) or Cys30 (P672<sub>21-32</sub>) (Figure 2.8 A). These peptides and BK1.1 were tested (single concentration of 100  $\mu$ M) in AS displacement assay looking at their potential of disruption of the CCL8/P672 complex. All three peptides showed significant disruption of the CCL8/P672 complex with P672<sub>21-32</sub> and BK1.1 having a much stronger effect (Figure 2.8 B). The same peptides were then tested in similar AS displacement assays set up against the CCL2/P672 and CCL3/P672 complex (Figure 2.8 C and D). P672<sub>21-32</sub> and BK1.1 showed moderate but significant disruption of the

CCL2/P672 complex and only P672<sub>21-32</sub> showed disruption of the CCL3/P672 complex. These results confirmed the importance of the *N*-terminal acidic patch for chemokine/P672 disruption activity and that the Cys30 was also important.

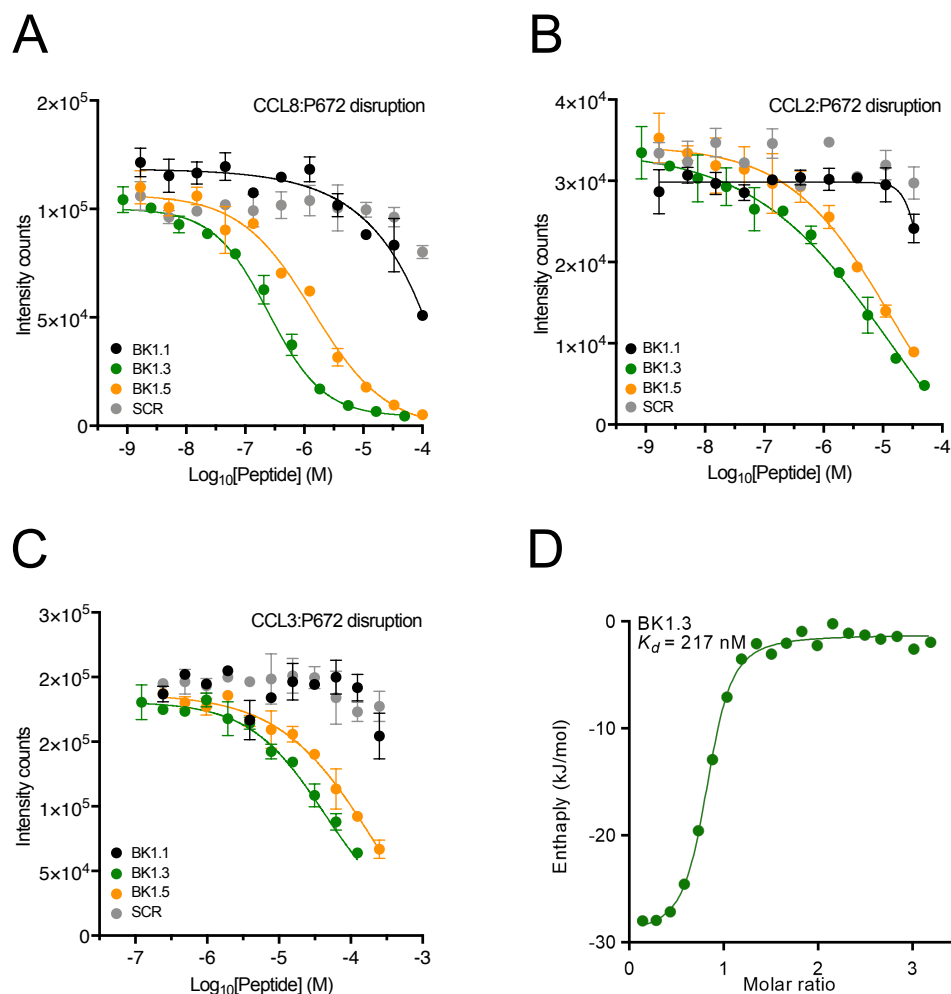


**Figure 2.8** BK1.1 derivatives design

**(A)** Sequences of peptides studied. **(B)-(D)** AlphaScreen displacement assay representing the effect of peptides (100  $\mu$ M) on the His<sub>6</sub>-P672 (CCL8: 2.5 nM, CCL2: 5 nM, CCL3: 1.25 nM) and CCL8-biotin (1.25 nM), CCL2-biotin (5 nM) or CCL3-biotin (2.5 nM). Biotinylated CCL2 and CCL3 were purchased (Almac). Data represented as mean  $\pm$  SEM of three biological replicates. Statistical significance (compared with chemokine-biotin + His<sub>6</sub>-P672) was calculated using a one-way ANOVA with Sidak's multiple comparisons test are indicated by asterisks: \*\*\*\*,  $p \leq 0.0001$ ; \*\*\*,  $p \leq 0.001$ ; \*\*,  $p \leq 0.01$ ; \*,  $p \leq 0.05$ . Figure adapted from reference<sup>1</sup>

This guided the design and synthesis of further peptides BK1.3 and BK1.5 based on BK1.1 (Figure 2.8 A), that contains the acidic patch and a Cysteine residue. All three peptides were synthesised using solid phase peptide synthesis (SPPS) with standard Fmoc chemistry supported on rink amide resin (Section 6.2.7). All peptides were obtained with C-terminal amide, purified using preparative HPLC (>90% purity, identity confirmed using HRMS, see Section 6.2.7) and lyophilised. For accurate peptide quantitation by NMR, peptide stocks were dissolved in  $d_6$ -DMSO. Each stock was then quantified by NMR, using  $d_4$ -3-(trimethylsilyl)propionic acid ( $d_4$ -TSP) as an internal standard. To measure their affinity for CCL8, the peptides were tested in a dose-response AS displacement assay against the CCL8/P672 complex. BK1.1 inhibited CCL8/P672 complex formation with  $^{AS}IC_{50} = 53 \pm 14 \mu M$  (unless otherwise stated data is represented as mean  $\pm$  SEM of three biological replicates, Figure 2.9 A), which was lower in affinity than anticipated from the FP data of BK1.1<sub>FITC</sub> ( $K_d = 160$  nM, Figure 2.4 C). BK1.5 showed a >30-fold improvement in binding affinity ( $^{AS}IC_{50} = 1.6 \pm 0.2 \mu M$ ), confirming the importance of conserving the wild-type Cys at position 30 for affinity. Interestingly, BK1.3 was the most potent binder ( $^{AS}IC_{50} = 480 \pm 60$  nM), indicating that the *N*-terminal Tyr increased its binding to CCL8 (Figure 2.9 A). Further AS displacement assays were established and conducted on CCL2/P672 and CCL3/P672 complexes, showing BK1.1 was unable to disrupt either interaction, consistent with previous FP experiment (Figure 2.5 A and Figure 2.9 B and C). BK1.3 (CCL2/P672  $^{AS}IC_{50} = 6 \pm 4 \mu M$  and CCL3/P672  $^{AS}IC_{50} = 90 \pm 40 \mu M$ ) and BK1.5 (CCL2/P672  $^{AS}IC_{50} = 3.1 \pm 0.8 \mu M$  and CCL3/P672  $^{AS}IC_{50} = 140 \pm 40 \mu M$ ) showed disruption of both complexes although moderate, and not significantly different from each other implying that the *N*-terminal Tyr does not play a major role in binding to CCL2 and CCL3 (Figure 2.9 B and C).

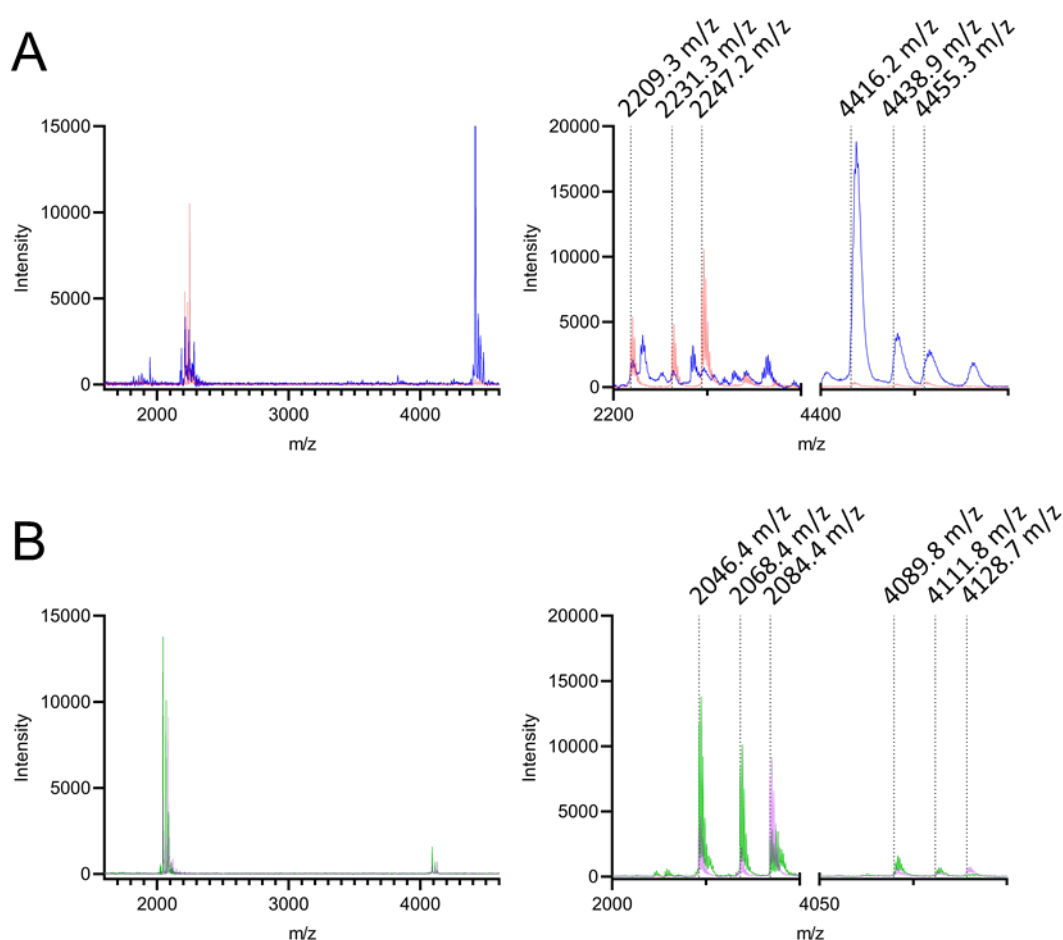
To validate these observations, isothermal titration calorimetry (ITC) was used. This technique is considered as the gold-standard for measuring biomolecular interactions, allowing the determination of thermodynamic parameters for a specific binding event in a label-free manner.<sup>210</sup> Binding of BK1.3 to CCL8 (produced in house with Avi-tag) was assessed using ITC, which confirmed direct binding of BK1.3 to CCL8 ( $K_d = 217$  nM, stoichiometry = 0.78) (Figure 2.9 D). These results are important validation of AS observation as ITC analysis enable a more accurate representative measurement of protein/peptide interactions as this study is label free and independent of a secondary binder, particularly in comparison to AS that requires donor-acceptor bead complex formation and is rank order based.



**Figure 2.9** Biophysical analysis of BK1.3 and BK1.5

**(A)-(C)** Representative dose-response AlphaScreen displacement assay for the disruption of His<sub>6</sub>-P672 (CCL8: 2.5 nM, CCL2: 5 nM, CCL3: 1.25 nM) and CCL8-biotin (1.25 nM), CCL2-biotin (5 nM) or CCL3-biotin (2.5 nM) by BK1.1, BK1.3 and BK1.5 with SCR as a negative control. Biotinylated CCL2 and CCL3 were purchased (Almac). Data represented as mean  $\pm$  SEM of two technical replicates. Data fitted to four-parameters model. **(D)** Isothermal calorimetry of BK1.3 binding to CCL8. Single site model was fitted to the data (solid line). Calculated binding thermodynamic values ( $\pm$  95% confidence interval) are  $K_d = 217 \pm 83$  nM, stoichiometry  $n = 0.776 \pm 0.024$ , enthalpy  $\Delta H = -28.12 \pm 1.59$  kJ/mol and entropy  $\Delta S = 33.26$  J/mol.K. Blank model:  $0.309 \pm 0.109$   $\Delta$ J. Figure adapted from reference<sup>1</sup>

Additionally, as BK1.3 and BK1.5 both have free cysteine residues, their potential to dimerise via disulfide bridge was tested using matrix-assisted laser desorption/ionisation coupled to time of flight mass spectrometry (MALDI-TOF MS) analysis after incubating these peptides in AlphaScreen buffer for 1.5h at room temperature (RT), recreating the conditions of the AlphaScreen assay. Interestingly, it was found that only BK1.3 formed almost completely dimeric species after this incubation, and only a limited portion of BK1.5 dimer was observed (Figure 2.10).



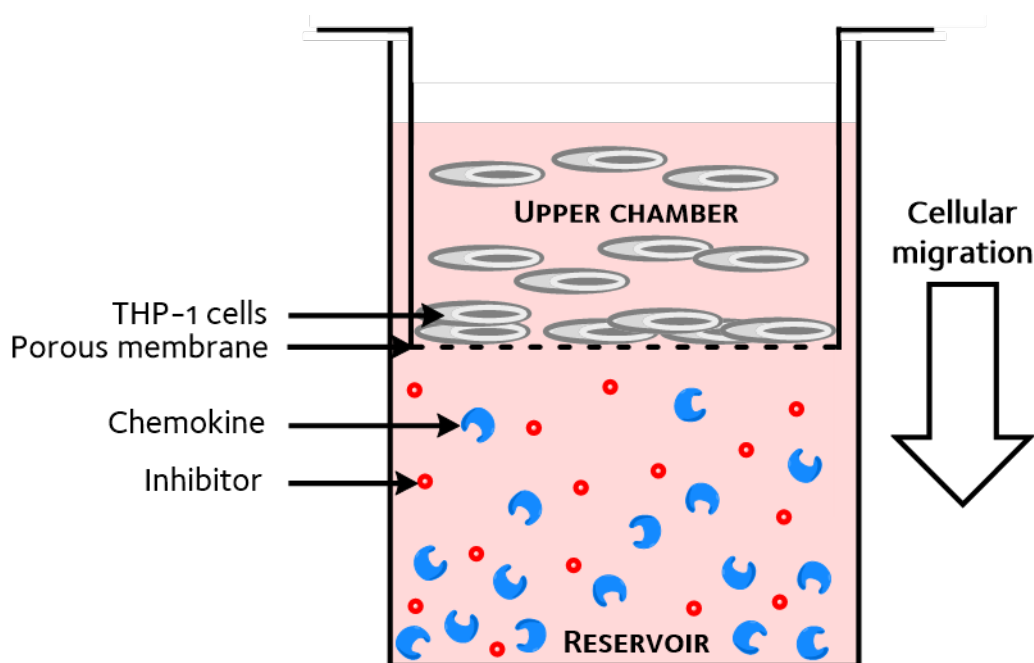
**Figure 2.10** MALDI-TOF MS of peptide dimer identification

**(A)** MALDI-MS spectrum of BK1.3 (20  $\mu$ M) powder stock (red) and after incubation in AS buffer at room temperature for 2h (blue). BK1.3  $[M+H]^+_{exp} = 2209.3$  m/z and  $[M-S-S-M+H]^+_{exp} = 4416.2$  m/z.

**(B)** MALDI-MS spectrum of BK1.5 (20  $\mu$ M) powder stock (purple) after incubation in AS buffer at room temperature for 2h (green). BK1.5  $[M+H]^+_{exp} = 2046.4$  m/z and  $[M-S-S-M+H]^+_{exp} = 4089.8$  m/z.

### 2.3.3 Evasin inspired peptide: BK1.3 neutralise chemokine activity

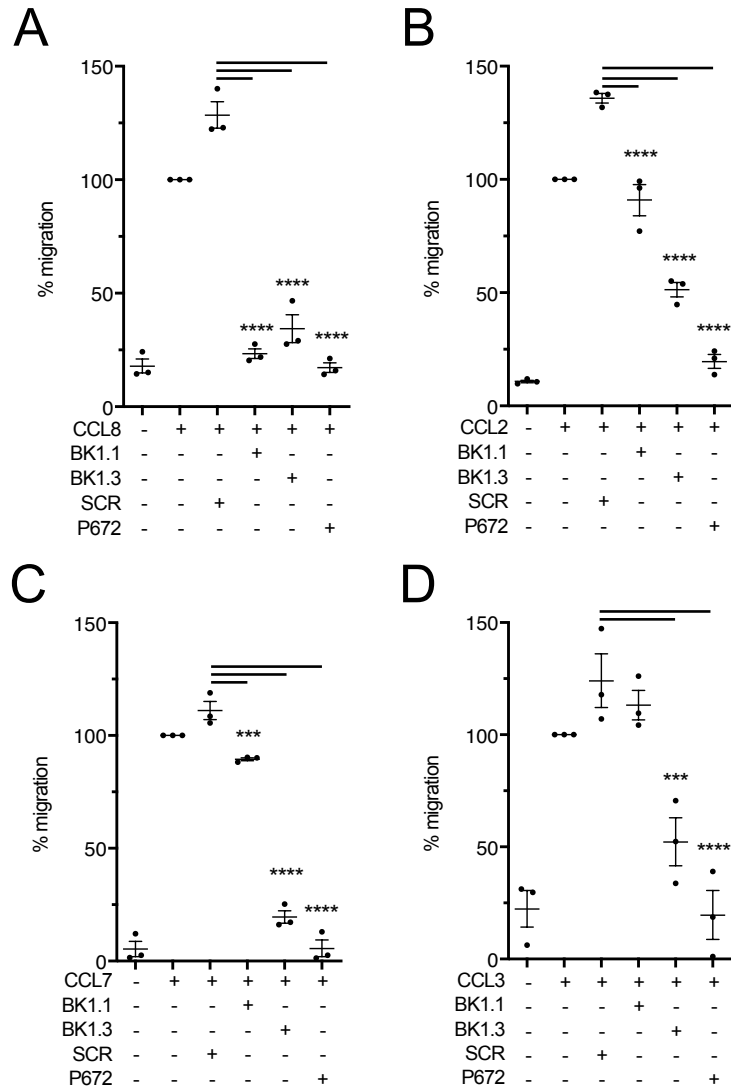
Next, the most potent hit peptide BK1.3 was tested in a cellular migration assay, alongside BK1.1 and P672 on CCL8-, CCL7-, CCL3- and CCL2-induced cell migration (Figure 2.11). Evasin P672 was previously shown to neutralise all four chemokines in similar assays.<sup>198</sup> Human acute monocytic leukemia cells THP-1<sup>211</sup> were used as they express CCR1, CCR2 and CCR5 on their surface<sup>212,213</sup> which are receptors activated by CCL2, CCL7 and CCL8 (though CCL3 activates only CCR1 and CCR5).<sup>214</sup>



**Figure 2.11** Chemokine induced THP-1 cell migration assay principle

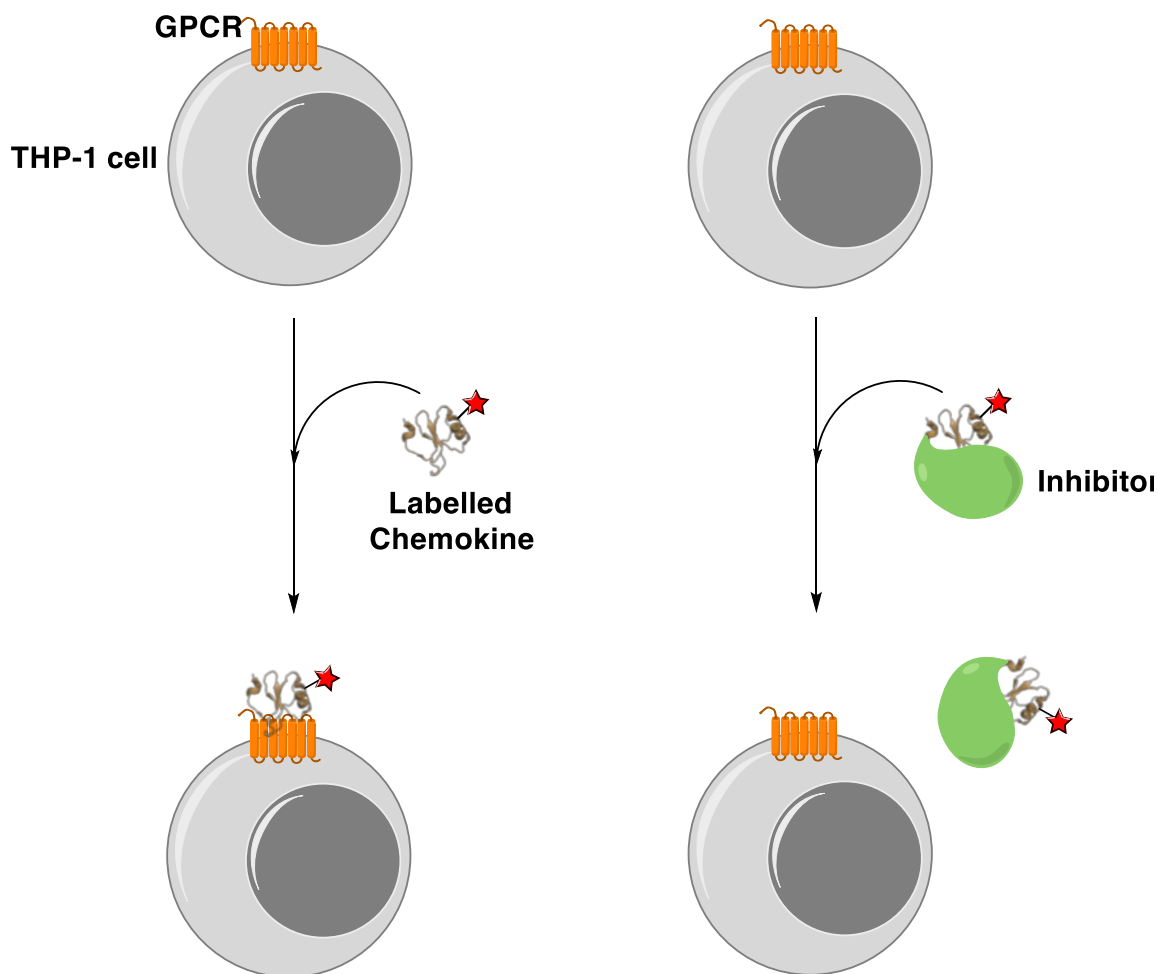
*In the upper chamber THP-1 cells are deposited in suspension in an appropriate cell media. In the reservoir an optimum concentration of chemokine preincubated with varying concentrations of inhibitors in matched cell media. THP-1 cells can migrate in response to chemokine stimulus through the porous membrane of the upper chamber. After a set amount of time, cells that migrated to the reservoir are counted using flow cytometry. This number of cells is representative of the chemotaxis strength of the studied chemokine and the inhibitory effect of the inhibitor molecule present.*

Peptides were tested at a single concentration (10  $\mu$ M) in parallel with P672 (300 nM) as a positive control and SCR as a negative control (Figure 2.12). BK1.1 was shown to neutralise CCL8 chemotactic activity effectively by reducing the number of cells attracted to the reservoir compared to the control without BK1.1. However, it only moderately neutralised CCL7-induced cell migration (Figure 2.12 A and C). This correlated with the ability of BK1.1<sub>FITC</sub> to bind those chemokines by FP (Figure 2.5). It had no significant effect on CCL3-induced cell migration (Figure 2.12 D). However, it showed a modest but significant neutralisation of CCL2-induced cell migration (at 10  $\mu$ M) (Figure 2.12 B). Similar to BK1.1, BK1.3 was able to inhibit CCL8-induced cell migration (Figure 2.12 A) but had a much stronger effect than BK1.1 on CCL2- and CCL7-induced cell migration (Figure 2.12 B and C). Additionally, BK1.3 was able to inhibit CCL3-induced cell migration to a lesser extent (Figure 2.12 D). These results are in line with the biophysical results previously obtained for BK1.3, confirming its increased potency and promiscuity in binding and neutralising chemokines.



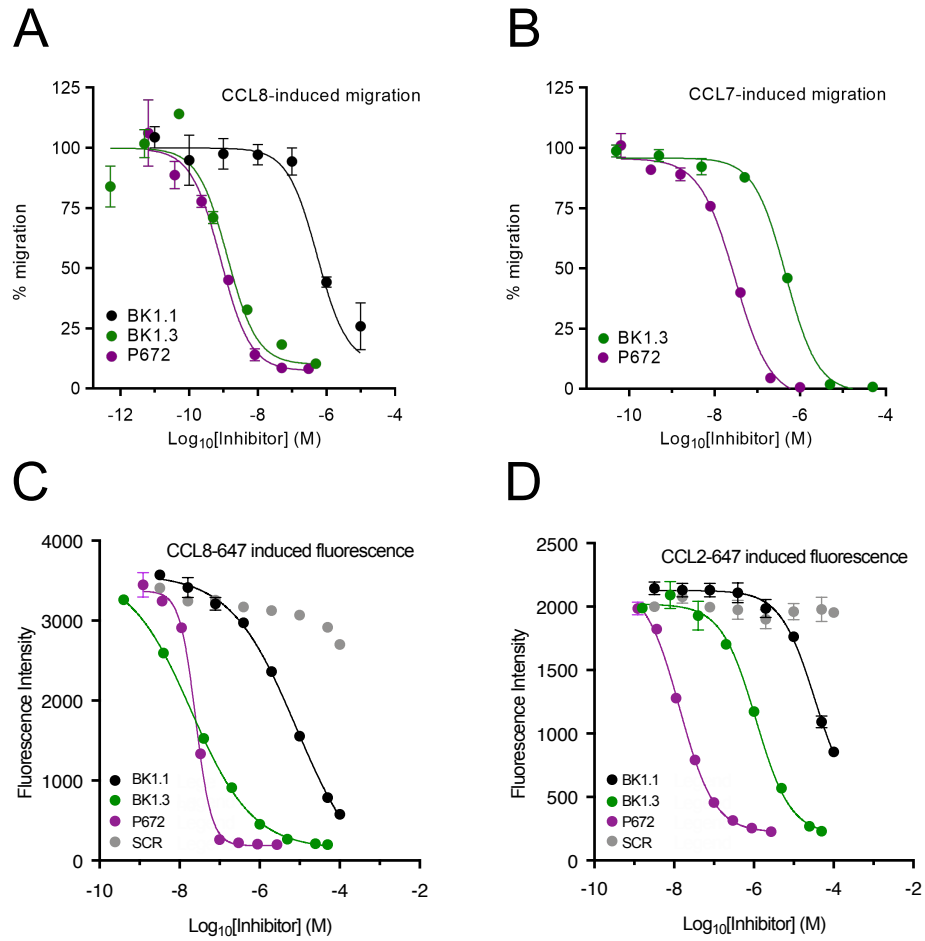
**Figure 2.12** Cell based assessment of BK1.3 chemokine neutralising potential  
**(A)-(D)** Boyden chamber chemokine driven THP-1 cell migration assays looking at the effect of a single dose of chemokine neutralising peptide (10  $\mu$ M) and Evasin P672 (300 nM) as a positive control. All data normalised to cell count of chemokine stimulation alone and compared to cell count of SCR arm (negative control). Experiment conducted for, respectively, CCL8 (5.8 nM), CCL2 (1.2 nM), CCL7 (7.2 nM) and CCL3 (3.5 nM) at their  $EC_{80}$ . Data represented as mean  $\pm$  SEM of three biological replicates. Statistically significant differences (compared with chemokine alone) was calculated using a one-way ANOVA with Sidak's multiple comparisons test are indicated by asterisks: \*\*\*\*,  $p \leq 0.0001$ ; \*\*\*,  $p \leq 0.001$ ; \*\*,  $p \leq 0.01$ ; \*,  $p \leq 0.05$ . Figure adapted from reference<sup>1</sup>

To explore the chemokine-neutralisation potential of BK1.3, dose-response experiments were performed. Against CCL8-driven cell migration BK1.1, BK1.3 and P672 were found to have  $^{THP-1}IC_{50}$  values of  $460 \pm 70$ ,  $7 \pm 4$  and  $2.6 \pm 0.9$  nM respectively (Figure 2.14 A). Against CCL7-driven cell migration, BK1.1 was shown to have no effect in the single dose experiment and therefore only BK1.3 and P672 were tested for dose-response, with  $^{THP-1}IC_{50}$  values of  $500 \pm 100$  and  $30 \pm 10$  nM respectively (Figure 2.14 B). Furthermore, to investigate the effect of the BK peptides on the chemokine ligand-cell interaction, a novel assay was developed (by Mr. Graham Davies) where a site-selective fluorophore (AlexaFluor647) labelled chemokine was used (commercially sourced at Almac, site selectively labelled with AlexaFluor647 at Lys75 on both CCL8 and CCL2). The labelled chemokine binds to THP-1 cells and therefore increases cellular fluorescence that can be quantitatively measured using flow cytometry techniques (Figure 2.13). The dose-response experiments of the BK peptides against CCL8-647 and CCL2-647 found that increasing dose of peptides decreased cellular fluorescence (Figure 2.14 C and D).  $^{CCL8-647}IC_{50}$  values for BK1.1 and BK1.3 in CCL8-647 inhibition assay were found to be respectively of  $5.8 \pm 0.8$   $\mu$ M and  $48 \pm 6$  nM. In the same assay P672 was found to have an  $^{CCL8-647}IC_{50}$  of  $21 \pm 2$  nM (Figure 2.14 C). In an analogous assay, BK1.1 and BK1.3 were found to have respectively  $^{CCL2-647}IC_{50}$  values of  $60 \pm 10$   $\mu$ M and  $2.3 \pm 0.1$   $\mu$ M against CCL2-647, whereas P672  $^{CCL2-647}IC_{50}$  value was  $12.9 \pm 0.7$  nM (Figure 2.14 D). Altogether these results show that BK1.3 not only neutralises several chemokines but also neutralises their chemotactic function by blocking chemokines binding to cells. These experiments were carried out by Dr. James Eaton.



**Figure 2.13** Labelled chemokine/receptor inhibition assay principle

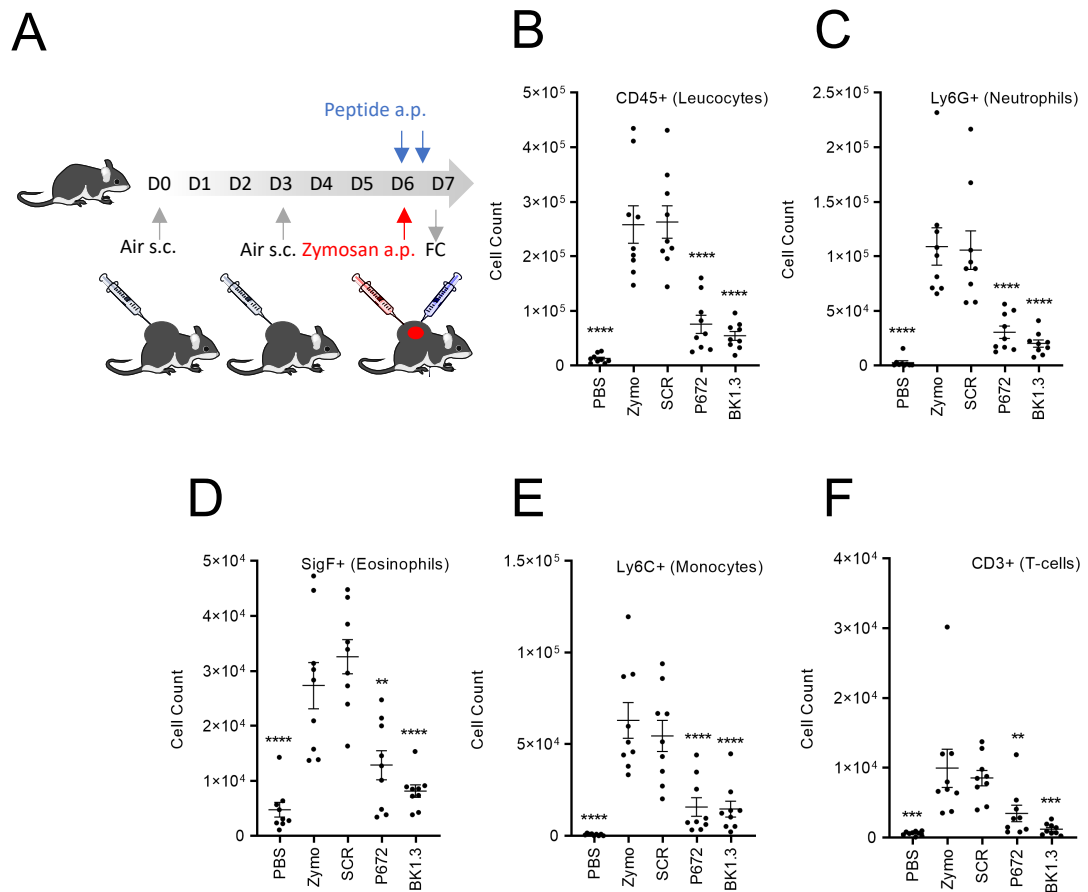
*THP-1 cells are incubated with a set optimised concentration of fluorescently labelled chemokine and varying concentration of inhibitor. After a set amount of time, a set number of THP-1 cells are counted using FACS and the sum of their surface fluorescence is recorded. High amount of fluorescence correlates with high amount of labelled chemokine bound to GPCRs on THP-1 cell surface. Inhibitor of this interaction therefore displays much lower cell surface fluorescence in a dose dependent manner.*



**Figure 2.14** Cell based dose-response experiments assessing BK1.3 potency  
**(A)-(B)** Representative dose-response experimental curve for inhibition of CCL8 and CCL7 induced THP-1 cell migration. Data represented as mean  $\pm$  SEM of three technical replicates. **(C)-(D)** Representative dose-response experimental curve for inhibition of CCL8-647 and CCL2-647 induced cellular fluorescence. Data represented as mean  $\pm$  SEM of two technical replicates. Figure adapted from reference<sup>1</sup>

### 2.3.4 *In vivo* anti-inflammatory activity of BK1.3

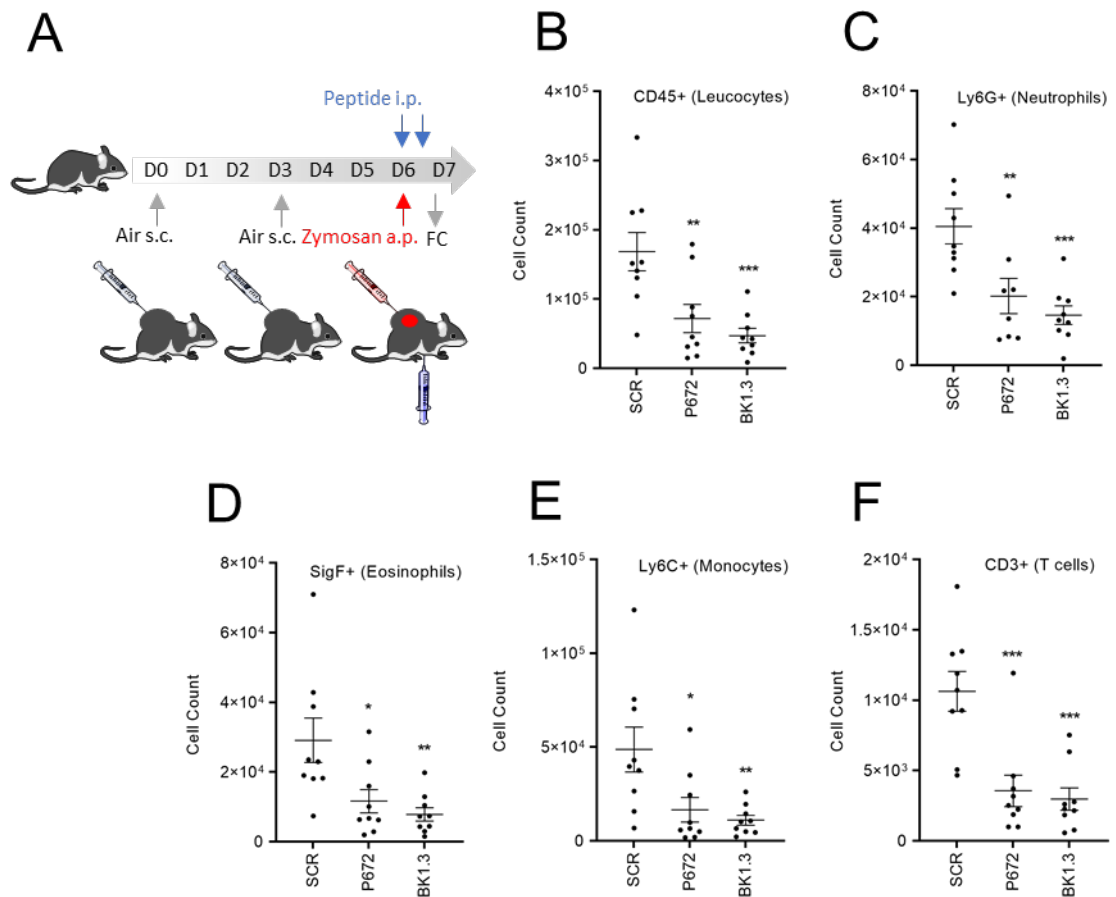
The overall *in vitro* results for BK1.3 suggested that BK1.3 had the potential to exhibit anti-inflammatory properties *in vivo*. BK1.3 was therefore tested in a mouse short-term inflammation model (work carried out by Dr. Gopala Yakala and Dr. Kalimuthu Karuppanan). The assay involves the injection of zymosan, a yeast derived PAMP that stimulates the release of cytokines and chemokines, causing leukocyte migration, in an artificial subcutaneous air pouch.<sup>215,216</sup> This model was characterised and showed that mouse mCCL9 is expressed at high level but not induced by zymosan. Mouse mCCL2, 5, 11, 12, 20, 22 and 24 as well as mCXCL1, 2, 4, 5, 11, 13, and 16 are significantly expressed at 4 h after zymosan injection and mouse mCCL2, 5 and 12 and mCXCL2, 4, 13 and 16 are significantly expressed at 24 h.<sup>1</sup> BK1.3 (5 mg/kg), SCR control (5 mg/kg) and P672 positive control (5 mg/kg) were injected at 0 and 9 h after zymosan injection directly in the air pouch. The dose (5 mg/kg) was chosen as a standard starting point to see effect. Further experiments to investigate optimal dose are underway. The air pouch exudate after 24 h was analysed using flow cytometry to assess the severity and nature of inflammation. BK1.3 and P672 showed a strong significant reduction in the number of neutrophils, eosinophils, monocytes, and T-cells infiltrated in the air pouch (Figure 2.15)



**Figure 2.15** *In vivo* local anti-inflammatory activity of BK1.3

**(A)** Design of the experiment where a dorsal air pouch is created by subcutaneous injection of air at day 0 and day 3. Zymosan or PBS was injected in the air pouch at day 6. BK1.3, SCR or P672 were injected in the air pouch at time of injection of zymosan and 9h later. Exudate from the air pouch were analysed by flow cytometry. **(B)-(F)** Flow cytometry data summary for total leukocytes, neutrophils, eosinophils, monocytes, and T-cells. Data presented as mean  $\pm$  SEM of 9 mice per arm. All datasets compared to Zymosan + SCR arm, statistically significance calculated using a one-way ANOVA with Dunnett's correction for multiple comparisons: \*\*\*\*,  $p \leq 0.0001$ ; \*\*\*,  $p \leq 0.001$ ; \*\*,  $p \leq 0.01$ ; \*,  $p \leq 0.05$ . Figure adapted from reference<sup>1</sup>

A similar experiment, where BK1.3, SCR and P672 were injected intraperitoneally at 0 and 9 h after zymosan injection in the air pouch, was conducted to assess systemic anti-inflammatory effect of BK1.3 and P672. Both compounds displayed similar activity compared to the local injection experiment with substantial reduction in the number of neutrophils, eosinophils, monocytes, and T-cells recruited in the air pouch (Figure 2.16). These results show that BK1.3 has *in vivo* local and systematic activity against inflammation.



**Figure 2.16** *In vivo* distal anti-inflammatory activity of BK1.3

**(A)** Design of the experiment where a dorsal air pouch is created by subcutaneous injection of air at day 0 and day 3. Zymosan or PBS was injected in the air pouch at day 6. BK1.3, SCR or P672 were injected intraperitoneally at time of injection of zymosan and 9h later. Exudate from the air pouch were analysed by flow cytometry. **(B)-(F)** Flow cytometry data summary for total leukocytes, neutrophils, eosinophils, monocytes, and T-cells. Data presented as mean  $\pm$  SEM of nine mice per arm. All datasets compared to Zymosan + SCR arm, statistically significance calculated using a one-way ANOVA with Dunnett's correction for multiple comparisons: \*\*\*\*,  $p \leq 0.0001$ ; \*\*\*,  $p \leq 0.001$ ; \*\*,  $p \leq 0.01$ ; \*,  $p \leq 0.05$ . Figure adapted from reference<sup>1</sup>

## 2.4 Discussion

To date, the development of peptides able to bind and neutralise multiple chemokines have largely focussed on chemokine receptor *N*-terminal tails<sup>217–219</sup> and some high throughput screening using phage display of peptides binding anti-receptor antibodies.<sup>220</sup> Peptides discovered using those methods have yielded relatively poor binding peptides (low micromolar to millimolar affinity) with fairly restricted promiscuity. Recently, a high throughput mRNA display screening including codon reprogramming to incorporate sulfotyrosines known to increase chemokine binding affinity of receptor terminal tails,<sup>89</sup> lead to the discovery of fairly tight binding cyclic peptides to CCR3-activating chemokines.<sup>221</sup> Some peptides derived from the study of CCL5 heterodimer interface were developed and could disrupt heterodimerization of CCL5 with CCL17, CXCL14 and CXCL12.<sup>66</sup> However, these peptides were only able to interact with these specific pairs and were not able to target chemokines in a promiscuous manner. The experiments described herein *vide supra* used the study of the chemokine-binding interface of a novel Evasin: P672, as the starting point for the design of smaller peptides able to bind chemokines promiscuously and capable of producing anti-inflammatory activity *in vivo*.

Previous work from Dr. Eaton and Dr. Asteggianti was critical to identify P672/CCL8 interface. HDxMS was used to identify a stretch of P672 (Glu22-Phe32) that was protected from deuterium exchange upon complexing with CCL8. Swapping that domain in Evasin-1, a CC chemokine binding Evasin unable to bind CCL8, transferred CCL8 binding ability to the chimeric Evasin asserting that this 11-residue region was responsible for CCL8 binding. Although previous homology modelling and domain swap experiments directed attention to the *N*-terminus tail of P672 as being responsible for CCL8 binding, this HDxMS study

highlighted this specific 11-residue region. This study also unveiled a CCL8 region (Arg19-Ser27) protected from deuterium exchange upon complexing with P672. This region covers some of CCL8 N-loop (Cys12-Arg24)<sup>208</sup> known to be involved in receptor binding.<sup>88</sup> This N-loop is targeted by several pathogenic chemokine-binding proteins like the viral VV-35kDa that targets Lys19 and Arg24 on CCL2,<sup>222</sup> and viral vCCI that targets Arg18 and Arg24 on CCL2.<sup>223</sup> As this chemokine neutralising strategy is shared between several species, it shows convergent evolution of these proteins to target this N-loop region. P672 binding to this region would prevent CCL8 binding to its cognate receptors and explain P672 CCL8-neutralising activity. Additionally, the N-loop on CCL8 and other CC chemokines is involved in homodimerization and glycosaminoglycan (GAG) binding<sup>74,88,208</sup> therefore explaining the disruption of CCL8 dimers by P672 previously reported.<sup>198</sup>

Following identification of the chemokine binding region of P672, a library of tiled peptide fragments was screened, revealing that the four acidic residues and Tyr21 are important for CCL8 binding in these conditions or that FITC labelling is hindering binding of shorter peptides. Alanine scan of BK1.1 suggested that Tyr and Phe residues as well as the *N*-terminal acidic residues mediate binding to CCL8. Tyr and Phe are known to be found in protein-protein interactions<sup>224,225</sup> and the charge complementarity that would be mediated by acidic residues on the peptides and basic residues presents on the N-loop region of CCL8 can modulate protein-peptide interaction.<sup>226,227</sup> More interestingly, the sole Pro27 residue in BK1.1 was found to be critical for CCL8 binding. Proline residues are frequently found in turns and are usually known to provide structural scaffolding for proteins and peptides.<sup>228</sup> BK1.1 was then found to bind CCL7 and CCL18 as well as CCL8. It is yet unclear how this promiscuous chemokine-binding mechanism is conveyed in this peptide and will require further structural analysis to understand it fully.

A series of peptides were then designed and revealed that the serendipitous addition of an *N*-terminal Tyr enhanced potency. This could be explained as Tyr are found in protein-protein interaction surfaces,<sup>225</sup> and that sY have been shown to enhance binding of chemokine receptor tails to N-loop of chemokines.<sup>89</sup> Cys30 (involved in a disulfide bridge in P672) reintroduced in BK1.5 showed a significant increase in chemokine affinity compared to BK1.1. Therefore, the enhancement of activity from BK1.3 compared to BK1.5 is most probably due to the introduction of this *N*-terminal Tyr. The spontaneous dimerisation of BK1.3 in aqueous buffer is a strange event which will need further experiments to ascertain this dimer formation (Figure 2.10). The role of the *N*-terminal Tyr is unclear but might provide favourable secondary structure to the peptide for the formation of a dimer. However, if this dimer formation event is real, it might explain the enhanced binding properties of BK1.3 over BK1.5, providing the BK1.3 dimer some avidity. ITC evaluation of BK1.3 binding to CCL8 showed an unusual stoichiometry of binding ( $n = 0.78$ ). As BK1.3 can form dimers with two presumed CCL8-binding sites, it was hypothesised that after the first binding event the second binding site might be partially sterically hindered preventing effective binding of a second unit of CCL8. This might explain an unusual stoichiometry ( $n = 0.78$ ), which lies in between BK1.3/CCL8 1/1 ( $n = 1$ ) and 2/1 ( $n = 0.5$ ).

BK1.3 and BK1.5 showed increased ability to disrupt P672/chemokine interactions by AlphaScreen assays (CCL8, CCL2 and CCL3), which showed promise of chemokine neutralization potential. Cell-based assays confirmed that lead peptide BK1.3 had increased inhibitory effect on CCL8-, CCL2-, CCL3- and CCL7-driven human monocytic THP-1 cell migration. BK1.1 was predicted to only bind to CCL8 and CCL7 by fluorescence polarisation assay, showing that BK1.3 had an increased promiscuity in binding CC chemokines.

Moreover, BK1.3 was shown to inhibit chemokine binding to cells, indicating that the mode of action for chemokine activity neutralisation for these peptides is likely by preventing receptor binding.

Clinical translation for novel anti-inflammatory peptide requires the demonstration of *in vivo* efficacy using preclinical model where several components of the immune-inflammatory network are activated. A short-term inflammation model utilizing Zymozan, a yeast cell surface PAMP, which activates TLR2 signalling<sup>1</sup> resulting in the release of cytokines, chemokines and complement, was developed.<sup>215,216</sup> Experiments using BK1.3 in this model revealed significant inhibition of inflammation when administered locally or distally. The most likely mechanism of action of BK1.3 *in vivo* is the inhibition of signalling by CC chemokines, which are not only involved in the direct recruitment of leukocyte but also can heterodimerise and synergise with a subset of CXC chemokines.<sup>69</sup>

## 2.5 Conclusions

This body of work established the chemokine binding site of Evasin P672 and elucidated its CCL8 neutralisation mechanism. This information was then used to design small peptides with promiscuous chemokine-binding properties able to neutralise several chemokines. BK1.3 was found to have potent anti-inflammatory activity *in vivo* in a short-term preclinical inflammation model. Therefore, this work demonstrates that small peptides designed from studying Evasin/chemokine interaction can be a promising strategy to develop promiscuous chemokine-binding anti-inflammatory therapeutics. The proof of concept that such peptides provide solid *in vivo* anti-inflammatory activity paves the way for further generations of potent anti-inflammatory peptides, against not only chronic inflammatory diseases like atherosclerosis, but also acute inflammation such as in influenza or COVID19-induced cytokine storm.

# Chapter 3 Enhancing clinical tractability of lead peptide

## 3.1 Introduction

Although well validated therapeutic targets, chemokine targeting has mostly failed to deliver active molecules in the clinic.<sup>229</sup> This has been imputed to the robustness of the chemokine network as one ligand can bind to multiple receptors and one receptor can be activated by multiple ligands.<sup>104</sup>

The discovery of a peptide binding multiple chemokines, BK1.3, in Chapter 2, provides a promising starting point for developing a second generation of promiscuous chemokine-binding peptides with *in vivo* efficacy. There are two major routes to improving BK1.3: modifying ADME (Absorption, Distribution, Metabolism, Excretion) properties and increasing binding affinity or specificity.

There are several ways to improve peptide ADME properties, either by modifying the peptide itself or by modifying the mode of administration.<sup>230</sup> Peptides therapeutics are known to have very limited oral bioavailability (typically 1% oral bioavailability with rare exceptions) and therefore require subcutaneous or intravenous injection.<sup>231</sup> After injection peptides enter the blood circulation (peptides with molecular weight <1 kDa) or the lymphatic vessels (molecular weight between 16 and 22 kDa). With most therapeutic peptides being between 1 kDa and 10 kDa, both routes are expected with uptake in blood capillaries as the predominant path.<sup>232</sup> Peptides have therefore been mostly limited to extracellular targets and most of the chemical research has focussed on enhancing their

metabolic stability and reducing their clearance time. Many methods have been used to reduce the proteolysis of peptides with the most favoured routes consisting of modifying *N*- and *C*-termini, insertion of *D*-amino acids, pseudo-peptide bonds, cyclisation and stapling.<sup>233,234</sup> All of these strategies require careful design and often involve a trade-off with peptide-target affinity. Clearance time, mostly renal clearance, is responsible for low *in vivo* half-life of many peptides. Due to renal glomeruli pore size of 8 nm, peptides with molecular weights of 2 to 25 kDa are filtered fairly rapidly. Therefore, strategies for increased clearance time focus on increasing the apparent size of the peptide. This can be achieved by covalently linking peptides to albumin-binding small molecules to increase their apparent size (bound to albumin) in the blood stream, conjugating peptides to long lived plasma proteins (such as fragments of immunoglobulin or albumin) or to large units of polymers (such as polyethylene glycol, hydroxyethyl starch or polysialic acid).<sup>235</sup> Together, these strategies can help tune ADME properties to deliver strong therapeutic peptides in the clinic.

Improving affinity is target dependant. In this particular case, the affinity to particular chemokines is important but maintaining the binding promiscuity is also crucial, as previously exemplified by the high therapeutic potential of Evasins.<sup>236</sup> One avenue for the amelioration of BK1.3 chemokine affinity would be the insertion of sulfated tyrosine (sY) in its sequence. sY is a known post-translational modification of chemokine receptors found at their *N*-terminal tail, which increases chemokine binding affinity.<sup>89</sup> Moreover, it was recently discovered that sY can play a significant role in Evasin binding to chemokines.<sup>235</sup> This discovery, as well as sulfation prediction software, predicted that Evasin P672 could be sulfated at position Tyr21,<sup>235</sup> a Tyr buried in an acidic patch characteristic of tyrosine sulfonation sites.<sup>237</sup> Furthermore, *de novo* sY containing peptides

binding several CCR3-activating chemokines were discovered using reprogrammed genetic code mRNA display, confirming that sY are key residues in chemokine-binding entities.<sup>221</sup>

## 3.2 Aims

In this chapter BK1.3 will be optimised to improve its ADME properties and potency against several chemokines.

Aims of the work described in this chapter are to:

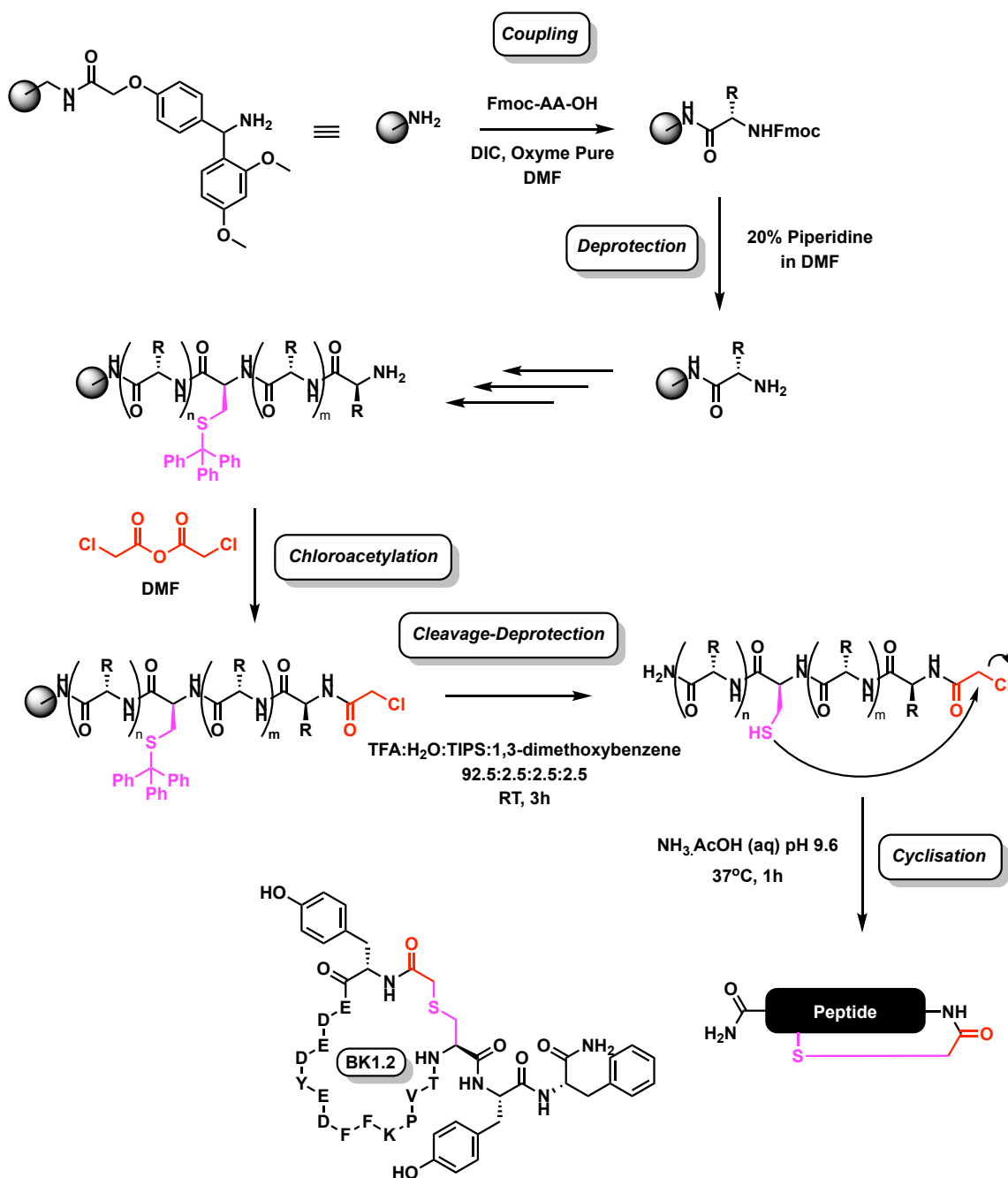
- a. Design cyclic peptides based on BK1.3 and BK1.5
- b. Evaluate the chemokine binding profile of such cyclic peptides *in vitro*
- c. Study the metabolic stability of these peptides
- d. Investigate presence of Tyrosine sulfation in P672
- e. Design sY variants of BK1.3
- f. Evaluate the effect of Tyrosine sulfation on chemokine binding affinity

## 3.3 Results

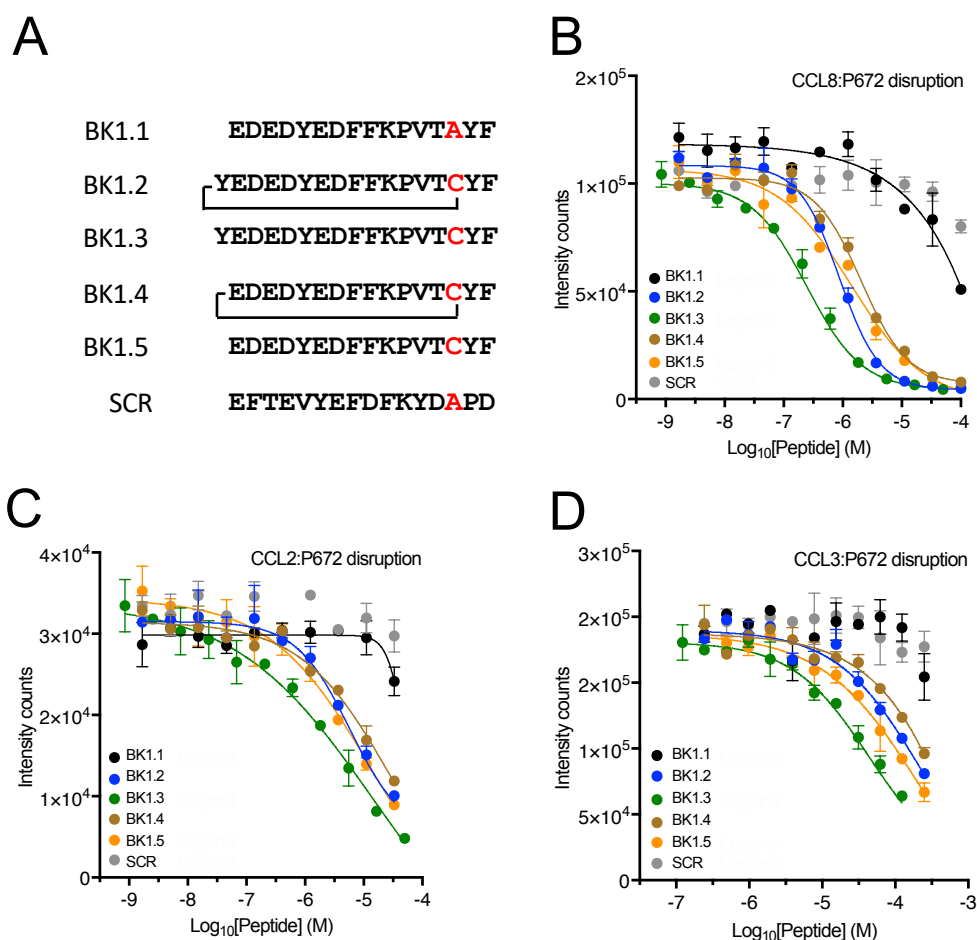
### 3.3.1 Cyclic derivatives of BK1.3 and BK1.5

Cyclisation is well known to increase conformational stability and the previously reported importance of Pro27 in BK1.1 for CCL8 binding indicated that conformational constraint could be beneficial for chemokine binding. Thus, cyclic versions of BK1.3 and BK1.5 were synthesised using thioether cyclisation and denoted as BK1.2 and BK1.4 respectively (Figure 3.1 and Figure 3.2 A).<sup>238</sup>

BK1.2 and BK1.4 were then tested on analogous AlphaScreen displacement assays against P672/CCL8, CCL2 and CCL3 interaction (Figure 3.2 B to D). BK1.2 and BK1.4 showed similar  $^{AS}IC_{50}$  values for the disruption of the P672/CCL8 interaction compared to their linear counterpart,  $730 \pm 80$  nM and  $1.8 \pm 0.2$   $\mu$ M respectively (unless otherwise specified data is presented as mean  $\pm$  SEM of three biological replicates, Figure 3.2 B). This indicates that cyclisation does not increase binding affinity to CCL8 as was intended but confirms the importance of the *N*-terminal Tyrosine residue to increase CCL8 binding as previously observed with BK1.3. Furthermore, both cyclic peptides displayed similar  $^{AS}IC_{50}$  values for the disruption of the P672/CCL2 and P672/CCL3, with  $6 \pm 1$   $\mu$ M and  $160 \pm 30$   $\mu$ M for BK1.2 and  $3.1 \pm 0.8$   $\mu$ M and  $140 \pm 40$   $\mu$ M for BK1.4 respectively (Figure 3.2 C and D). Their activity showed no statistically significant difference between themselves and compared to their linear versions, indicating as previously shown that the *N*-terminal Tyrosine present in BK1.2 and BK1.3 does not improve CCL2 and CCL3 affinity. BK1.2 has analogous P672/chemokine disruption profile to BK1.3.



**Figure 3.1** Schematic summarising the synthesis of cyclic peptide variants



**Figure 3.2** Design and biophysical analysis of BK1.2 and BK1.4

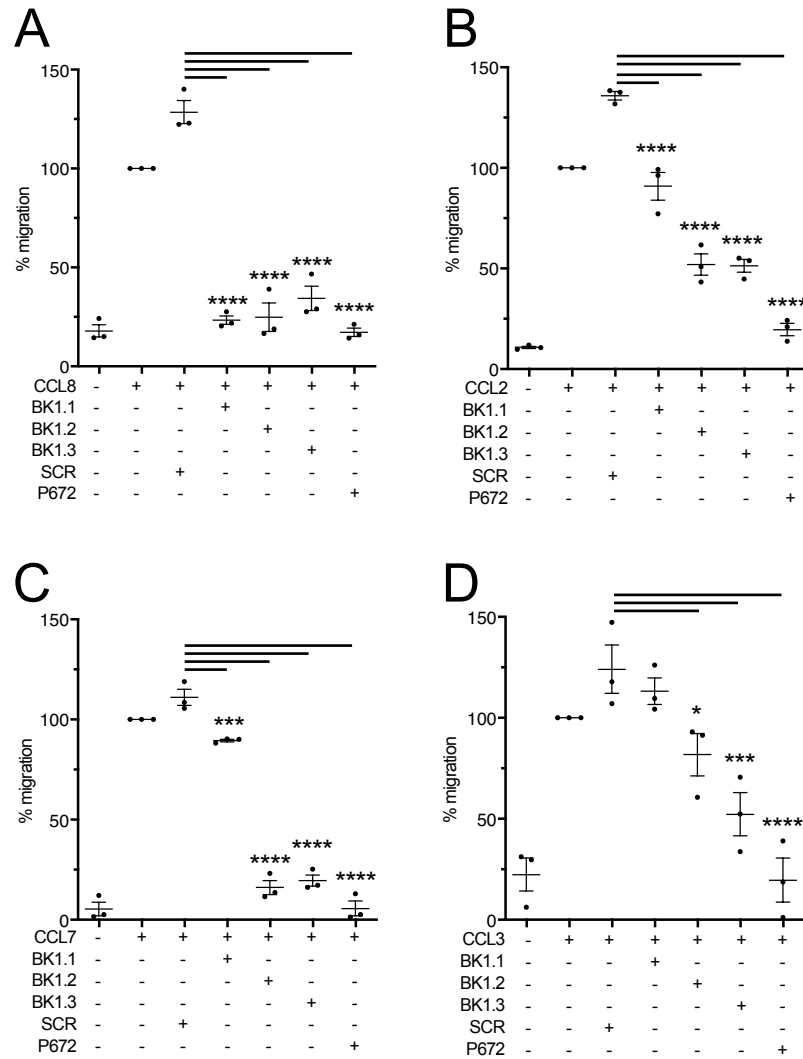
**(A)** Sequences of peptides studied. Cyclisation using chloroacetylation of N-terminal residue and thioether formation. Linked residues indicated. **(B)-(D)** Representative dose-response Alphascreen displacement assay for the disruption of His<sub>6</sub>-P672 (CCL8: 2.5 nM, CCL2: 5 nM, CCL3: 1.25 nM) and CCL8-biotin (1.25 nM), CCL2-biotin (5 nM) or CCL3-biotin (2.5 nM) by BK1.1, BK1.2, BK1.3, BK1.4 and BK1.5 with SCR as a negative control. Biotinylated CCL2 and CCL3 were purchased (Almac). Data represented as mean  $\pm$  SEM of two technical replicates. Data fitted using four-parameters binding model. Figure adapted from reference<sup>1</sup>

| Peptides | CCL8<br>$ASIC_{50}$   | CCL2<br>$ASIC_{50}$   | CCL3<br>$ASIC_{50}$  |
|----------|-----------------------|-----------------------|----------------------|
| BK1.1    | 53 $\pm$ 14 $\mu$ M   | ND                    | ND                   |
| BK1.2    | 730 $\pm$ 80 nM       | 6 $\pm$ 1 $\mu$ M     | 160 $\pm$ 30 $\mu$ M |
| BK1.3    | 480 $\pm$ 60 nM       | 6 $\pm$ 4 $\mu$ M     | 90 $\pm$ 40 $\mu$ M  |
| BK1.4    | 1.8 $\pm$ 0.2 $\mu$ M | 6 $\pm$ 4 $\mu$ M     | 170 $\pm$ 20 $\mu$ M |
| BK1.5    | 1.6 $\pm$ 0.2 $\mu$ M | 3.1 $\pm$ 0.8 $\mu$ M | 140 $\pm$ 40 $\mu$ M |

**Table 3.1** Summary of Alphascreen displacement assays  $IC_{50}$  for each peptide  $IC_{50}$  values presented as mean  $\pm$  SEM of three biological replicates. ND: not determined, showing no significant inhibition at concentration over 100  $\mu$ M.

### 3.3.2 Cellular efficacy of BK1.2

As BK1.2 has similar properties to BK1.3, it was further evaluated using Boyden chamber human monocytic THP-1 cell migration assays (as previously described in Section 2.3.3). Firstly, on a single dose experiment, BK1.2 showed strong inhibition of CCL8 and CCL7 induced cell migration at 10  $\mu$ M (Figure 3.3 A, and C). It also had a moderate but statistically significant effect on CCL2 induced cell migration (Figure 3.3 B). These results are in line with BK1.3 activity, reinforcing the notion that cyclisation does not affect the chemokine binding affinity of these peptides. However, BK1.2 showed a more moderate inhibition of CCL3 driven cell migration compared to BK1.3 (Figure 3.3 D).

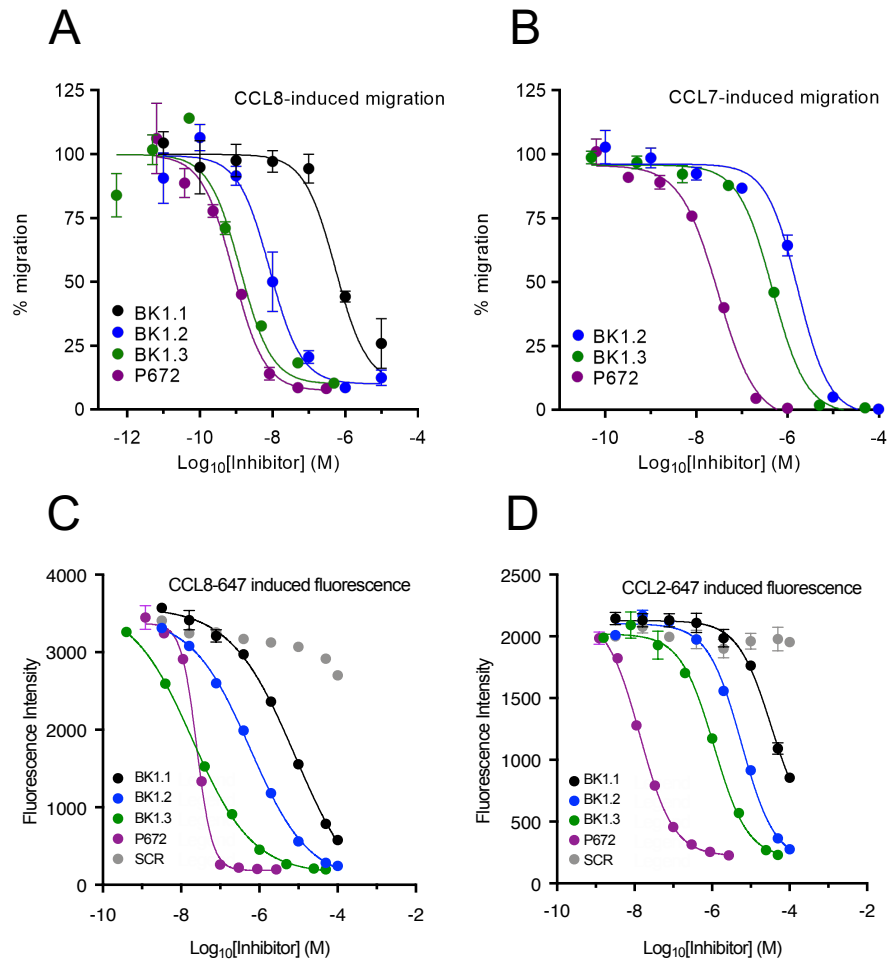


**Figure 3.3** Cell based assessment of BK1.2 chemokine neutralising potential  
**(A)-(D)** Boyden chamber chemokine driven THP-1 cell migration assays looking at the effect of a single dose of chemokine neutralising peptide (10  $\mu$ M).Evasin P672 (300 nM) is used as a positive control. All data normalised to cell count of chemokine stimulation alone and compared to cell count of SCR arm (negative control). Experiment conducted for, respectively, CCL8 (5.8 nM), CCL2 (1.2 nM), CCL7 (7.2 nM) and CCL3 (3.5 nM) at their  $EC_{80}$ . Data represented as mean  $\pm$  SEM of three biological replicates. Statistically significant differences (compared with chemokine alone) using a one-way ANOVA with Sidak's multiple comparisons test are indicated by asterisks: \*\*\*\*,  $p \leq 0.0001$ ; \*\*\*,  $p \leq 0.001$ ; \*\*,  $p \leq 0.01$ ; \*,  $p \leq 0.05$ . Figure adapted from reference<sup>1</sup>

BK1.2 was then tested in dose response experiment and displayed  $^{THP-1}IC_{50}$  values against CCL8- and CCL7-induced cell migration of respectively  $19 \pm 8$  nM and  $1.7 \pm 0.5$   $\mu$ M (Figure 3.4 A and B). Although less potent than BK1.3 ( $^{THP-1}IC_{50}$  respectively against CCL8 and CCL7,  $7 \pm 4$  nM and  $500 \pm 100$  nM, Figure 2.14), BK1.2 still showed strong inhibition in the same order of magnitude as BK1.3 making it a promising candidate as a lead peptide. Following these results, BK1.2 was tested in our newly developed chemokine-driven cellular fluorescence assay against CCL8-647 and CCL2-647. It yielded  $IC_{50}$  values for CCL8-647 and CCL2-647 induced cellular fluorescence of  $620 \pm 80$  nM and  $6.8 \pm 0.7$   $\mu$ M respectively (Figure 3.4 C and D). These results show that BK1.2 acts similarly to BK1.3 and inhibits chemokine induced cell migration most probably by blocking receptor interaction. Although, BK1.2 appears less potent than BK1.3 in cellular assays, these  $IC_{50}$  values demonstrates promiscuous chemokine binding and good *in vitro* affinity.

| Peptides | CCL8<br>$^{THP-1}IC_{50}$ | CCL7<br>$^{THP-1}IC_{50}$ | CCL8-647<br>$^{THP-1}IC_{50}$ | CCL2-647<br>$^{THP-1}IC_{50}$ |
|----------|---------------------------|---------------------------|-------------------------------|-------------------------------|
| BK1.1    | $460 \pm 70$ nM           | ND                        | $5.8 \pm 0.8$ $\mu$ M         | $60 \pm 10$ $\mu$ M           |
| BK1.2    | $19 \pm 8$ nM             | $1.7 \pm 0.5$ $\mu$ M     | $620 \pm 80$ nM               | $6.8 \pm 0.7$ $\mu$ M         |
| BK1.3    | $7 \pm 4$ nM              | $500 \pm 100$ nM          | $48 \pm 6$ nM                 | $2.3 \pm 0.1$ $\mu$ M         |
| P672     | $2.6 \pm 0.9$ nM          | $30 \pm 10$ nM            | $21 \pm 2$ nM                 | $12.9 \pm 0.7$ nM             |

**Table 3.2** Summary of THP-1 cellular assays  $IC_{50}$  for each peptide and P672  
 $IC_{50}$  values presented as mean  $\pm$  SEM of three biological replicates. ND: not determined, showing no significant inhibition at concentration over 10  $\mu$ M.



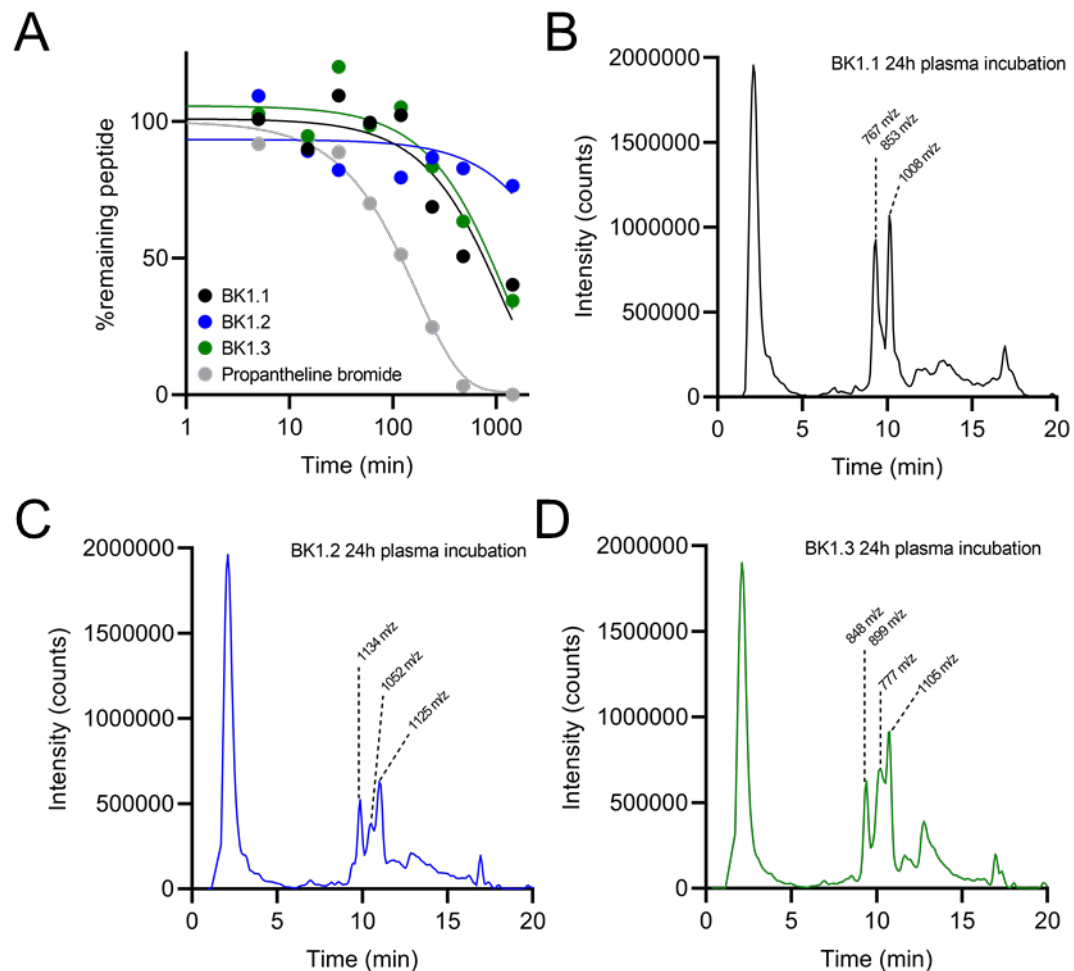
**Figure 3.4** Cell based dose-response experiments assessing BK1.2 potency  
**(A)-(B)** Representative dose-response experimental curve for inhibition of CCL8 and CCL7 induced THP-1 cell migration. Data represented as mean  $\pm$  SEM of three technical replicates. **(C)-(D)** Representative dose-response experimental curve for inhibition of CCL8-647 and CCL2-647 induced cellular fluorescence. Data represented as mean  $\pm$  SEM of two technical replicates. Data fitted with four-parameters binding model. Figure adapted from reference<sup>1</sup>

### 3.3.1 Human plasma stability of the BK series

To assess the stability of the BK peptide series, a human plasma stability assay was performed, where peptide is incubated at 37°C in reconstituted citrated human plasma and samples are analysed at different time points after protein precipitation by liquid chromatography coupled with mass spectroscopy (LCMS). Spectra are then analysed, looking at remaining intact peptide and then plotted as percentage of time point 0 min (Figure 3.5 A). This experiment revealed that, using a one-phase decay model, BK1.1, BK1.2 and BK1.3 had half-lives of 1730 min, 3430 min and 928 min respectively. Propantheline Bromide was used as a positive control and showed a half-life of 116 min, in accordance with previously published results.<sup>221</sup> All peptides were fairly resistant to plasma proteolysis, but cyclic peptide BK1.2 was clearly the most resistant (Figure 3.5 A).

Interestingly, the spectra of samples after 24 h incubation in human plasma, revealed some of the proteolytic metabolites of each peptide (Figure 3.5 B to D). BK1.1 ( $[M+2H]^{2+} = 1108 \text{ m/z}$ ) showed proteolysis of C-terminal Tyr and Phe (BK1.1(1-14),  $[M+2H]^{2+} = 853 \text{ m/z}$ ) and also a smaller peptide showing C-terminal proteolysis of the last four residues (BK1.1(1-12),  $[M+2H]^{2+} = 767 \text{ m/z}$ ) (Table 3.3). For BK1.2 ( $[M+2H]^{2+} = 1125 \text{ m/z}$ ), the most stable of the three peptides, only the proteolytic cleavage of the C-terminal Phe, which is not part of the macrocyclic portion of the peptide was observed ( $[M+2H]^{2+} = 1052 \text{ m/z}$ ) as well as a potential ring opening hydrolysis (indicated by a mass increase of 18 Da, ( $[M+2H]^{2+} = 1134 \text{ m/z}$ )), needing further MS/MS to understand which site, if any specifically, is targeted (Table 3.3). Finally, BK1.3 ( $[M+2H]^{2+} = 1105 \text{ m/z}$ ) showed C-terminal proteolysis of its last three or four residues (BK1.3(1-14),  $[M+2H]^{2+} = 899 \text{ m/z}$  and BK1.3(1-13),  $[M+2H]^{2+} = 848 \text{ m/z}$  respectively) (Table 3.3). The

777  $m/z$  mass observed is likely a more complex proteolytic product of BK1.3 but could not be explained without further MS/MS. These results together show that most of the proteolytic cleavages happen at the C-terminus and therefore could inform some further C-terminal modifications to increase stability.



**Figure 3.5** Plasma proteolytic stability of BK series

**(A)** Time course experiment showing remaining intact peptide. Propantheline bromide used as a positive control. Data presented as a single data point and fitted with one-phase decay model. **(B)-(D)** LCMS trace of 24h incubation in human plasma samples from BK1.1, BK1.2 and BK1.3 showing mass of major peaks.

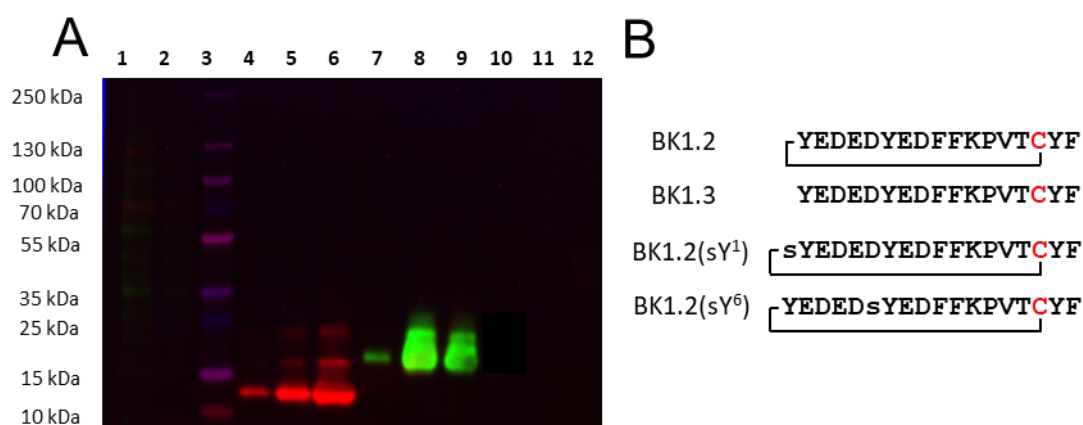
| PEPTIDE           | SEQUENCE                                      | [M+2H] <sup>2+</sup> (m/z) |
|-------------------|-----------------------------------------------|----------------------------|
| BK1.1             | EDEDYEDFFKPVTA <del>Y</del> F–NH <sub>2</sub> | 1108                       |
| BK1.1(1-14)       | EDEDYEDFFKPVTA–OH                             | 853                        |
| BK1.1(1-12)       | EDEDYEDFFKPV–OH                               | 767                        |
| BK1.2             | <u>YEDEDYEDFFKPVTCY</u> F–NH <sub>2</sub>     | 1125                       |
| BK1.2(1-16)       | <u>YEDEDYEDFFKPVTCY</u> –OH                   | 1052                       |
| BK1.2(hydrolysis) | <del>YEDEDYEDFFKPVTCY</del> F–NH <sub>2</sub> | 1134                       |
| BK1.3             | YEDEDYEDFFKPVTCYF–NH <sub>2</sub>             | 1105                       |
| BK1.3(1-14)       | YEDEDYEDFFKPV <del>T</del> –OH                | 899                        |
| BK1.3(1-13)       | YEDEDYEDFFKPV–OH                              | 848                        |

**Table 3.3** Summary of the species observed at 24h of the plasma stability assay  
*Underlined residues indicate cyclic portion of the peptide following thioether cyclisation procedure previously described (Figure 3.1). Barred residues indicate cyclic portion hydrolysed at an unknown site.*

### 3.3.2 Investigation of sulfotyrosine variants of BK1.2

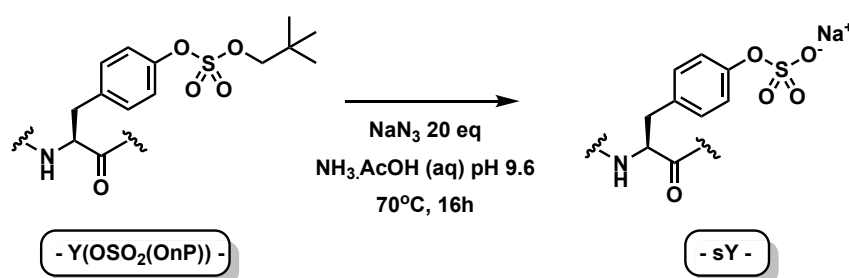
Sulfation of Tyrosine is a post-translational modification that plays a role in Evasin binding, and P672 was predicted to be sulfated on Tyr21.<sup>238</sup> In order to confirm the presence of sY on P672, P672 and deglycosylated P672 (both expressed in HEK293 cells as previously described,<sup>198</sup> provided by Dr. James Eaton) were probed by Western Blot using a mouse anti-sY primary antibody. Biotinylated CCL8 was used as a negative control and probed using Streptavidin-AlexFluor647 conjugate (Figure 3.6 A). Only deglycosylated P672 showed presence of sY, which was expected according to previously reported Western Blot experiment indicating that glycosylation might block antibody recognition of sY in P672.<sup>238</sup> Biotinylated CCL8 showed no sY signal. It confirmed that P672 contained sY as predicted.<sup>238</sup> Interestingly, HEK293 cell lysate showed faint but visible protein bands containing sY and biotin. Following these results, a new series of BK1.2 variants was designed including sY residues (Figure 3.6 B). Sulfotyrosine residue was added using neopentyl (nP) protected sulfotyrosine Fmoc protected amino acid (Bachem). This nP protecting group is resistant to acidic treatment and 20% piperidine treatments, it is removed after cyclisation is basic

condition with 50 equivalents of sodium azide ( $\text{NaN}_3$ ) at 70°C over-night (Figure 3.7). The predicted position sTyr21 was synthesised as a priority (BK1.2(sY6)) and as the *N*-terminal Tyrosine is also close to an acidic patch (a known Tyrosine sulfonation sequence requirement<sup>237</sup>) this peptide was also synthesised (BK1.2(sY1)).



**Figure 3.6** Sulfotyrosine variant design

**(A)** Western Blot of biotinylated CCL8, deglycosylated P672 and P672 probed with mouse anti-sY (green) and Streptavidin-AlexaFluor647 (red). Lanes - 1: HEK293 lysate (20  $\mu\text{g}$ ); 2: HEK293 lysate (10  $\mu\text{g}$ ); 3: protein ladder; 4: biotinylated CCL8 (50 ng); 5: biotinylated CCL8 (250 ng); 6: biotinylated CCL8 (500 ng); 7: deglycosylated P672 (50 ng); 8: deglycosylated P672 (250 ng); 9: deglycosylated P672 (500 ng); 10: P672 (50 ng); 11: P672 (250 ng); 12: P672 (500 ng). **(B)** Sequences of peptides studied. Cyclisation using chloroacetylation of *N*-terminal residue and thioether formation. Linked residues indicated. sY indicating sulfoTyrosine.



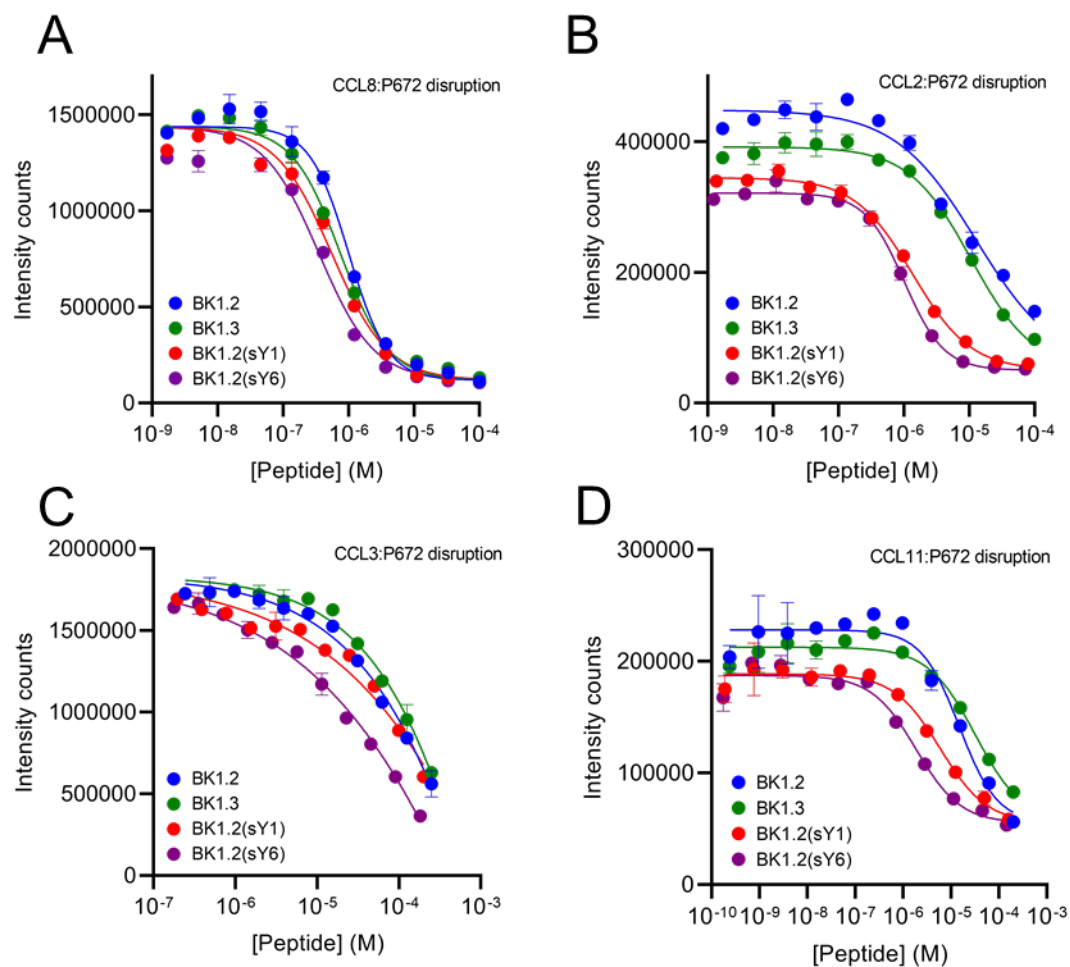
**Figure 3.7** Neopentyl protected sulfotyrosine deprotection

sY cyclic peptide variants were then tested in an AlphaScreen displacement assay for their inhibitory effects on P672/CCL8, CCL2, CCL3 and CCL11 complex formation. For P672/CCL8, both BK1.2(sY<sup>1</sup>) and BK1.2(sY<sup>6</sup>) exhibited similar but lower  $\text{ASIC}_{50}$  values than the

parent peptides (BK1.2(sY<sup>1</sup>): 490 ± 30 nM, BK1.2(sY<sup>6</sup>): 348 ± 3 nM and BK1.2: 730 ± 80 nM), indicating that the introduction of sY has minimal impact on binding affinity to CCL8 (Figure 3.8 A). However, disruption of P672/CCL2 and CCL3 interaction activity of BK1.2(sY1) and BK1.2(sY6) was significantly higher than for BK1.3 (CCL2<sub>BK1.3</sub> = 6 ± 4 μM, CCL3<sub>BK1.3</sub> = 90 ± 40 μM) and BK1.2 (CCL2<sub>BK1.2</sub> = 6 ± 1 μM, CCL3<sub>BK1.2</sub> = 160 ± 30 μM) with <sup>AS</sup>IC<sub>50</sub> values of CCL2<sub>BK1.2(sY1)</sub> = 1.1 ± 0.1 μM, CCL2<sub>BK1.2(sY6)</sub> = 800 ± 100 nM, CCL3<sub>BK1.2(sY1)</sub> = 80 ± 1 μM, and CCL3<sub>BK1.2(sY6)</sub> = 25 ± 2 μM (Figure 3.8 B and C). This suggests that sY insertion especially at position Tyr21 (BK1.2(sY6)) greatly increases peptide disruption of CCL2 and CCL3 interaction with P672. Finally, against the P672/CCL11 interaction BK1.2, BK1.3, BK1.2(sY1) and BK1.2(sY6) showed <sup>AS</sup>IC<sub>50</sub> values of 15.8 ± 0.5 μM, 36 ± 2 μM, 6.1 ± 0.7 μM and 2.9 ± 0.9 μM respectively (Figure 3.8 D). The results further indicate that sY insertion may play a role in increased binding to chemokines, therefore broadening chemokine binding promiscuity.

| Peptides   | CCL8<br><sup>AS</sup> IC <sub>50</sub> | CCL2<br><sup>AS</sup> IC <sub>50</sub> | CCL3<br><sup>AS</sup> IC <sub>50</sub> | CCL11<br><sup>AS</sup> IC <sub>50</sub> |
|------------|----------------------------------------|----------------------------------------|----------------------------------------|-----------------------------------------|
| BK1.2      | 730 ± 80 nM                            | 6 ± 1 μM                               | 160 ± 30 μM                            | 15.8 ± 0.5 μM                           |
| BK1.2(sY1) | 490 ± 30 nM                            | 1.1 ± 1.1 μM                           | 80 ± 1 μM                              | 6.1 ± 0.7 μM                            |
| BK1.2(sY6) | 348 ± 3 nM                             | 800 ± 100 nM                           | 25 ± 2 μM                              | 2.9 ± 0.9 μM                            |
| BK1.3      | 480 ± 60 nM                            | 6 ± 4 μM                               | 90 ± 40 μM                             | 36 ± 2 μM                               |

**Table 3.4** Summary of Alphascreen displacement assays IC<sub>50</sub> for each peptide  
*IC<sub>50</sub> values presented as mean ± SEM of three biological replicates.*



**Figure 3.8** Biophysical characterisation of sulfotyrosine derivatives  
*Representative dose-response Alphascreen displacement assay for the disruption His-tagged P672 and biotinylated CCL8 (A), CCL2 (B), CCL3 (C) and CCL11 (D) by BK1.2, BK1.3, BK1.2(sY1), and BK1.2(sY6). Data represented as mean  $\pm$  SEM of two technical replicates. Data fitted using a four-parameters binding model.*

### 3.4 Discussion

The need for new and potent anti-inflammatory therapeutics is growing and the previous discovery of peptide BK1.3 that is able to bind several chemokines and exhibits *in vivo* anti-inflammatory efficacy, provides a starting point for therapeutical development. Refining a peptide into a strong clinical candidate usually involves an increase in metabolic stability and in clearance time.<sup>230</sup> Increasing metabolic stability is often achieved through peptide cyclisation which introduces conformational constraint and reduces the flexibility of peptides. Many proteases are exoproteases, therefore cyclisation masking *N*- and/or *C*-termini will inherently reduce their proteolytic activity on the peptide. Cyclisation can be achieved by several strategies including lactamisation, lactonisation and Cysteine-based bridges.<sup>239</sup> However, in a study of difference in stability between the linear epitope of glycoprotein-D herpes simplex virus protein and three different cyclisation methods found that thioether cyclisation provided the best stability profile in human serum and lysosomal preparation.<sup>240</sup>

In this chapter, the synthesis of cyclic versions of previously studied BK1.3 and BK1.5: BK1.2 and BK1.4 respectively was described. These cyclic versions were designed to potentially increase proteolytic stability of the peptides. Additionally, peptide cyclisation would help limit conformational dynamics and potentially help cyclic variants with their chemokine binding properties. However, these peptides showed very similar activity in disrupting the interaction between P672 and CCL8, CCL2 or CCL3 compared to their linear counterparts (as measured by AlphaScreen displacement assay). This indicates that cyclisation has very limited effect on chemokine binding affinity. Moreover, it was confirmed that the serendipitous insertion of the *N*-terminal Tyr has a beneficial effect on

P672/CCL8 disruption. The most active cyclic peptide: BK1.2, was then tested in cellular assays where it showed similar chemokine promiscuity compared to its linear form (BK1.3) in Boyden chamber assays against CCL8-, CCL2- and CCL7-induced THP-1 cell migration. It did however show decreased activity against CCL3-driven THP-1 cell migration compared to BK1.3 indicating that its chemokine binding affinity profile might be more restricted than its linear form. In dose-response Boyden chamber assays against CCL8- and CCL7-induced THP-1 cell migration, it was confirmed that  $^{THP-1}IC_{50}$  values for BK1.2 were higher than for BK1.3, although within the same order of magnitude. The same observation was made looking at chemokine driven cell fluorescence assay, which confirms that the mode of action of BK1.2 is most probably like BK1.3 and is mediated by receptor-binding inhibition. These observations all together suggest that BK1.2 is less potent than BK1.3 based on biophysical and cellular analysis of its chemokine binding and neutralisation properties but is still fairly potent in these settings.

Human plasma stability of BK1.1, BK1.2 and BK1.3 was then assessed using LCMS. Interestingly, all peptides seemed to be resistant to proteolysis, but BK1.2 was clearly the most stable with over 75% of intact peptide remaining after 24 h incubation in human plasma. This correlates well with previously observed improved stability of peptides upon thioether cyclisation strategy.<sup>240</sup> More importantly it was observed that the metabolic fragments observed after 24 h incubation in human plasma showed a similar proteolytic pattern, with C-terminus proteolysis in all three peptides up to Val28 in linear peptides and limited to Phe32 in the cyclic BK1.2. Additionally, a cyclic peptide opening hydrolytic product was observed as a proteolysis fragment of BK1.2. Unfortunately, in the absence of MS/MS data on this particular metabolite, it is impossible to decipher the hydrolytic position on the cyclic portion of BK1.2. BK1.2 has a clear advantage over BK1.3 in terms of metabolic

stability, although it is still unclear and would necessitate more *in vivo* studies to prove the therapeutic benefit of a cyclic version of BK1.3.

Furthermore, as recently described, sY is suggested to play a role in the chemokine binding activity of Evasins.<sup>238</sup> It is predicted that Evasin P672 is sulfated at Tyr21 and that this *N*-terminal post-translational modification plays a role similar to that of the chemokine receptor *N*-terminal sY-modified tail for chemokine binding.<sup>89,238</sup> The presence of sY in deglycosylated P672 was confirmed by Western Blot, although no signal was observed in glycosylated P672 as previously reported for Evasin ACA-01.<sup>238</sup> More importantly faint signal came from HEK293 lysate further proving that the cellular expression of P672 is able to contain this post-translational modification.<sup>241</sup> Following this observation sY derivatives of BK1.2 were synthesised with sY at Tyr21 (as predicted to be the sulfonated position in the parent Evasin P672) and on the *N*-terminal Tyr as it is also close to an acidic patch, known to be present near sY marks.<sup>237</sup> Both of these peptides performed better than their parent peptide BK1.2 and in all cases either equalled or surpassed the inhibitory activity of lead peptide BK1.3 in AlphaScreen displacement assays against P672/CCL8, CCL2, CCL3 and CCL11 interaction. BK1.2(sY6) displayed higher inhibitory activity compared to BK1.2(sY1), as was predicted previously and further indicating that Tyr21 might be sulfonated in P672.<sup>238</sup> Together, these results suggest that BK1.2(sY6) is a promising candidate for further investigation.

### 3.5 Conclusions

Development of therapeutically tractable peptides is a long process and necessitates cycles of iterations to refine the properties of each sequential series. After the discovery of BK1.3, a promiscuous chemokine neutralising peptide, BK1.2, cyclic derivative of BK1.3, was designed and was found to have moderate loss in chemokine neutralising activity while conserving chemokine binding promiscuity. It was shown to resist more readily to proteolytic cleavage in human plasma, an important factor in progressing towards clinically viable peptides. Further work is needed to fully understand the proteolytic fragments created during plasma incubation, but it seems that C-terminal residues are targeted first and in the case of BK1.2, hydrolytic cleavage of the ring occurs. MS/MS studies would be required to effectively guide the chemical design of more proteolytic resistant versions of BK1.2, potentially involving C-terminal modifications such as insertion of D-Phe or linkage to albumin binding small molecules which would likely increase *in vivo* half-life as well as protect from C-terminal degradation. Derivatives of BK1.2 were synthesised containing sY, inspired by the presence of this modification in its parent Evasin P672 and the predicted position of this modification. These peptides and especially BK1.2(sY6) showed increased biophysical activity compared to all peptides previously synthesised from P672. Thus, BK1.2(sY6), combining the increased proteolytic stability of BK1.2 and an enhanced biophysical chemokine affinity, is worth being further investigated.

# Chapter 4 Peptide display technologies to interrogate chemokine binding

## 4.1 Introduction

There is a growing interest for peptide chemical probes and therapeutics, as evidenced from the increasing number of peptide drugs approved over the past two decades.<sup>242</sup> In order to sustain this effort to find *de novo* peptide sequences viable for pharmaceutical development, several peptide display technologies have been established to screen libraries against protein targets of interest.<sup>243</sup> These display techniques were designed based on two major requisites: linking genotype to phenotype by coupling encoded material to the peptides for easy peptide sequence determination, and library diversity to allow as many unique sequences to be screened at once against a protein target.

The most well-known and well-established peptide display technique is phage display, where small filamentous bacteriophages are genetically modified to express a protein or peptide on their surface.<sup>244–246</sup> The capsid protein pIII, of which there are 5-7 copies expressed on the bacteriophage surface, is typically used as it can accommodate large protein fusions. DNA libraries of bacteriophage genomes with pIII fusions can be designed, and the phage library is constructed by transforming the DNA library in bacteria. The modified bacteriophage genomes are then expressed in bacteria and viral particles are secreted with peptide or protein displayed on its surface. The expression on the surface of 5-7 copies of the displayed peptide is advantageous as the avidity created by the local high peptide concentration allows for weaker affinity binders to be picked up during the

selection process. While phage display has been a very successful peptide display technique, there remains some limitations concerning the library diversity. These limitations come from the maximum efficiency of bacteria transformation which limits the number of transformants ( $\sim 10^9$  transformants maximum).<sup>247</sup> It also comes as the bacteriophage are assembled in bacteria, therefore some replication biases can happen with expressed peptides promoting or slowing bacterial growth and in some cases peptides that can be lethal for the bacterium, which would therefore reduce library diversity.<sup>248</sup> Finally, this technique is limited to the use of the 20 natural amino acids therefore lacking the option to insert unnatural amino acids.

Other well-known display technologies are yeast<sup>249</sup> and bacteria<sup>250</sup> display. DNA library encoding peptides or proteins in fusions of endogenous cell surface proteins are transformed into cells. Many carrier surface proteins have been used but Aga2p agglutinin subunit in yeast display and flagella for bacteria display are good examples. Yeast display is a proven viable method to display peptides and large proteins, especially for antibody discovery.<sup>194,196,198,251,252</sup> Yeast display, using eukaryotic single-cell microorganisms, has an advantage over phage and bacteria display because yeast can express well-folded and post-translationally modified mammalian peptides and proteins. Both yeast and bacteria display have multiple copies of peptides on their surface, similar to phage display, with the avidity advantage that it brings. However, a few drawbacks remain for yeast and bacteria display: there is a transformation limitation for the library diversity; the library is limited to natural amino acids; and the surface of the carrier of the peptide is much more complex, displaying large numbers of proteins, some of which are glycosylated, which thus requires careful counter screening methods to remove non-specific binding hits.

An *in vitro* based peptide display technique was developed, which overcomes some of these issues: mRNA display.<sup>253,254</sup> It is a simpler display technique as it consists of the displayed peptide linked to its encoding mRNA. It relies on the use of *in vitro* transcription translation (IVTT). Here, a DNA library containing as much as  $\sim 10^{12}$  diversity, is transcribed to mRNA that is then linked to a modified primer containing puromycin. Puromycin mimics a tyrosine residue linked to tRNA, but is non-hydrolysable, and so when it enters the ribosome at the C-terminus of the peptide, covalent linkage between the peptide and encoding mRNA is formed. The peptide-mRNA construct is then released and can be used to screen against target protein of interest, hits being identified by simple sequencing of the cDNA that has been reverse transcribed from the mRNA attached to the recovered peptides. The key advantage of the mRNA peptide display technique compared to the ones previously described is that it is not limited by transformation efficiency or replication biases as it uses IVTT. Therefore, mRNA display is amenable to very high library diversity ( $>10^{12}$ ) and has a low rate of non-specific binding because of the low complexity of the peptide carrier. Moreover, the use of IVTT allows for customisation of IVTT reagents and therefore insertion of different chemistry, formation of macrocycles and insertion of unnatural amino acids. In fact, the mRNA display technology has been shown to be a very effective system for the introduction of unnatural amino acids in displayed peptides, especially since the development of the Flexizyme system by Suga.<sup>255,256</sup> However, one drawback is that peptides are displayed individually, and consequently weak binders are usually quickly eliminated in the early selection rounds and are rarely identified using this technique.

Finally, the use of chemically synthesised peptide arrays on cellulose support or SPOT peptide arrays<sup>257,258</sup> is a very efficient way of testing relatively large amounts of peptides (about 8 spots/cm<sup>2</sup> containing  $\sim 20$  nmol of peptide) where genotype is linked to

phenotype by the position of the peptide spot on the cellulose membrane.<sup>259</sup> This can be especially useful for more targeted library screening and epitope mapping. It is a well-established technique for high-throughput investigations of protein-protein interactions.<sup>260</sup> The synthesis is initiated by activating the membrane with a flexible ligand (typically Fmoc- $\beta$ -Ala-OH) linking the nascent peptide chain by esterification on the free hydroxyl residues of the cellulose membrane. Peptides are then elongated, using standard Fmoc peptide synthesis, manually or using automation methods, by dispensing sub- $\mu$ L volume of reagents on a cellulose based membrane creating individual spot areas containing defined peptides. Peptide arrays built in this way provide a very high local concentration of peptide per spot, allowing for weaker binders to be identified. Such membrane arrays can be probed with a target protein of interest and then hybridised with a secondary antibody to reveal binding peptides.

Protein-protein interactions are critical for chemokine biology and their inhibition by Evasins. While the chemokine network is highly complex, understanding the molecular details of these interactions is crucial in order to further develop effective chemokine inhibitors. However, characterising such PPIs is challenging and using proven biophysical methods such as HDxMS can be time-consuming and require significant resources. Therefore, alternative methods using peptide display techniques described above could lead to high-throughput chemokine PPI characterisation. This would enable a faster pipeline for the discovery, development and validation of novel Evasin-inspired chemokine neutralising peptides.

## 4.2 Aims

This chapter will focus on the establishment of peptide display and array methods to characterise Evasin/chemokine interactions to pioneer high-throughput chemokine PPI characterisation.

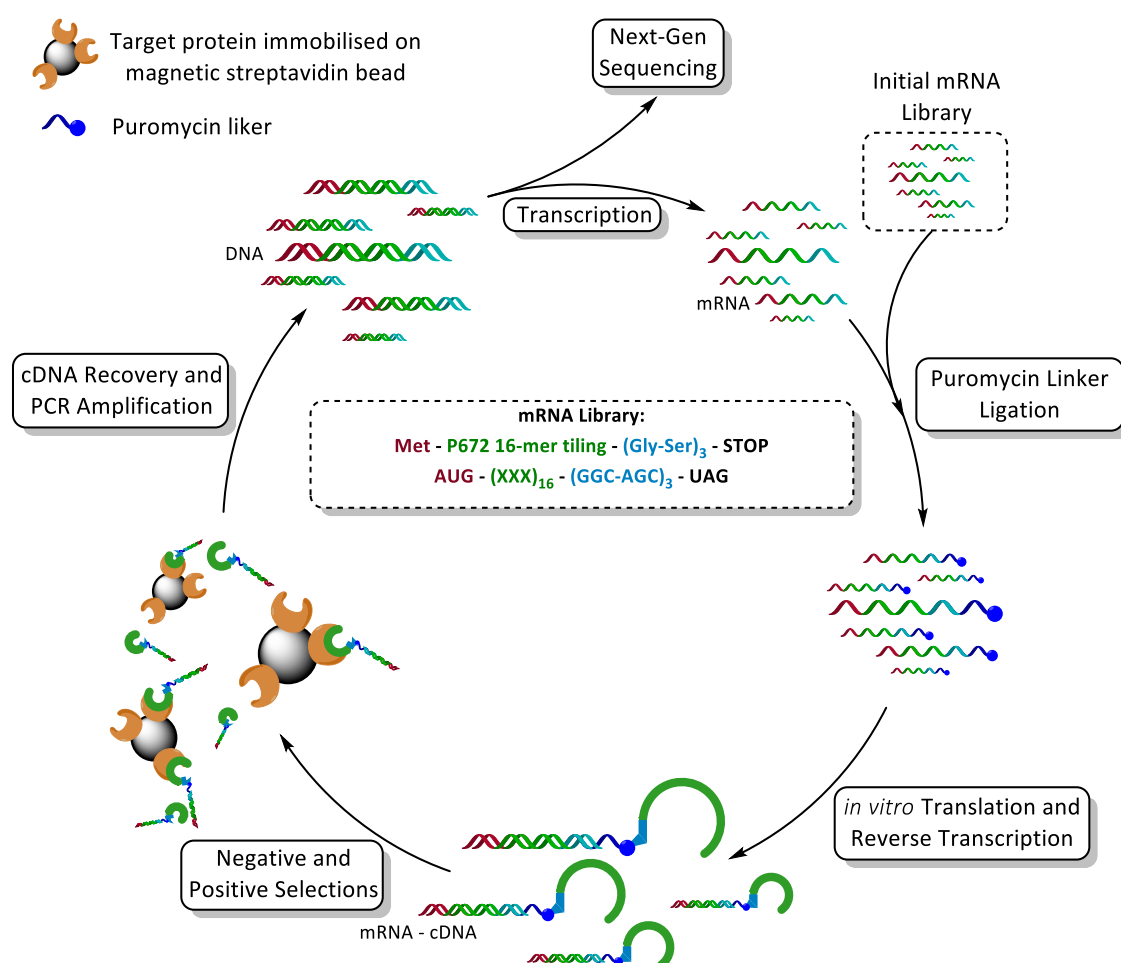
Aims of the work described in this chapter are:

- a. Mapping P672/chemokine protein-protein interaction using mRNA display
- b. Mapping P672/chemokine protein-protein interaction using peptide arrays
- c. Probing BK1.3 interaction with chemokines using peptide arrays

## 4.3 Results

Previously collected data by HDxMS enabled the development of anti-inflammatory BK1.1 derivatives in Chapter 2 and Chapter 3. Despite the success with P672/CCL8, HDxMS studies are challenging and are highly dependent on high affinity ligands and protease cleavage sites. Several other P672/chemokine and BK1.1/chemokine HDxMS studies were attempted but yielded no usable data (not shown). Thus, new methods were tested to establish their reliability in studying chemokine PPIs with Evasins and peptides.

### 4.3.1 Interrogating P672/chemokine interaction surface using mRNA display



**Figure 4.1** Schematic summarising P672 tiling mRNA peptide display experiment

A library of DNA oligos coding for 16-mer tiling of P672 with all cysteine residues mutated to serine was designed. Using standard mRNA peptide display technology and IVTT material, this linear, tiled P672 peptide library was tested for binding enrichment against biotinylated CCL8, CCL2, CCL3 and CCL11 immobilised on magnetic streptavidin beads. These chemokines were chosen as they were shown to bind to P672 in previous experiments.<sup>1,198</sup> After four rounds of enrichment, encoding DNA was recovered and sequenced along with the initial library to assess sequence enrichment. Each peptide was assigned an E (enrichment) value as a comparison between the fraction of sequence counts of a said sequence in the initial library and the fraction of sequence counts of the same sequence in the fourth round such as:

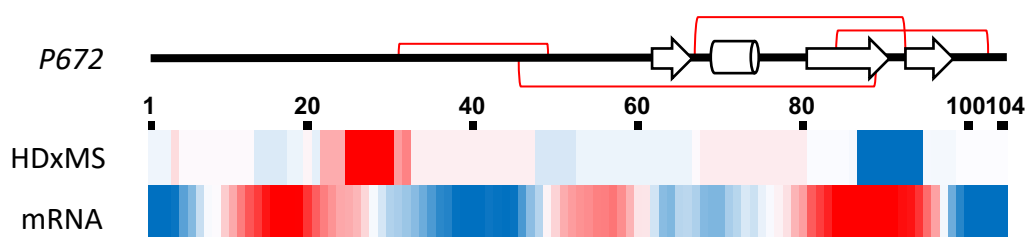
$$E_{peptide} = \frac{f_{final\ round}}{f_{initial\ library}}$$

Then each residue in P672 was assigned a score (e) as the sum of contributions from each peptide containing this residue such as:

$$e_{residue} = \sum_{\substack{peptides \\ containing \\ residue}} \frac{E_{peptide}}{length_{peptide}}$$

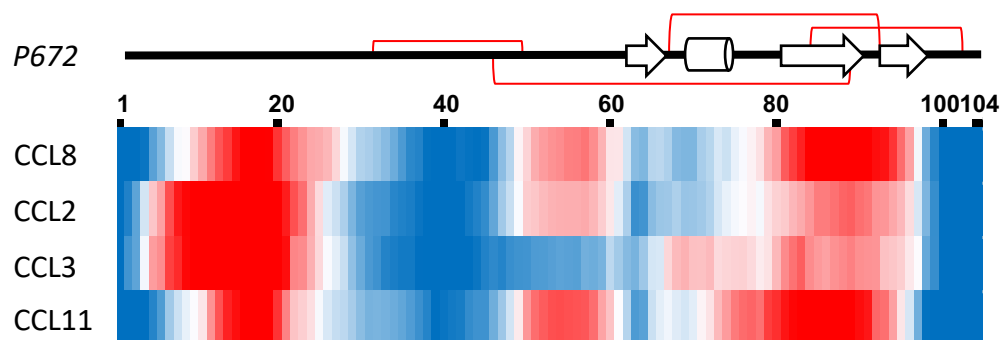
The mRNA display screening of this P672 tiling library against CCL8 is shown in Figure 4.2. The selection data revealed a higher score (a higher contribution of these residues towards peptide enrichment, here interpreted as binding regions) for the Gly11-Lys26 and Arg79-Ala96 regions (Figure 4.2). The *N*-terminal region Gly11-Lys26 overlaps with the P672/CCL8 interface previously described using HDxMS (Tyr21-Phe32)<sup>1</sup>, although these results seems to indicate a higher importance of the *N*-terminal tail of P672 (Figure 4.2). A region ranging from Arg79-Ala96 appears to provide some binding and overlaps with the previously described region of P672 which was more prone to deuterium exchange upon

binding to CCL8 (Gly87-Cys94). It is however unclear why this region would show higher enrichment through mRNA peptide display screening (Figure 4.2). This might be due to the amount of acidic residues present in this region, potentially pointing out to electrostatic non-specific interactions.



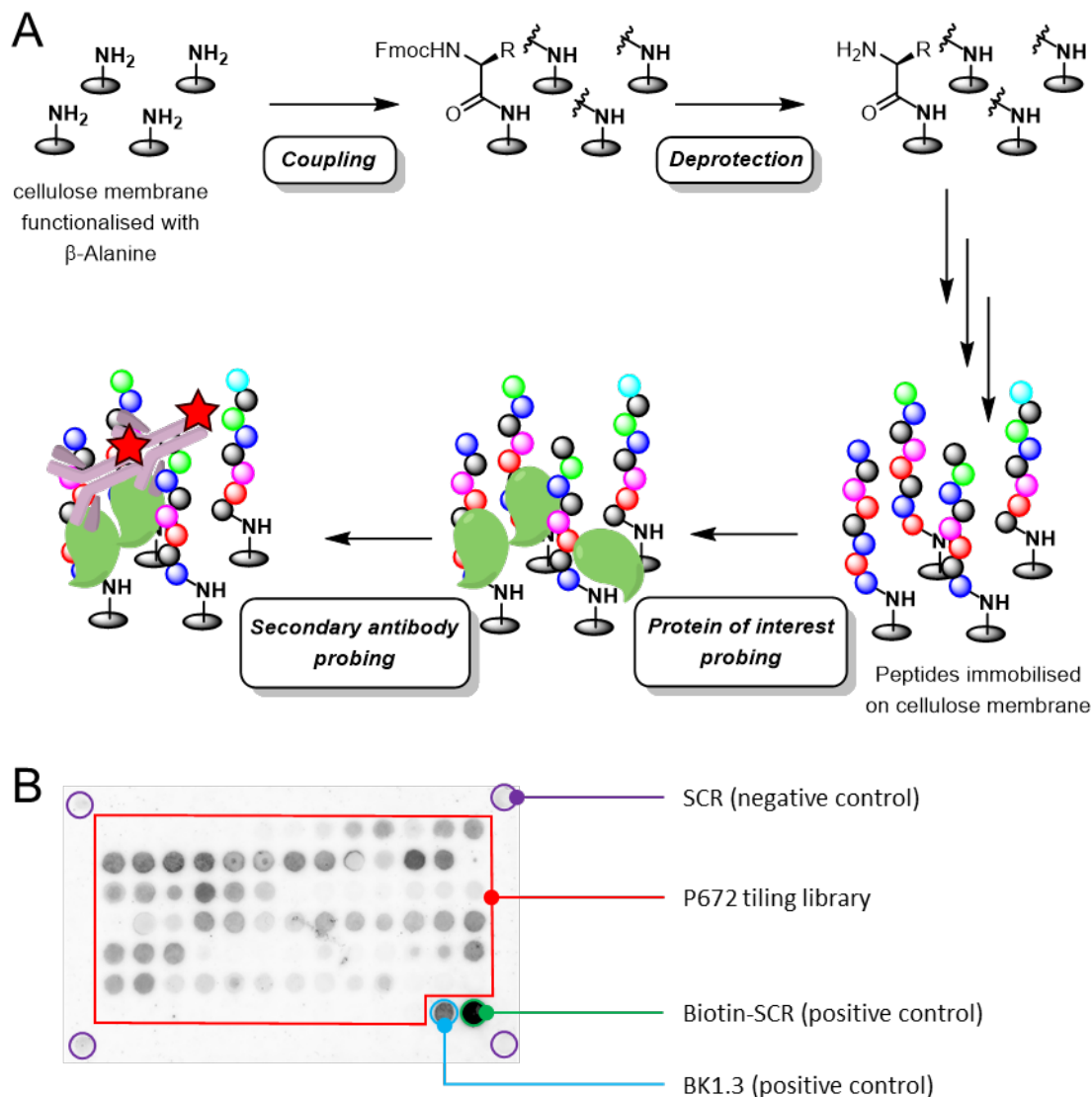
**Figure 4.2** Mapping of the P672/CCL8 interface using mRNA display  
*Top panel: representation of P672 secondary structure (arrow:  $\beta$ -sheet, cylinder:  $\alpha$ -helix) including disulfide bridges in red. Bottom panel: heat map summarising HDxMS and mRNA display results. P672 sequence from N-terminus (left) to C-terminus (right). HDxMS key: lower (red) to higher (blue) deuterium exchange. mRNA key: high (red) to low (blue) residue enrichment score.*

Analogous screening was carried out on CCL2, CCL3 and CCL11 in parallel with CCL8 screening, treated and analysed the same way. These mRNA display screens confirmed the importance of the N-terminal region of P672 for chemokine binding (Figure 4.3).<sup>198</sup> In more detail, the Glu6-Phe24 region showed significant enrichment against CCL2 (Figure 4.3), similarly the Ser5-Phe24 region showed significant enrichment against CCL3 (Figure 4.3). Interestingly, the screen against CCL11 showed two clusters of enrichment for Glu12-Tyr21 at the N-terminus and Glu73-Cys94 at the C-terminus in a similar fashion to the CCL8 screen (Figure 4.3). Together, these results display the difference in the likely mode of binding for P672 peptide fragments to different chemokines and highlight the importance of the N-terminal tail of P672 for chemokine binding, as confirmed previously by HDxMS.



**Figure 4.3** Mapping of the P672/chemokine interface using mRNA display  
*Top panel: representation of P672 secondary structure (arrow:  $\beta$ -sheet, cylinder:  $\alpha$ -helix) including disulfide bridges in red. Bottom panel: heat map summarising mRNA display results for CCL8, CCL2, CCL3 and CCL11 screens (from top to bottom). P672 sequence from N-terminus (left) to C-terminus (right). Key: high (red) to low (blue) residue enrichment score.*

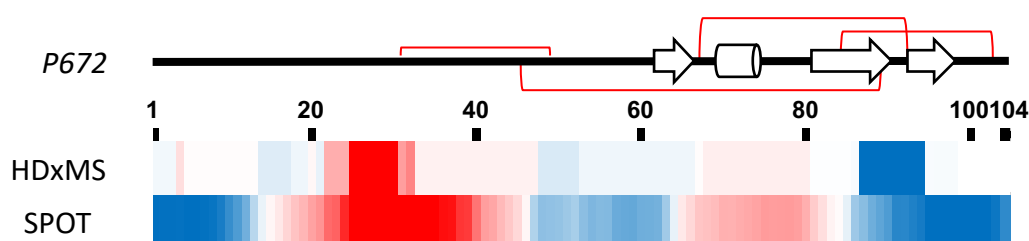
### 4.3.2 Interrogating the P672/chemokine interaction surface using SPOT peptide arrays



**Figure 4.4** Schematic summarising P672 tiling SPOT peptide array experiment **(A)** General schematic of SPOT peptide array synthesis and probing. **(B)** Example of raw data image from P672 tiling library SPOT peptide array (red) probed using CCL8-biotin then streptavidin-AlexaFluor647 conjugate including positive (blue and green) and negative (purple) controls. Fluorescence quantification and calculations are explained in details in Section 6.2.12

In order to study the P672/chemokines interaction an orthogonal method was used. An analogous library of 16-mer peptide tiling of P672 was printed on a cellulose membrane using a SPOT synthesis method according to the manufacturer's protocol on a Multiprep synthesiser (CEM). The membrane was hybridised with biotinylated CCL8, CCL2, CCL3 or CCL11 and then visualised with streptavidin-Alexafluor647 conjugate for read-out. Each spot intensity was used to create a rolling average of intensity attributed to each P672 residue, creating a similar heat map as previously shown (Figure 4.3).

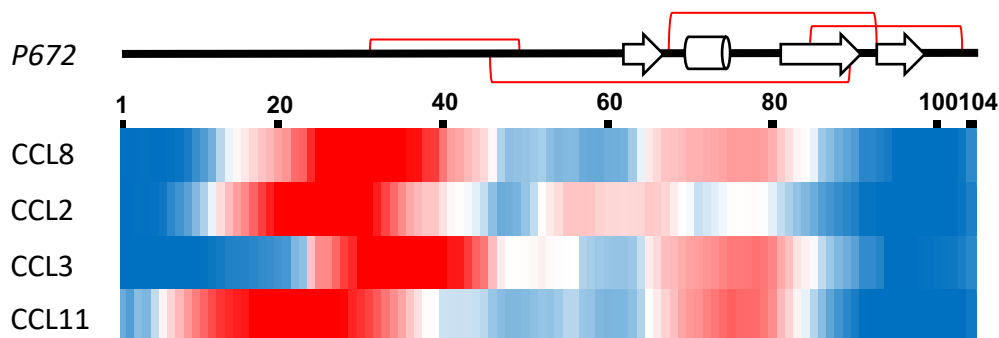
Hybridising the SPOT membrane with biotinylated CCL8 showed the Glu22-Leu40 region of P672 binding to CCL8 (Figure 4.5). This correlates well with the previous PPI region Tyr21-Ph32 identified by HDxMS (Figure 4.5).<sup>1</sup> However, this surface only slightly covers the interaction region, Glu6-Phe24, found using mRNA display (Figure 4.2).



**Figure 4.5** Mapping of the P672/CCL8 interface using a SPOT membrane  
*Top panel: representation of P672 secondary structure (arrow:  $\beta$ -sheet, cylinder:  $\alpha$ -helix) including disulfide bridges in red. Bottom panel: heat map summarising HDxMS and SPOT membrane probing results. P672 sequence from N-terminus (left) to C-terminus (right). HDxMS key: lower (red) to higher (blue) deuterium exchange. SPOT key: high (red) to low (blue) residue average intensity.*

Further experiments using biotinylated CCL2, CCL3 and CCL11 hybridised to the SPOT membrane revealed different interaction patches on P672 (Figure 4.6). The Glu17-Asn34 region seems to bind to CCL2 (Figure 4.6), where the Pro27-Cys45 stretch binds to CCL3 (Figure 4.6) and the larger surface, Gly11-Tyr31, binds to CCL11 (Figure 4.6). These discrete regions are all located on the solvent-exposed N-terminal tail of P672. It is

also important to note that there appears to be residual binding on a C-terminal loop of P672, potentially pointing towards a transient or weak conformational epitope.



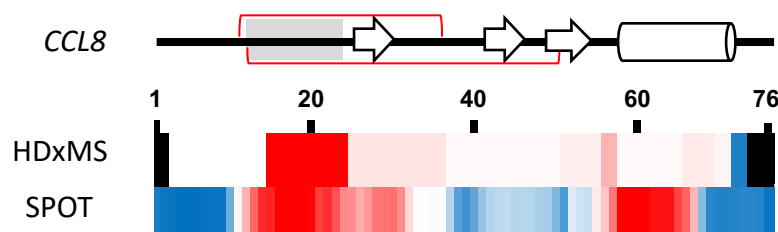
**Figure 4.6** Mapping of the P672/chemokine interface using a SPOT membrane  
*Top panel: representation of P672 secondary structure (arrow:  $\beta$ -sheet, cylinder:  $\alpha$ -helix) including disulfide bridges in red. Bottom panel: heat map summarising SPOT membrane probing results for CCL8, CCL2, CCL3 and CCL11 screening (from top to bottom). P672 sequence from N-terminus (left) to C-terminus (right). Key: high (red) to low (blue) residue average intensity.*

### 4.3.3 Interrogating the chemokine/P672 interaction surface using SPOT peptide arrays

Similarly, to the study carried out on the chemokine/P672 interaction, a library of 10-mer peptides tiling of CCL8, CCL2, CCL3 and CCL1 was printed on a cellulose membrane following the same SPOT synthesis method. This membrane was probed by hybridising with His<sub>6</sub>-P672 and then a secondary antibody, anti-His<sub>6</sub>-AlexaFluor647 conjugate, for visualisation. Each spot intensity was used to create a rolling average of intensity attributed to each chemokine residue.

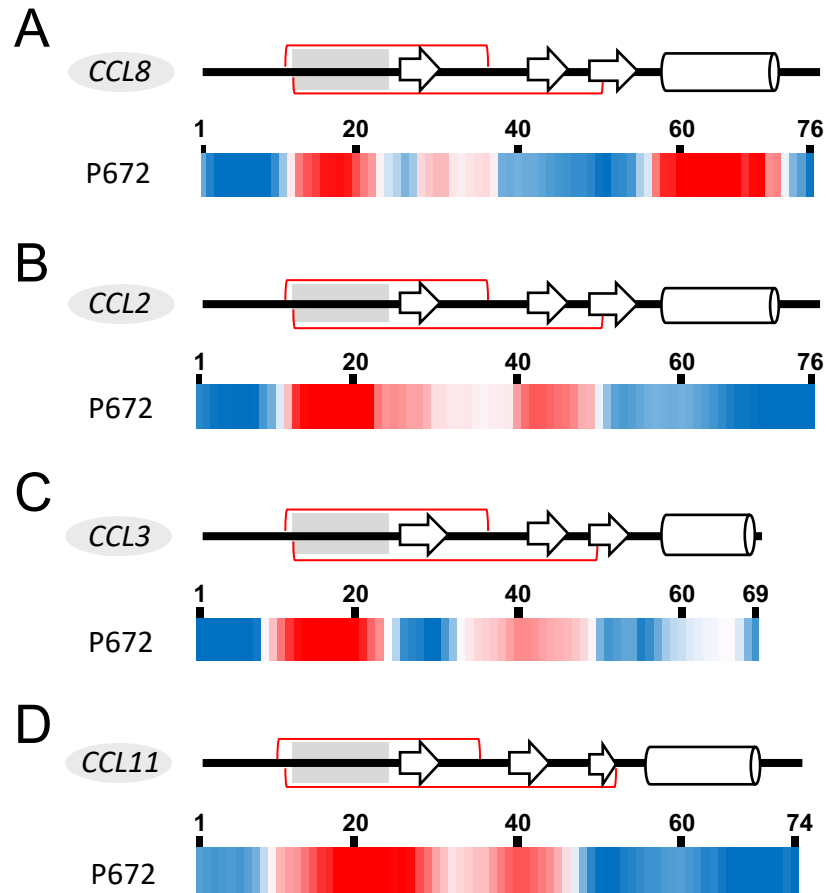
His<sub>6</sub>-P672 probing of the CCL8 10-mer peptide tiling SPOT membrane revealed a large CCL8 N-loop region interacting with P672 (Phe13-Ile31) that aligns with the previously described HDxMS CCL8/P672 interface (Arg18-Ser27)<sup>1</sup> (Figure 4.7). However, the C-terminal

region, Glu57-His66, also showed significant signal indicating potential binding of His<sub>6</sub>-P672 to the C-terminal  $\alpha$ -helix of CCL8 (Figure 4.7).



**Figure 4.7** Mapping of the CCL8/P672 interface using a SPOT membrane  
*Top panel: representation of CCL8 secondary structure (arrow:  $\beta$ -sheet, cylinder:  $\alpha$ -helix, grey area: N-loop region) including disulfide bridges in red. Bottom panel: heat map summarising HDxMS and SPOT membrane probing results. CCL8 sequence from N-terminus (left) to C-terminus (right). HDxMS key: lower (red) to higher (blue) deuterium exchange and missing coverage (black). SPOT key: high (red) to low (blue) residue average intensity.*

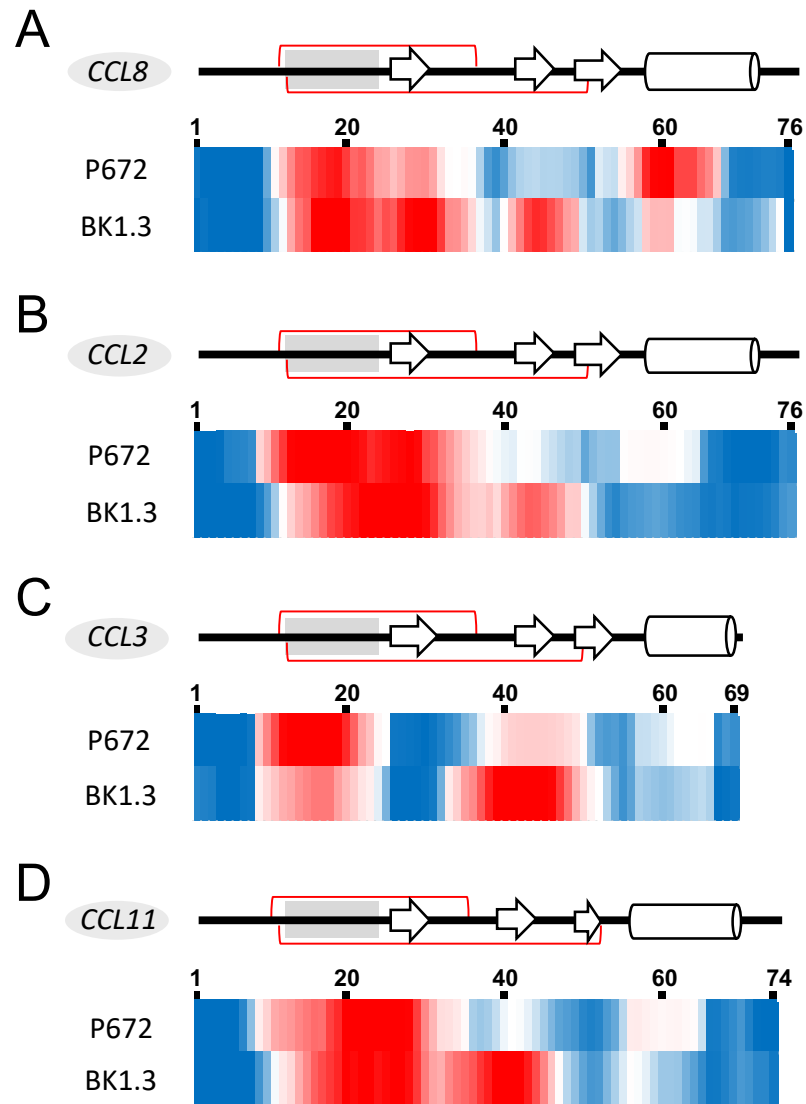
Further experiments on CCL2, CCL3 and CCL11 10-mer peptide tiling SPOT membranes using the same hybridisation method as for the CCL8 screen, displayed similar interaction patterns for chemokine/P672 (Figure 4.8 A). Chemokine/P672 interactions seem to cluster on the N-loop of all chemokines tested with interaction patches at Cys11-Ser33 for CCL2 (Figure 4.8 B), Cys10-Gln21 for CCL3 (Figure 4.8 C) and Asn15-Thr30 for CCL11 (Figure 4.8 D). These results together show that P672 is likely to bind to similar regions on the chemokines it neutralises.



**Figure 4.8** Mapping of the chemokine/P672 interface using a SPOT membrane  
**(A)-(D)** Top panel: representation of CCL8, CCL2, CCL3, and CCL11 secondary structure (arrow:  $\beta$ -sheet, cylinder:  $\alpha$ -helix, grey area: N-loop region) including disulfide bridges in red. Bottom panel: heat map summarising SPOT membrane probing results. CCL8, CCL2, CCL3 and CCL11 sequence from N-terminus (left) to C-terminus (right). SPOT key: high (red) to low (blue) residue average intensity.

### 4.3.4 Interrogating the chemokine/BK1.3 interaction surface using SPOT peptide arrays

In Chapter 2, it was demonstrated that peptide BK1.3 can displace CCL8, CCL2, CCL3 and CCL11 binding to P672 in AlphaScreen displacement assays. Thus, the chemokine 10-mer peptide tiling SPOT membrane was probed using BK1.3<sub>FITC</sub> for peptide/peptide interaction mapping and for a direct read-out of fluoresceine fluorescence. Interestingly, BK1.3 seem to interact in a similar fashion as P672 with CCL8 (Figure 4.9 A) with a more diffuse cluster on the N-loop, Asn14-Ile34 and some binding towards the second and third  $\beta$ -strand of the CCL8  $\beta$ -sheet, Ile42-Arg47 (Figure 4.9 A). The interacting region on the  $\alpha$ -helix of CCL8 that was seen with P672 (Glu57-His66) is only very weak with BK1.3. The interaction area of CCL2 to BK1.3 (Asn17-Thr32) and P672 (Cys11-Ser33) are very similar but there is some weak binding of BK1.3 to CCL2  $\beta$ -sheet (Figure 4.9 B). A very similar pattern was observed with CCL11 binding to BK1.3 (Asn15-Thr30) and P672 (Asn15-Thr30) with a significant interaction of BK1.3 with the CCL11  $\beta$ -sheet (Cys34-Leu45) (Figure 4.9 D). For CCL3, the N-loop binding region to BK1.3, although in line with the interacting region to P672 (Cys10-Gln21), showed less significant signal intensities compared to the CCL3  $\beta$ -sheet interaction of BK1.3 (Lys37-Arg48) (Figure 4.9 C). This CCL3  $\beta$ -sheet interaction was observed with His<sub>6</sub>-P672 probing but was much weaker (Figure 4.9 C). Together, these results indicate that BK1.3 mostly binds in a similar fashion as P672 to chemokines, targeting the N-loop of chemokines. BK1.3 binding observed at the  $\beta$ -sheets of chemokines is yet unclear.



**Figure 4.9** Mapping of the chemokine/BK1.3 interface using a SPOT membrane **(A)-(D)** Top panel: representation of *CCL8*, *CCL2*, *CCL3*, and *CCL11* secondary structure (arrow:  $\beta$ -sheet, cylinder:  $\alpha$ -helix, grey area: N-loop region) including disulfide bridges in red. Bottom panel: heat map summarising SPOT membrane probing results for P672 and BK1.3<sub>FITC</sub>. *CCL8*, *CCL2*, *CCL3* and *CCL11* sequence from N-terminus (left) to C-terminus (right). SPOT key: high (red) to low (blue) residue average intensity.

## 4.4 Discussion

Understanding PPIs is a powerful way to design inhibitors, and a variety of biophysical methods have been developed so far, such as crystallography, NMR, cryo-EM, or HDxMS, yet these methods can be time consuming and challenging. Here, we explored two alternative methods to map PPIs in the context of Evasin P672 binding to chemokines: mRNA peptide display and SPOT peptide arrays.

mRNA display is a technique that has previously been used to map physiologically relevant PPIs with some success using careful library design.<sup>261</sup> The library design for this work involved 16-mer tiling of P672 (cysteine codons replaced by serine codons) flanked by a T7 promoter site with an amber start codon at the 5' end and a (GlySer)<sub>3</sub> coding motif followed by a stop codon at the 3' end. This library was then screened against CCL8, CCL2, CCL3 and CCL11 over four cycles of enrichment as they were previously shown to bind to P672.<sup>198</sup> In order to check the data produced, the CCL8 mRNA display results were compared to previously obtained HDxMS P672/CCL8 PPI mapping (Figure 4.2). This gave a general sense of the reliability of this method to map PPIs, showing that the mRNA display screening results using this library against CCL8 revealed similar regions of interaction, although shifted to the P672 *N*-terminus. Additionally, strong peptide enrichment at the *C*-terminus of P672 binding to CCL8 was observed, although unexpected. These peptide regions should be synthesised and validated as PPI regions in biophysical assays. The mRNA data for the CCL2, CCL3 and CCL11 screens looked fairly similar to the CCL8 screening, displaying higher enrichment in variable interacting regions of the *N*-terminal tail of P672 (Figure 4.3). This is consistent with previous results showing the importance of the P672 *N*-terminus for chemokine binding.<sup>198</sup> Nevertheless, enrichment was also observed at the

P672 C-terminal region for the chemokine and CCL8 screens. Therefore, these peptide regions should also be tested against CCL2, CCL3 and CCL11 to be validated as true PPI regions or artefacts of the screening method.

SPOT peptide arrays are a well-established method to study PPIs,<sup>260</sup> although they cannot deal well with conformational epitopes formed by distant secondary structures of proteins. In this work, a library identical to the previous mRNA display experiment, consisting of 16-mer P672 tiling peptides (cysteine mutated to serine) was synthesised on a cellulose membrane. This library was then hybridised using biotinylated CCL8 (Figure 2.6), CCL2, CCL3 and CCL11 (ALMAC) to look at the P672 PPI with these chemokines. The CCL8 probing results were compared to the previous HDxMS PPI data (Figure 4.5).<sup>1</sup> The data here aligns well at the *N*-terminal region of P672 between the SPOT peptide array and the HDxMS PPI. The interaction area detected is more diffuse in the SPOT peptide array data, probably due to a loss in sensitivity using long peptides (16-mer) in the P672 tiling library. Nevertheless, an interaction motif towards the *C*-terminus of P672 was observed upon hybridising of the membrane with biotinylated CCL8, potentially indicating weak conformational binding to this P672 core loop. Incubation of the SPOT peptide array membrane with biotinylated CCL2, CCL3 and CCL11 revealed similar patterns of interaction (Figure 4.6), although distinct from chemokine to chemokine. P672 interacting region with CCL2 and CCL11 is located further towards the *N*-terminus of P672 compared to the CCL8 interacting region, while the P672 interacting region with CCL3 is located slightly more towards the *C*-terminus of P672. These incubations also revealed a similarly located weak interaction patch on the P672 core loop towards the *C*-terminus of P672. These interaction stretches need to be synthesised as free peptides and properly validated as PPIs in biophysical assays. However, the collection of data obtained from the SPOT peptide arrays

indicates that the Evasin P672 plasticity for binding to several chemokines lies in several discreet overlapping binding patches displayed along its *N*-terminal tail.

Following these results, a new SPOT peptide array membrane was designed containing 10-mer tiling (cysteine mutated to serine) of CCL8, CCL2, CCL3 and CCL11. Hybridisation of the membrane with His<sub>6</sub>-P672 looked at chemokine interaction regions with P672. Results for the CCL8 tiling library were comparable to previously recorded HDxMS data on the CCL8/P672 PPI (Figure 4.7).<sup>1</sup> The data here aligns mostly at the N-loop region of CCL8 between the SPOT peptide array and the HDxMS PPI region. The interaction patch on the SPOT peptide array is more diffuse than in the HDxMS experiment probably due to a loss in sensitivity using 10-mer peptides in the chemokine tiling library. However, a second unexpected cluster of interaction appeared on the C-terminal  $\alpha$ -helix of CCL8 that was not present in the HDxMS results. It is yet unclear how this region shows a different binding site to P672 in SPOT peptide array than in HDxMS analysis, but it could be hypothesised that the linear 10-mer peptides expose residues that would otherwise be buried in the CCL8  $\alpha$ -helix in the native protein. This peptide should be synthesised and validated in biophysical assays in order to assess if it is a genuine PPI region on CCL8 or an artefact of the SPOT peptide array. For the CCL2, CCL3 and CCL11 libraries results are fairly similar, showing a P672 interaction region ranging on the N-loop and extending in the case of CCL2, CCL8 and CCL11 to the first  $\beta$ -strand of the chemokine  $\beta$ -sheet (Figure 4.8). These peptides would need to be properly validated using biophysical assays after synthesis. The N-loop of chemokines is critical for signalling as it is involved in binding specificity in the first binding events with their cognate receptors.<sup>87</sup> Additionally, the N-loop of chemokines is involved in GAG binding as well as their C-terminal  $\alpha$ -helix.<sup>74</sup> Therefore, this data strongly suggests that P672 is neutralising chemokine activity through inhibiting receptor

engagement via its binding to the N-loop of chemokines. Moreover, this data also indicates that P672, through tight binding to the N-loop of chemokines, would be able to disrupt chemokine/GAG interactions, disturbing the critical chemokine gradient at the surface of the blood vessel endothelium at the site of inflammation.<sup>262</sup> Disrupting this interaction has already proven to significantly reduce inflammation.<sup>263,264</sup> This could add to the therapeutic potential of Evasins such as P672 as they would be able to suppress chemokine signalling through two different modes of action.

Next the lead peptide BK1.3 from previous study was hybridised with the same SPOT peptide array membrane displaying 10-mer peptide tiling of CCL8, CCL2, CCL3 and CCL11. BK1.3<sub>FITC</sub> (*N*-terminal 6-aminohexanoic acid linker) using it to directly probe this SPOT peptide array. The data was compared to P672 binding sites on CCL8, CCL2, CCL3 and CCL11 (Figure 4.9). Interestingly, BK1.3<sub>FITC</sub> was able to mostly replicate P672 binding to the N-loop of all these chemokines. However, it showed significant binding further in the core of CCL8, CCL2, CCL3 and CCL11 with interacting regions being displayed on the second  $\beta$ -strand of the chemokine  $\beta$ -sheet (Figure 4.9). This could be due to the addition of the FITC moiety to BK1.3 forming non-specific binding to those residues but is yet unclear and will need further experiments to confirm. This data indicates that BK1.3 interacts with chemokines in a similar fashion as P672 by binding to their N-loop, blocking receptor engagement and potentially disrupting chemokine/GAG binding.

## 4.5 Conclusions

Development of PPI inhibitors is challenging and requires accurate mapping of such PPIs. This work explored Evasin/chemokine interactions using mRNA peptide display and SPOT peptide array techniques. While the screening methods use peptide-peptide interactions and further experiments are needed to validate the Evasin and chemokine interacting regions, this initial set of data provided important information on the mode of binding of Evasin P672 to a set of chemokine binding partners. It provided further evidence of the role of the *N*-terminal tail of P672 for chemokine binding but also supplied a more detailed view on the incredible plasticity of P672 in binding different chemokines, at discreet sites on its *N*-terminal tail. It was also observed that Evasin P672 was binding in a very similar fashion to all chemokines tested, targeting their N-loop, providing more general molecular evidence of the binding of Evasin P672 to chemokines. Furthermore, a peptide derived from P672 and previously described, BK1.3, showed very similar mode of binding to the chemokines, translating characteristics of P672 into a smaller molecular entity.

This study is a proof of concept for the potential of peptide display (especially SPOT peptide arrays) to study and map the binding of CKBPs to chemokines in detail. More work is needed to validate interaction regions described above, but it shows the potential to accelerate the development of anti-inflammatory peptides like BK1.3 by high-throughput identification of CKBP binding sites to chemokines.

## Chapter 5 Conclusions and Future work

### 5.1 Summary

Evasins are tick saliva proteins able to neutralise a wide range of chemokines, and thus are able to feed on their host evading their immune response.<sup>182</sup> Evasins have been investigated as potential anti-inflammatory agents for their unique multiple chemokine properties.<sup>265</sup> A novel CC chemokine binding Evasin, P672, was discovered and its N-terminus was shown to be carrying its chemokine binding properties.<sup>198</sup> Further study on the PPI between P672 and CCL8 by HDxMS revealed a distinct 12 residue region of P672, Tyr21-Phe32, interacting with CCL8 (Figure 2.2). This interaction was then validated in a domain swap experiment in Evasin-1, showing that this region alone was able to transfer binding to CCL8 (Figure 2.3). These results led to the design of a peptide containing this P672 fragment, BK1.1, that was shown to bind CCL8 but also CCL7 and CCL18 (Figure 2.4 and Figure 2.5).

This work reports the design of a new series of peptides inspired by P672, which can bind to multiple chemokines such as CCL8, CCL2 and CCL3 (Figure 2.8 and Figure 2.9). BK1.3, the lead peptide, was shown to dimerise through the formation of disulfide bridge spontaneously in assay buffers (Figure 2.10). BK1.3 was further shown to neutralise CCL2-, CCL3-, CCL7- and CCL8-induced THP-1 cell migration (Figure 2.12). Furthermore, BK1.3 showed dose dependent inhibition of CCL7- and CCL8-driven THP-1 cell migration (Figure 2.14) as well as blocking CCL2 and CCL8 binding to THP-1 cell surface receptors (Figure 2.14). Critically, this peptide neutralises multiple chemokines. The chemokine network is extremely robust displaying “one-to-many” type of interaction, where one chemokine binds

to multiple receptors and *vice versa*.<sup>104</sup> This robustness explains in part the failure in the clinic of many single-chemokine or single-receptor-inhibiting agents for the treatment of inflammatory disorders. Moreover, this multiple chemokine-binding property displayed by Evasins is also present in many viral and parasitic-secreted proteins, showing that convergent evolution towards such a characteristic is beneficial to overcome chemokine signalling network robustness. Evasins, with their multiple chemokine-binding properties, have been shown to be efficacious in many pre-clinical studies against inflammatory disorders.<sup>265</sup> Therefore, neutralisation of multiple chemokines is a viable strategy for the design of agents against inflammatory disorders. Thus, it was later shown that BK1.3 displays local and distal anti-inflammatory activity in a short-lived *in vivo* inflammation model (Figure 2.15 and Figure 2.16).

In order to improve the pharmacokinetic properties of BK1.3 a series of cyclic derivatives were synthesised and showed similar biophysical activity against CCL8, CCL2 and CCL3 compared to their linear parent (Figure 3.2). BK1.2, the cyclic version of BK1.3 was then tested in cellular assays and showed mostly analogous chemokine neutralisation activity (Figure 3.3 and Figure 3.4), thus validating the potential of BK1.2 as anti-inflammatory peptide. Additionally, BK1.2 display a longer half-life in human plasma stability assay compared to the parent hit BK1.1 and its linear counterpart BK1.3 (Figure 3.5). Proteolytic fragments identified will drive the design of more resistant variants of BK1.2. To increase the potency of BK1.2, a series of sY variants were designed as sY is known to be a key PTM for chemokine receptors and Evasins binding to chemokines<sup>89,238</sup> and was shown to be present in P672 by western blot (Figure 3.6). These variants displayed increased biophysical activity in AlphaScreen displacement assay against CCL8, CCL2, CCL3 and CCL11 interaction with P672 compared to BK1.3 and BK1.2 (Figure 3.8). This provides a

way forward for the development of more potent chemokine modulatory peptides with increase pharmacokinetic profile.

Finally, better understanding of PPI involved in Evasin/chemokine binding is critical to developing anti-inflammatory peptides, such as BK1.3. While HDxMS was effectively used for BK1.3 design, the throughput and the level of reagent required can be limiting. Peptide display techniques were used to look at Evasin P672 tiling peptide library binding to several chemokines. Comparing the data produced for P672 binding to CCL8 to the previously described HDxMS interface showed loose correlation, implying that more work would be needed to validate those potential PPI interfaces (Figure 4.2). It was shown through mRNA display that the *N*-terminus of P672 plays an important role in binding, and potentially involves part of P672 *C*-terminal core when binding to CCL8, CCL2, CCL3 and CCL11 (Figure 4.3). Furthermore, SPOT peptide array membrane containing an analogous P672 tiling peptide library probed using biotinylated CCL8 showed a strong correlation with HDxMS data previously reported for the P672/CCL8 interface (Figure 4.5). The same SPOT peptide array membrane was then probed using biotinylated CCL2, CCL3 and CCL11 and displayed discreet binding sites located on the *N*-terminal tail of P672 (Figure 4.6). Further experiments, with SPOT peptide array of tiling peptide library of CCL8 and probed using His<sub>6</sub>-P672, revealed a CCL8 binding interface to P672 covering CCL8 *N*-loop in strong correlation with previously described HDxMS data on CCL8/P672 interface (Figure 4.7). SPOT peptide array membrane containing tiling peptide library of CCL2, CCL3 and CCL11 probed with His<sub>6</sub>-P672, showed very similar mode of binding with *N*-loop of chemokines being the primary target for P672 and some interaction with the  $\beta$ -sheet core of chemokines (Figure 4.8). To assess the mode of binding of BK1.3 to chemokines, the previous SPOT peptide arrays of chemokine tiling peptide libraries were probed using BK1.3<sub>FLTC</sub> and

revealed a very similar pattern of binding compared to P672 binding interactions with chemokines (Figure 4.9), showing mostly direct translation of P672 binding regions to BK1.3.

## 5.2 Conclusions

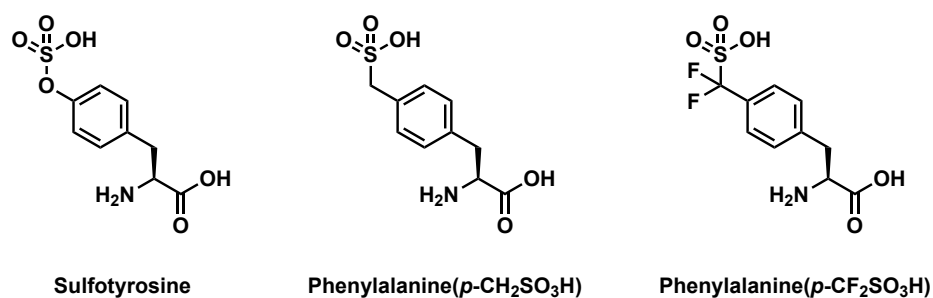
This work has shown that it is possible to design chemokine binding peptides by identifying PPIs between Evasins and chemokines. Work described in Chapter 2 showed that linear peptide derived from Evasin P672 binding site to CCL8, termed BK1.3, was able to bind and neutralise several chemokines and *in fine* produce anti-inflammatory activity *in vivo*. Moreover, work developed in Chapter 3 revealed that cyclic peptide, termed BK1.2, derived from BK1.3 hit was able to retain *in vitro* activity and displayed increased human plasma stability. Moreover, in Chapter 3 it was also shown that sY cyclic derivatives of BK1.2 showed increased activity and thus these results pave the way for further development of P672-derived peptides towards serious clinical candidates. Additionally, in Chapter 4, further study of Evasin P672/chemokine PPIs using peptide display techniques revealed that P672 and its derived linear peptide, BK1.3, both target the N-loop in several chemokines and that chemokines bind in discreet binding sites on the N-terminal tail of P672. This body of work showcases the power of understanding PPIs to design chemokine inhibitors, showing promising implications in the further identification and design of peptide agents in the future.

## 5.3 Future work

Evasin P672 is a novel Evasin discovered from a yeast display screen of putative Evasins against CCL8. The library contained around 350 Evasins and only 15 have been to date fully characterised. Therefore, many more Evasins remain to be characterised and may contain, as P672, binding sites to chemokines which could be translated into similar peptides as in Chapter 2 and Chapter 3. Moreover, systematic characterisation of the interaction of Evasins and chemokines could draw general rules for chemokine-binding peptides. From the results gathered in Chapter 4, SPOT peptide membranes or even CelluSpot glass slide peptide arrays may be suitable for high-throughput screening of such Evasin tiling library. Peptides are more tractable as therapeutics than Evasins because they are unlikely to produce immunogenicity and are chemically synthesisable permitting easier scale up and batch to batch reproducibility. Once several motifs are identified with high-throughput chemokine PPI characterisation method, more peptide design will be considered potentially involving multiple chemokine binding motif tethering, different stappling sites stabilising secondary structure and non-proteogenic amino-acids derivatives. This flexibility in the chemistry that can be applied to the design, fine-tuning of peptide affinity and physico-chemical properties, is also a major advantage of peptides over Evasins in developing attractive clinical candidates for inflammatory disorders.

Cyclic peptides BK1.2, BK1.2(sY1) and especially BK1.2(sY6) should lead to further series of promiscuously binding peptides. This new series should be informed by the human plasma stability assay from Chapter 3 to provide optimum protease resistance. Additionally, BK1.2(sY1, sY6) should be synthesised and characterised to see if the improved binding seen with each sY could be additive or synergistic. However, due to the susceptibility of sY for

hydrolysis in slightly acidic conditions, non-hydrolysable isosteres should be investigated, such as derivatives of 4-methylphenylalanine (Figure 5.1). Moreover, further information on P672 binding sites to CCL2, CCL3 and CCL11 in Chapter 4 should provide insight either on the design of slightly longer peptides or new candidates for targeted chemokines. These new peptides will need to be validated in biophysical assays, in this case AlphaScreen displacement assays or ITC if sufficient amount of chemokine target can be sourced or produced. Furthermore, extensive *in vivo* testing should be performed to assess full pharmacokinetic properties of the peptides and then testing in pre-clinical disease models should be performed to see activity in disease relevant settings such as murine models of atherosclerosis, pulmonary fibrosis or myocarditis.



**Figure 5.1** Structure of sulfotyrosine and potential non-hydrolysable isosteres

BK1.3 was shown to readily dimerise, which is unexpected. Further studies on dimerization kinetic must be performed to understand this phenomenon. For example, kinetic studies on the dimerization of P672(21-32) peptide, could provide an insight in the role of the acidic patch and *N*-terminal Tyr onto the dimerization of BK1.3. Furthermore, with thermodynamic data obtained by ITC for the binding of BK1.3 to CCL8 as well as the HDxMS data providing a reasonable binding region on CCL8, computational modelling of this interaction could be performed. Using such technique, deep mutational analysis of BK1.3 and its predicted effect on CCL8 binding could be obtained, informing further on

potential new candidates able to accommodate non proteogenic amino acids. Additionally, the use of fluorinated amino acid, such as 4-fluorophenylalanine, could help screen new candidates using fluorine NMR for binding studies against a range of chemokines, which would allow for a fast and relatively cheap pipeline for the discovery of the next generation of Evasin-inspired anti-inflammatory peptides.

Chapter 4 provided important information about the plasticity of binding of P672. Unfortunately, due to time constraints, validation of PPI regions on P672 and on several chemokines were not possible. More work needs to be carried out to synthesise these in small scale and evaluate their affinity. It is especially crucial for the mRNA display results on P672 interface with CCL8, CCL2, CCL3 and CCL11 as the regions identified on P672 for CCL8 binding diverge from previous HDxMS data. These experiments would be invaluable to validate the use of SPOT peptide membrane array and potentially mRNA display to screen Evasin/chemokine interactions.

Finally, data from the chemokine tiling SPOT peptide membrane array in Chapter 4 showed that PPIs mapped using HDxMS can be identified and reproduced using this method. Chemokines are known to activate their receptors, bind to GAGs and oligomerise. There are many interactions happening on their surface, and although they have all been shown to be important for their biology, they have not been systematically studied at a molecular level. Using techniques such as SPOT peptide membrane arrays or CelluSpot glass slide peptide arrays could be an efficient way to map these interactions on chemokines in a high-throughput manner. Deeper understanding of these interactions would provide the community with better tools to design effective and innovative agents to modulate the complex chemokine network, sustaining the effort to reduce the burden of inflammation on our communities.

## Chapter 6 Materials and Methods

### 6.1 Materials

#### 6.1.1 General chemicals, oligonucleotides, enzymes and proteins

Chemicals were purchased from Merck, Thermo Fisher or Fluorochem. Solvents were purchased from Thermo Fisher or Merck. Enzymes were purchased from New England Biolabs, Thermofisher or Biotline. Chemokines were purchased from Peprotech. Antibodies were purchased from Merck and Abcam. Streptavidin-AlexaFluor647 conjugate was purchased from Thermo Fisher. Biotinylated chemokines (site selective biotinylation at C-terminus with linker: 8-amino-3,6-dioxaoctanoic acid) were purchased from Almac. Custom oligonucleotides were purchased from Merck or IDT.

| AMINO ACID         | FMOC-PROTECTED AMINO ACID                     | MOLECULAR FORMULA                                             | SUPPLIER   |
|--------------------|-----------------------------------------------|---------------------------------------------------------------|------------|
| Alanine (A)        | Fmoc-L- <b>Ala</b> -OH                        | C <sub>18</sub> H <sub>17</sub> NO <sub>4</sub>               | CEM        |
| β-Alanine (βA)     | Fmoc-β- <b>Ala</b> -OH                        | C <sub>18</sub> H <sub>17</sub> NO <sub>4</sub>               | Alfa-Aesar |
| Aspartic acid (D)  | Fmoc-L- <b>Asp</b> (OtBu)-OH                  | C <sub>23</sub> H <sub>25</sub> NO <sub>6</sub>               | CEM        |
| Cysteine (C)       | Fmoc-L- <b>Cys</b> (Trt)-OH                   | C <sub>37</sub> H <sub>31</sub> NO <sub>4</sub> S             | CEM        |
| Glutamic acid (E)  | Fmoc-L- <b>Glu</b> (OtBu)-OH                  | C <sub>24</sub> H <sub>27</sub> NO <sub>6</sub>               | CEM        |
| Lysine (K)         | Fmoc-L- <b>Lys</b> (Boc)-OH                   | C <sub>26</sub> H <sub>32</sub> N <sub>2</sub> O <sub>6</sub> | CEM        |
| Phenylalanine (F)  | Fmoc-L- <b>Phe</b> -OH                        | C <sub>24</sub> H <sub>21</sub> NO <sub>4</sub>               | CEM        |
| Proline (P)        | Fmoc-L- <b>Pro</b> -OH                        | C <sub>20</sub> H <sub>19</sub> NO <sub>4</sub>               | CEM        |
| Threonine (T)      | Fmoc-L- <b>Thr</b> (tBu)-OH                   | C <sub>23</sub> H <sub>27</sub> NO <sub>5</sub>               | CEM        |
| Tyrosine (Y)       | Fmoc-L- <b>Tyr</b> (tBu)-OH                   | C <sub>28</sub> H <sub>29</sub> NO <sub>5</sub>               | CEM        |
| Sulfotyrosine (sY) | Fmoc-L- <b>Tyr</b> (SO <sub>2</sub> (OnP))-OH | C <sub>29</sub> H <sub>31</sub> NO <sub>8</sub> S             | Bachem     |
| Valine (V)         | Fmoc-L- <b>Val</b> -OH                        | C <sub>20</sub> H <sub>21</sub> NO <sub>4</sub>               | CEM        |

**Table 6.1** List of protected amino acids used in peptide synthesis

### 6.1.2 Buffer list

Binding buffer (BB): 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 500 mM NaCl, pH 7.5

Washing buffer (WB): 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 M NaCl, 20 mM imidazole, pH 7.5

Elution buffer (EB): 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 500 mM NaCl, 500 mM imidazole, pH 7.5

PBS: 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, 2.7 mM KCl, 137 mM NaCl, pH 7.5

PBS-500: 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, 2.7 mM KCl, 500 mM NaCl, pH 7.5

AlphaScreen buffer (ASB): 50 mM HEPES, 1% DMSO, 0.1% BSA, 0.01% Tween20, 150 mM NaCl, pH 7.5

Migration media (MM): RPMI-1640, 2 mM L-Glutamine + 0.5% heat treated FBS

LB media (LB): 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl

TB media (TB): 11.8 g/L tryptone, 23.6 g/L yeast extract, 9.4 g/L K<sub>2</sub>HPO<sub>4</sub>, 2.2 g/L KH<sub>2</sub>PO<sub>4</sub>, 0.4% v/v glycerol

TBE buffer: 89 mM Tris-borate and 2 mM EDTA, pH 8.3

TGS buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3

ITC buffer: 50 mM HEPES, 150 mM NaCl, pH 7.5

TBS: 20 mM Tris, 150 mM NaCl, pH 7.5

TBS-T: 20 mM Tris, 150 mM NaCl, 0.1% Tween20, pH 7.5

Transfer buffer: TBS in 20% methanol

### 6.1.3 Cell lines

Human monocytic THP-1 cells (ECACC 88081201) were used in this work. They were propagated in suspension culture and kept at  $\sim 0.3 \times 10^6$  cells/mL in culture media (RPMI-1640, 4 mM L-Glutamine + 10% heat treated FBS). THP-1 cells were kept in a

humidified chamber at 37°C and 5% CO<sub>2</sub>. Cultures were tested for mycoplasma regularly using MycoAlert test kit (Lonza).

#### 6.1.4 Synthetic peptides

P672(C30A)<sub>21-32</sub> and P672<sub>21-32</sub> were purchased from GL Biochem (Shanghai) as C-terminal amide products. BK1.3<sub>FITC</sub> was purchased from GL Biochem as a C-terminal amide product and with a 6-aminohexanoic acid linker to the FITC moiety at the N-terminus. They were synthesised using standard Fmoc solid phase peptide synthesis, deemed >90% pure via HPLC analysis and their mass verified using LCMS by GL Biochem. Peptides were dissolved in d<sub>6</sub>-DMSO and their accurate concentration was established using quantitative NMR with d<sub>4</sub>-TSP as internal standard.<sup>266</sup>

Synthetic biotinylated chemokines were purchased from Almac. Biotin moiety was selectively inserted at the C-terminus using 8-amino-3,6-dioxaoctanoic acid as a linker. They were deemed >95% purity by HPLC analysis and their mass were verified with MS analysis by Almac. Biotinylated chemokines were dissolved in 20 µM stocks in pure milliQ water as recommended by the manufacturer.

#### 6.1.5 Plasmid and PCR product purification kits

Macherey-Nagel NucleoSpin Plasmid kit was used to isolate plasmid DNA using the manufacturer's instructions. Final PCR products for sequencing were purified using NucleoSpin Gel and PCR Clean-up kit from Macherey-Nagel according to manufacturer's instructions.

## 6.2 Methods

### 6.2.1 PCR

Polymerase chain reaction was performed using either Q5 DNA polymerase (New England Biolabs) or BioTaq polymerase (Bioline) using the manufacturer's buffers and instructions. PCR primers were designed with an annealing temperature of 55-60°C. An initial heating step (1 min at 95°C) was performed to heat activate the polymerase. Then 15 to 30 cycles of 95°C denaturation step (40 seconds), 50°C annealing step (40 seconds), and 72°C elongation step (40 seconds). The number of cycles was adjusted depending on the amount of input DNA.

### 6.2.2 Transformation

Propagation of plasmids was carried out using DH5- $\alpha$  (Invitrogen) *E. coli* bacteria cell line. Chemically competent DH5- $\alpha$  *E. coli* were kept on ice and incubated with 50-100 ng of the plasmid for 30 minutes. Cells were then heat shocked at 42°C for 45 seconds and placed back on ice for one minute. Pre-warmed (1 mL, 37°C) LB media was added to the cells, which were then incubated under vigorous shaking at 37°C for one hour. This cell suspension (100  $\mu$ L) was plated on agar plates containing the appropriate antibiotic. The remaining suspension was gently spun down (4000  $\times g$ , 5 min, RT), supernatant was discarded, and pellets were resuspended in 50  $\mu$ L of fresh LB media, then plated on another agar plate containing the appropriate antibiotic. Both plates were incubated overnight at 37°C. Single colonies were picked and left to grow in 5 mL of LB media with the appropriate antibiotic at 37°C overnight. Cells were spun down (4000  $\times g$ , 5 min, RT), the supernatant was discarded,

pellets were lysed, protein and cell debris were precipitated, and plasmid DNA was purified using NucleoSpin Plasmid kit (Macherey-Nagel) following manufacturer's instructions.

### 6.2.3 Agarose gel electrophoresis

DNA fragments and PCR products were analysed with a 3% agarose gel. TBE buffer (50 mL) combined with 1.5 g of agarose was heated until all solid was dissolved in a microwave and then complemented with SYBR Safe gel stain (LifeTechnologies). The gel was set, DNA was loaded, bands were separated on a horizontal gel electrophoresis apparatus (Bio-Rad) by application of a potential difference of 150 V for 30 minutes and finally visualised using a ChemiDoc imager (Bio-Rad).

### 6.2.4 SDS-PAGE

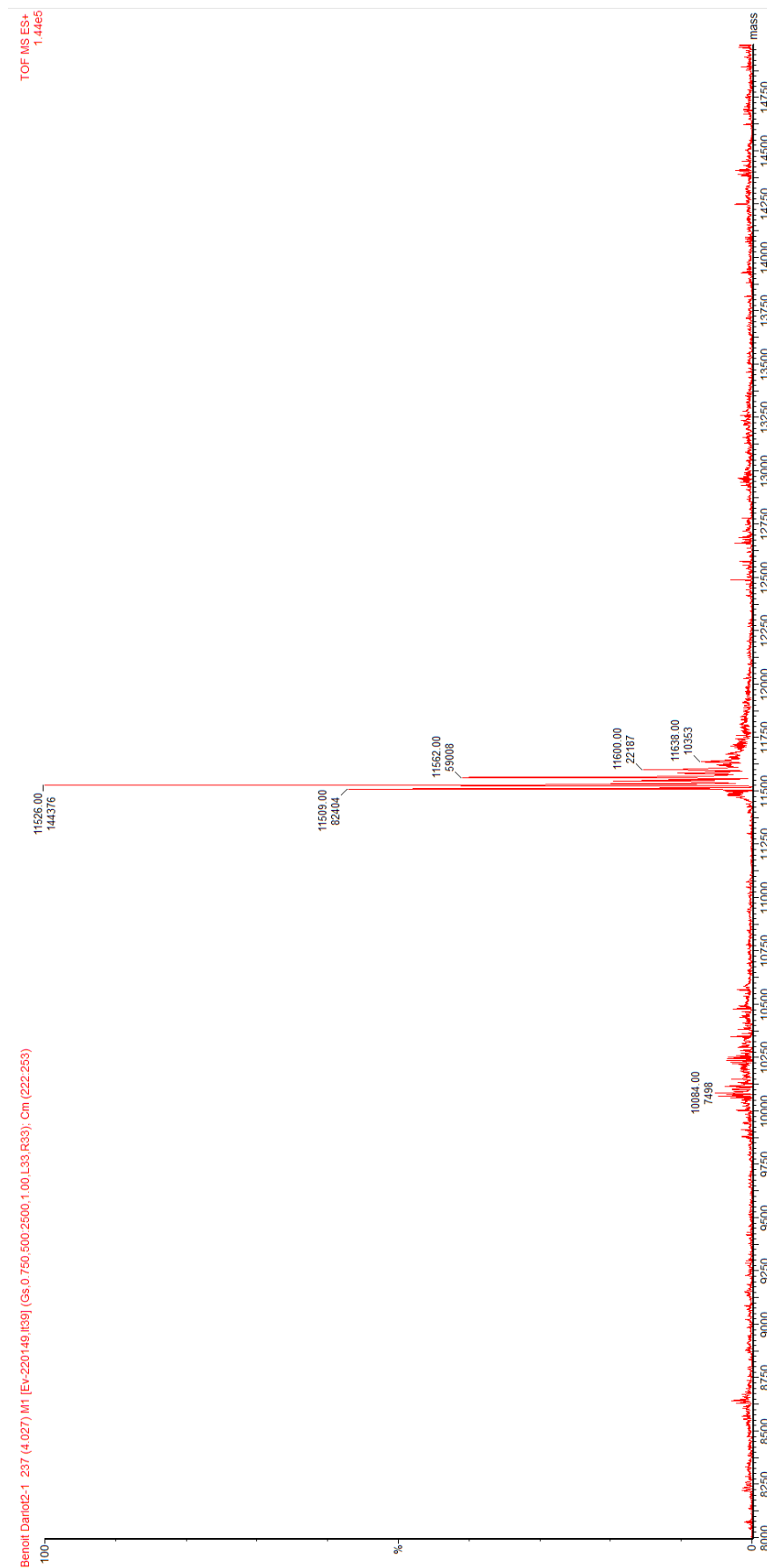
SDS-PAGE was performed using 4-15% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad). Gels were ran using TGS buffer for 25-30 minutes at 200 V. Gels were then washed with water and stained using colloidal Coomassie blue (InstantBlue™) for at least one hour. The protein gel was then thoroughly washed with water several times before imaging using a ChemiDoc imager (Bio-Rad).

### 6.2.5 Protein expression in *E. coli*

Human CCL8 expression plasmid was provided as a gift from Dr. James Eaton. It was constructed with the PCR amplification of a SUMO-CCL8 cassette using ligation-independent cloning into a pNIC-BIO3 vector (GenBank: JN792439) to create a His<sub>6</sub>-SUMO-CCL8 expression vector containing a C-terminal GSKGGYGLNDIFEAQKIEWHE (Avi) tag.

Human CCL8 (residues 24-99) was co-expressed with BirA (UniProtKB - P06709) in *E. coli* RosettaGami™ 2 (DE3) cells (Novagen). Initial bacterial culture was grown overnight at 37°C in LB media containing chloramphenicol (25 µM/mL), tetracycline (10 µM/mL), kanamycin (50 µM/mL) and Spectinomycin (100 µM/mL). This initial culture was then diluted 1 in 30 in TB media and incubated at 37°C until the culture reached  $A_{600}$  of ~0.8. Then Isopropyl β-d-1-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 1 mM as well as biotin to a final concentration of 500 mM, allowing the culture to incubate overnight at 18°C. The resulting cells were pelleted using centrifugation (4000  $xg$ , 30 minutes, 4°C) and pellets were stored at -80°C.

Pellets (10-20 g) were suspended in BB and sonicated on ice to lyse the cells. Few crystals of DNase I were added to degrade genomic DNA. Soluble and insoluble fractions were separated using centrifugation (30000  $xg$ , 45 minutes, 4°C). Soluble fraction was loaded by gravity flow on nickel-charged IMAC Sepharose 6 Fast Flow resin (Cytivia), equilibrated with BB, washed after binding with WB, and finally eluted in 3 mL fractions with EB. Elution fractions were desalted using PD-10 desalting column (Cytivia) according to manufacturer's instruction and eluted in PBS-500. DTT was added to the eluate to a final concentration of 1 mM as well as SUMO-protease (gift from Dr. Chiara Maniaci) and was left to react overnight at 4°C. The cleaved chemokine was finally purified using size exclusion chromatography on a HighLoad Superdex 16/600 75 µg column (Cytivia) equilibrated in final buffer PBS-500.



**Figure 6.1** CCL8-Avi-Biotin protein MS spectrum deconvoluted  
 Mass expected  $[M+H]^+ = 11526$  m/z (biotinylated construct). Mass observed:  $[M+H]^+ = 11509$  m/z (pyroglutamine formation),  
 and  $[M+K]^+ = 11562$  m/z.

### 6.2.6 Cell migration

The concentration of chemokine which is able to produce 80% of maximal migration ( $EC_{80}$ ) was determined using 96-well transwell migration plates (HTS Transwell® 96 well permeable supports, 5  $\mu$ m pore polycarbonate membrane, Corning) in three technical replicates and three biological replicates, as previously described.<sup>70</sup> Chemokines (0-100 nM (Peprotech or produced in house) in 150  $\mu$ L MM) were dispensed in the reservoir. Cells (THP-1, 300,000 cells in 50  $\mu$ L MM) were placed in the upper chamber and were incubated at 37°C, 5% CO<sub>2</sub> in humidified chamber for four hours. The upper chamber insert was then removed and the plate was shaken at 850 r.p.m. for 10 minutes. Media from the reservoir was transferred to a V-bottomed 96 well plate. Cells were counted using an ATTUNE flow cytometer (Life Technology) using forward versus side scatter (FSC vs SCC) dot plot and data was finally analysed using GraphPad Prism fitting to an agonist response curve with four parameters.

IC<sub>50</sub> values for peptides and Evasin P672 were determined in three technical replicates and three biological replicates as above. Chemokine (at  $EC_{80}$ : CCL8 (5.8 nM), CCL2 (1.2 nM), CCL7 (7.2 nM) and CCL3 (3.5 nM)) and inhibitor (at varying doses) were added to the reservoir and pre-incubated for 30 minutes at 37°C before beginning the cell migration assay described as before. In all peptide IC<sub>50</sub> determination assay all MM was complemented with 0.2% DMSO to insure peptide solubility. Data was analysed in GraphPad prism fitting an inhibitor response curve with four parameters.

## 6.2.7 Peptide synthesis

All peptides in this work were synthesised with an amidated C-terminus on either a 50  $\mu\text{mol}$  (BK1.1, BK1.2, BK1.3, BK1.4, BK1.5 and SCR) or 100  $\mu\text{mol}$  (BK1.2(sY1) and BK1.2(sY1)) scale using NovaPEG Rink Amide resin (Merck) and following standard Fmoc protection chemistry. *N,N'*-Diisopropylcarbodiimide (DIC) (Fluorochem) was used as a coupling agent, Oxyma Pure (CEM) as the activator, 20% piperidine in dimethylformamide (DMF) (Merck) as the deprotection mix and DMF (Merck) as solvent. Synthesis was performed either on an automated LibertyBlue™ (CEM) microwave peptide synthesiser or an automated PurePep® Chorus (Gyros) peptide synthesiser following manufacturer's instructions. Typically coupling of amino acid was performed in 5-molar excess at 90°C (2 min in LibertyBlue™ (CEM) and 5 min in PurePep® Chorus (Gyros)) except for Cysteine, Histidine and Fmoc-Tyr(SO<sub>2</sub>(OnP))-OH (protected sY, Bachem) couplings performed at 50°C (5 min in LibertyBlue™ (CEM) and 15 min in PurePep® Chorus (Gyros)). Linear precursors of cyclic peptides were finally capped using a 5-molar excess of chloroacetic anhydride at 60°C (automated: 5 min in LibertyBlue™ (CEM) and 15 min in PurePep® Chorus (Gyros)).

BK1.1, BK1.2, BK1.3, BK1.4, BK1.5 and SCR were synthesised using LibertyBlue™ (CEM) microwave peptide synthesiser. BK1.2(sY1) and BK1.2(sY6) were synthesised using PurePep® Chorus (Gyros) peptide synthesiser.

The resin was then thoroughly washed with dichloromethane (DCM) (Fisher) and dried. Peptides were then cleaved and deprotected using typically 3-5 mL of deprotection mix: TFA:water:TIPS:1,3dimethoxybenzene (92.5:2.5:2.5:2.5) with gentle mixing at RT for three hours. Peptides were then precipitated by mixing this concentrated acidic liquor into cold diethyl ether (Fisher). Pellets were recovered by centrifugation (14000  $xg$ , 10 min, 4°C),

discarding supernatant and resuspended in fresh cold diethyl ether. This process was repeated four times, then pellets were left to air dry overnight.

*Linear peptides:* Peptide dried pellets were then dissolved in the minimum amount of water and acetonitrile (Fisher) and were purified by preparative HPLC (JASCO system with Gemini-NX 5 C18 110A (250-30mm) column, 20 mL/min varying gradient of either water (0.1% TFA) and acetonitrile (0.1% TFA) or 95:5 water:acetonitrile (10 mM  $\text{NH}_4\text{HCO}_3$ ) and 5:95 water:acetonitrile (10 mM  $\text{NH}_4\text{HCO}_3$ )). Fractions were analysed using MALDI (microflex MALDI-TOF, Bruker), and fractions containing pure peptides were then lyophilised.

*Cyclic peptides:* Peptide dried pellets were then dissolved in 1 mL of triethylammonium acetate (1 M, pH 9.6) and minimum amount of acetonitrile (Fisher), pH was adjusted to 9.6. Cyclisation was then performed by incubating this solution at 37°C for one hour, confirmation of completion of cyclisation was obtained by MALDI analysis. The resulting solution was then lyophilised. Lyophilised powder was then dissolved in minimum amount of water (0.1% TFA) and acetonitrile (0.1% TFA) and purified by preparative HPLC as previously described above.

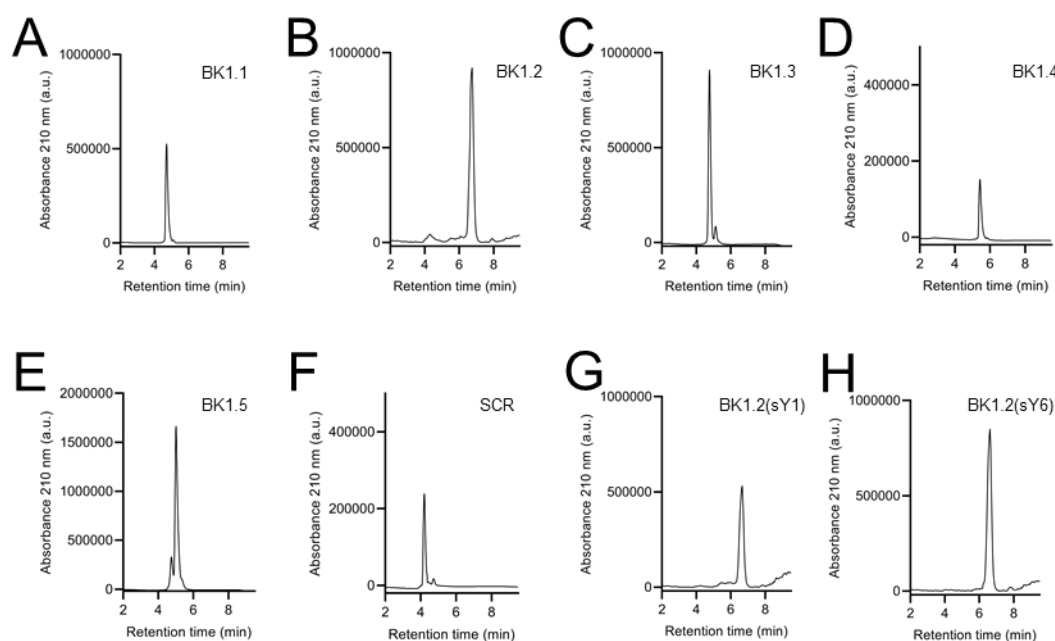
*Cyclic sY derivative peptides:* Cyclisation and deprotection of neopentyl group were carried out simultaneously as previously described<sup>221</sup> from linear precursor. Briefly, 20 equivalents of  $\text{NaN}_3$  were added to the cyclisation mix after completion of cyclisation and was further incubated at 70°C overnight. This mix was directly purified by preparative HPLC (JASCO system with Gemini-NX 5 C18 110A (250-30mm) column, 20 mL/min varying gradient of 95:5 water:acetonitrile (10 mM  $\text{NH}_4\text{HCO}_3$ ) and 5:95 water:acetonitrile (10 mM  $\text{NH}_4\text{HCO}_3$ )). Fractions were analysed using MALDI (microflex MALDI-TOF, Bruker), and fractions containing pure peptides were then lyophilised.

Pure peptide lyophilised powder were then typically dissolved in d<sub>6</sub>-DMSO to form stocks at 100 mM that would be stored at -20°C. Accurate stock concentration was established using quantitative NMR with d<sub>4</sub>-TSP as internal standard.<sup>266</sup>

| Peptide    | Formula                                                                          | Exact mass ( <i>m/z</i> )        | HRMS ( <i>m/z</i> ) | Δ (ppm) | Yield* |
|------------|----------------------------------------------------------------------------------|----------------------------------|---------------------|---------|--------|
| BK1.1      | C <sub>95</sub> H <sub>124</sub> N <sub>18</sub> O <sub>31</sub>                 | [M+H] <sup>+</sup> = 2013.87527  | 2013.87025          | 2.5     | 8%     |
| BK1.2      | C <sub>106</sub> H <sub>133</sub> N <sub>19</sub> O <sub>34</sub> S              | [M+H] <sup>+</sup> = 2248.90558  | 2248.90576          | 0.1     | 16%    |
| BK1.3      | C <sub>104</sub> H <sub>133</sub> N <sub>19</sub> O <sub>33</sub> S              | [M+H] <sup>+</sup> = 2208.91067  | 2208.91479          | 1.9     | 17%    |
| BK1.4      | C <sub>97</sub> H <sub>124</sub> N <sub>18</sub> O <sub>32</sub> S               | [M+H] <sup>+</sup> = 2085.84225  | 2085.83838          | 1.9     | 22%    |
| BK1.5      | C <sub>95</sub> H <sub>124</sub> N <sub>18</sub> O <sub>31</sub> S               | [M+H] <sup>+</sup> = 2045.84734  | 2045.84216          | 2.5     | 24%    |
| SCR        | C <sub>95</sub> H <sub>124</sub> N <sub>18</sub> O <sub>31</sub>                 | [M+H] <sup>+</sup> = 2013.8759   | 2013.8716           | 2.1     | 14%    |
| BK1.2(sY1) | C <sub>106</sub> H <sub>133</sub> N <sub>19</sub> O <sub>37</sub> S <sub>2</sub> | [M-2H] <sup>2-</sup> = 1162.9197 | 1162.9219           | 1.9     | 2.4%   |
| BK1.2(sY6) | C <sub>106</sub> H <sub>133</sub> N <sub>19</sub> O <sub>37</sub> S <sub>2</sub> | [M-2H] <sup>2-</sup> = 1162.9197 | 1162.9235           | 3.3     | 2.8%   |

**Table 6.2** Peptide characterisation summary

\*Yields calculated for the synthesis on 100 μmol scale (BK1.1, BK1.2, BK1.3, BK1.4, BK1.5 and SCR) and 50 μmol scale (BK1.2(sY1) and BK1.2(sY6)) adjusted after NMR quantification.



**Figure 6.2** LCMS spectra of each peptides

HPLC spectra for **(A)** BK1.1 (retention time: 4.71 min, purity<sub>A210</sub>: 98%), **(B)** BK1.2 (retention time: 6.74 min, purity<sub>A210</sub>: 93%), **(C)** BK1.3 (retention time: 4.76 min, purity<sub>A210</sub>: 94%), **(D)** BK1.4 (retention time: 5.43 min, purity<sub>A210</sub>: 99%), **(E)** BK1.5 (retention time: 5.01 min, purity<sub>A210</sub>: 90%, modelled on 2 gaussian curves overlapping), **(F)** SCR (retention time: 4.21 min, purity<sub>A210</sub>: 94%), **(G)** BK1.2(sY1) (retention time: 6.25 min, purity<sub>A210</sub>: 92%) and **(H)** BK1.2(sY6) (retention time: 6.63 min, purity<sub>A210</sub>: 98%). Samples were analysed with LCMS Agilent Infinity II 1260 HPLC + MSD XT, Raptor C-18 column (100 x 3.0 mm; 2.7 μm particle size), gradient of water (0.1% TFA) and acetonitrile (0.1% TFA) over 10 min except for BK1.2, BK1.2(sY1) and BK1.2(sY6) with gradient of water (10 mM NH<sub>4</sub>OH) and acetonitrile (10 mM NH<sub>4</sub>OH).

### 6.2.8 Alphascreen assay

Optimal concentration of biotinylated chemokine (Almac or in house produced) and His<sub>6</sub>-P672 (gift from Dr. James Eaton) were determined using AlphaScreen® Histidine detection kit from PerkinElmer (6760619M lot 2457886). The assay was set up in white-bottomed Proxiplate™ 384 Plus microplates (PerkinElmer) following the manufacturer's instructions. Varying concentration of His<sub>6</sub>-P672 in ASB were combined with several set concentrations of biotinylated chemokine in ASB and were allowed to pre-incubate at RT for 30 minutes. Finally, acceptor and receptor beads were added as a 1:1 suspension in ASB in each well and allowed to further incubate at RT for one hour away from light. Data was obtained using a Pherastar FSX plate reader (excitation, 680 nm; emission, 570 nm, BMG Labtech) and was analysed using GraphPad Prism. Optimal signal to amount of material used was determined for each P672/chemokine pair.

IC<sub>50</sub> values for peptides were obtained following the same assay described above. Different final concentrations of peptides in ASB (typically 0-250 µM) were incubated with optimal final concentration of biotinylated chemokine determined previously in ASB (1.25 nM (CCL8), 5 nM (CCL2), 2.5 nM (CCL3), and 5 nM (CCL11)) at RT for 15 minutes. Then final concentration of His<sub>6</sub>-P672 determined previously in ASB (2.5 nM (CCL8), 5 nM (CCL2), 1.25 nM (CCL3), and 0.625 nM (CCL11)) was added and further incubated at RT for 30 minutes. Finally, acceptor and receptor beads were added as a 1:1 suspension in ASB in each well and allowed to further incubate at RT for one hour away from light. Data was obtained using a Pherastar FSX plate reader (excitation, 680 nm; emission, 570 nm). Data was analysed in GraphPad prism fitting an inhibitor response curve with four parameters.

### 6.2.9 Isothermal titration calorimetry

Isothermal titration calorimetry was carried out on a TA Instruments Affinity ITC with low volume cells at 25°C and stirring at 250 r.p.m. in matched ITC buffer. Twenty sequential 2 µl injections of BK1.3 (125 µM) into CCL8 (10 µM) were used to generate a binding isotherm for the binding interaction. The isotherm was fitted to an independent (one site) model using NanoAnalyze (TA Instruments).

### 6.2.10 Peptide stability assay

Human citrated plasma (Sigma P9523) was reconstituted using PBS. Peptides and propantheline bromide were dissolved to 400 µM final concentration in a 200 µL stock of human reconstituted plasma. Stocks were incubated at 37°C and 20 µL samples were taken at different time points. Samples were immediately quenched with 60 µL of methanol:acetonitrile (1:1), centrifuged at 17,000 x *g* for 15 min and 50 µL of supernatant was stored for later analysis. Samples were analysed with LCMS (Agilent Infinity II 1260 HPLC + MSD XT, Raptor C-18 column (100 x 3.0 mm; 2.7 µm particle size), gradient of water (0.1% TFA) and acetonitrile (0.1% TFA)). Area under the curve of the peak of the right mass were compared to same peak in initial (0 minute) time point and converted in % of intact remaining peptide. Data was then analysed using GraphPad Prism and fitted with a one phase decay model to extract half-life values.

### 6.2.11 SPOT peptide array synthesis

SPOT peptide array synthesis was performed as described previously<sup>267</sup> using a MultiPep (CEM) automated peptide synthesiser. Briefly, cellulose membrane (Whatman Chromatography paper) were functionalised using 10 mL DMF solution containing 0.64 g of Fmoc- $\beta$ -alanine-OH, 374  $\mu$ L DIC and 317  $\mu$ L *N*-methylimidazole (NMI) (1:1:1), and incubated at RT overnight. The membrane was then thoroughly washed with DMF and then Fmoc-deprotection was performed by incubating the membrane in 20% piperidine in DMF at RT for 5 minutes, twice, and washed with DMF.

The membrane was then placed on the MultiPep automated peptide synthesiser and synthesis was carried out following manufacturer's instructions (sequence of coupling, deprotection, washing and drying: hardcoded in the ResPepSLi\_Spotter software). Protected amino acids were premixed with DIC, Oxyma Pure and *N*-methyl-2-pyrrolidone (NMP) (1:1:1:1) allowed to activate at RT for 15 minutes (pre-activated mix). Coupling was performed with pre-activated mix applied to specific SPOT positions (100 nL) and allowed to react 5 minutes at RT. The coupling was repeated a second time using the same pre-activated mix. Membrane was then covered with capping solution (2% acetic anhydride, 2% *N,N*-Diisopropylethylamine (DIPEA) in DMF) and left to react for 15 minutes at RT. The membrane was then dried, thoroughly washed with DMF, then washed with ethanol and finally dried. Deprotection solution (20% piperidine in DMF) was then added to cover the membrane and allowed to react for 15 minutes at RT. The membrane was then dried, thoroughly washed with DMF, then washed with ethanol and finally dried, ready for the next coupling cycle.

The first amino acid added was Fmoc- $\beta$ -alanine-OH to define the spot pattern on the membrane and then the synthesis of the peptides was carried out.

For the final step after the last deprotection, the membrane was capped one last time with capping solution (2% acetic anhydride, 2% DIPEA in DMF) and left to react for 15 minutes at RT. The membrane was then dried, thoroughly washed with DMF, then washed with ethanol and finally dried.

The membrane was treated with TFA:water:TIPS:1,3-dimethoxybenzene at 92.5:2.5:2.5:2.5 (deprotection mix) and allowed to incubate for two hours at RT. The membrane was carefully washed five times with DCM, then five times with ethanol and finally air dried. The membrane was then ready to use for probing.

#### 6.2.12 SPOT peptide array probing

Dried membrane was blocked using 5% skimmed milk in TBS-T for 2h at RT. The membrane was then washed three times with TBS-T. The membrane was then hybridised using either 0.1  $\mu$ M biotinylated chemokine (CCL8: in-house production; CCL2, CCL3, CCL11: Almac) or 0.1  $\mu$ M His<sub>6</sub>-P672 (gift from Dr. James Eaton) or 1  $\mu$ M BK1.3<sub>FITC</sub> (GL Biochem) in TBS-T containing 5% BSA for one hour at RT. The membrane was carefully washed three times with TBS-T. BK1.3<sub>FITC</sub> probing was directly imaged using a ChemiDoc imager (Bio-Rad), for biotinylated chemokines the membrane was further hybridised with 1:2000 Streptavidin-AlexaFluor-647 conjugate (Thermo Fisher) in TBS-T containing 5% BSA for one hour at RT and for His<sub>6</sub>-P672 probing the membrane was further hybridised with 1:2000 Rabbit monoclonal [EPR20547] to 6X His tag<sup>®</sup> (Alexa Fluor<sup>®</sup> 647) antibody conjugate (ab237337, Abcam) in TBS-T containing 5% BSA for one hour at RT. The membrane was finally washed three times with TBS-T and imaged using a ChemiDoc imager (Bio-Rad).

Signal intensity of each spot (Image Lab, Bio-Rad) was divided by the number of residues contained in the peptide probed (16 for P672 tiling library and 10 for chemokine tiling library) and each residue was assigned that value. Each residue was assigned a final score calculated as the average of each individual contribution for this residue. A scale was established from maximum residue score (1, red) to minimum residue score (0, blue) for each individual protein tiling experiment.

### 6.2.13 Western Blot

Protein separated on SDS-PAGE gel were transferred to nitrocellulose membrane (GE Healthcare) in transfer buffer at 90 V for two hours on ice. Membrane was then washed with water and blocked using 5% skimmed milk in TBS-T for 2h at RT. The membrane was then carefully washed three times with TBS-T. The membrane was hybridised with anti-sY primary antibody (Clone Sulfo-1C-A2 (05-1100X), Sigma) at 4°C overnight. The membrane was carefully washed three times with TBS-T. The membrane was finally incubated with 1:2000 Anti-mouse IgG HRP conjugate and 1:2000 Streptavidin-AlexaFluor-647 conjugate (Thermo Fisher) in TBS-T containing 5% BSA for one hour at RT. The membrane was then carefully washed three times with TBS-T. Finally, the membrane was developed using Pierce™ ECL Western Blotting Substrate (Thermo Scientific) following manufacturer's instructions. The membrane was then imaged using a ChemiDoc imager (Bio-Rad).

## 6.2.14 mRNA peptide display screening

DNA pooled library consisting of 89 sequences (5'- CTT TAA GAA GGA GAT ATA CAT ATG - (48 bases coding sequence) - GGC AGC GGT AGC GGG AGC TAG – 3') coding for 16mer tiling peptides of P672 (all Cysteine mutated to Serine) was purchased from IDT. The library was amplified by PCR using Q5 DNA polymerase and HS\_P1 (5' – TAA TAC GAC TCA CTA TAG GGT TAA CTT TAA GAA GGA GAT ATA CAT ATG – 3') and HS\_P2\_EVA (5' – TTT CCG CCC CCC GTC CTA GCT CCC GCT ACC GCT GCC – 3') as primers. The resulting DNA library product was purified using Phenol/Chloroform extraction and ethanol precipitation as previously described<sup>268</sup>, finally quantified using Qubit fluorometer (Thermo Fisher). The DNA library was then transcribed to mRNA using TranscriptAid T7 high yield kit (Thermo Fisher) following manufacturer's instruction for an overnight reaction at 37°C. The resulting mRNA library was purified using Phenol/Chloroform extraction and ethanol precipitation as previously described<sup>268</sup>, finally quantified using Qubit fluorometer.

### *Puromycin linker ligation to mRNA*

mRNA library (10 µM final concentration) was ligated to a puromycin linker (5' – CTC CCG CCC CCC GTC C – (SPC18)<sub>5</sub> – CC – puromycin – 3'; 30 µM final concentration) using T4 RNA ligase (Thermo Fisher) and following manufacturer's instruction at RT for one hour. Enzyme was heat inactivated at 70°C for 10 minutes.

### *In vitro translation*

IVTT kit was purchased from New England Biolab and used to translate the puromycin linked mRNA library (final concentration 2.5 µM) following manufacturer's instruction for a 5 µL reaction at 37°C for one hour. Reaction was incubated further 10 min at 60°C to inactivate the IVTT kit.

### *Reverse transcription*

M-MLV reverse transcriptase was purchased from Promega and used to reverse translate the now peptide linked mRNA library (final concentration 1.25  $\mu$ M) following manufacturer's instruction for a 10  $\mu$ L reaction at 42°C for one hour. The reaction was then diluted with ice-cold 2x PBS containing 0.1% Tween20.

### *Negative selection*

Dynabeads-Streptavidin M280 were purchased from Thermo Scientific. Dynabeads slurry (10  $\mu$ L) was thoroughly washed with PBS containing 0.05% Tween20 and finally resuspended in the 20  $\mu$ L of diluted reverse transcription reaction described above. This suspension was incubated on a shaker for 30 minutes at 37°C.

### *Positive selection*

Dynabeads slurry (10  $\mu$ L) was thoroughly washed with PBS containing 0.05% Tween20 and finally resuspended with 10  $\mu$ L of biotinylated chemokine (CCL8: in-house; CCL2, CCL3, CCL11: Almac) at 2  $\mu$ M in PBS containing 0.05% Tween20 (20 pmol). The biotinylated chemokine was allowed to incubate in the bead suspension on a shaker for 30 minutes at 37°C. The slurry was thoroughly washed with PBS containing 0.05% Tween20 and finally resuspended with the supernatant of the negative selection described above. The peptide linked mRNA/cDNA library was allowed to incubate with the chemokine loaded beads on a shaker for one hour at 37°C.

### *DNA recovery*

cDNA linked to peptides bound to beads were recovered by quickly washing the positive and negative beads with ice cold PBS containing 0.05% Tween20 and then resuspending them in 100  $\mu$ L of PCR buffer condition for BioTaq (BioLine) according to manufacturer's instructions containing HS\_P1 (5' – TAA TAC GAC TCA CTA TAG GGT TAA CTT

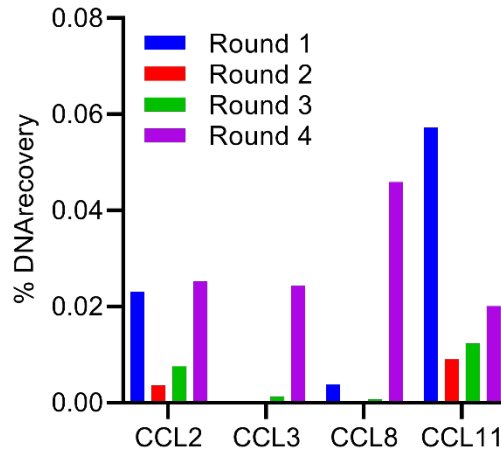
TAA GAA GGA GAT ATA CAT ATG – 3') and HS\_P2\_EVA (5' – TTT CCG CCC CCC GTC CTA GCT CCC GCT ACC GCT GCC – 3') as primers. Recovered DNA was quantified with qPCR analysis (CFX qPCR Instrument, Bio-Rad) using SYBR Green qPCR reagent mix (Thermo Scientific) following manufacturer's instructions.

DNA from positive beads was then amplified using PCR after addition of BioTaq DNA polymerase following manufacturer's instructions with a number of PCR cycle dependant on the concentration determined by qPCR. DNA product was analysed by 3% agarose gel electrophoresis to control the expected size of the products. Then DNA was purified using phenol/chloroform extraction and ethanol precipitation as previously described<sup>268</sup>, finally quantified using Qubit fluorometer.

#### *Transcription reaction*

The resulting selected DNA library was transcribed to mRNA using TranscriptAid T7 high yield kit (Thermo Fisher) following manufacturer's instruction for an overnight 20 µL reaction at 37°C. The resulting mRNA library was purified using Phenol/Chloroform extraction and ethanol precipitation as previously described<sup>268</sup>, finally quantified using Qubit fluorometer.

The resulting mRNA library after this round was fed again for further selection round up until the fourth round.



**Figure 6.3** DNA recovery summary from chemokine selections  
*DNA recovery converted in percentage of input library from qPCR quantification*

Resulting DNA recovered from selection round four and initial library were amplified using Q5 DNA polymerase following manufacturer's instructions with TEM\_junk\_fwd (5' – ATT GAC TAA CGT ACT GCA TAA TCT AAT ACG ACT CAC TAT AGG GT – 3') and TEM\_junk\_rev (5' – CAT TGA TAA CTG CAT GCA TAA TCT TTT CCG CCC CCC G – 3') as primers to create larger constructs. Then following the same PCR protocol using Q5 DNA polymerase the DNA library was further PCR amplified with either GW2\_F1.F64 (5' – ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT **CTA GCG CTA** TTG ACT AAC GTA CTG CAT AAT C – 3') or GW2\_F2.F62 (5' – ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT **TGC ATA TCA** TTG ACT AAC GTA CTG CAT AAT C – 3') as forward primer and either GW2\_R3.R63 (5' – GAC TGG AGT TCA GAC GTG TGC TCT TCC GAT CTC **GTC TGC** GCA TTG ATA ACT GCA TGC ATA ATC – 3') or GW2\_R4.R63 (5' – GAC TGG AGT TCA GAC GTG TGC TCT TCC GAT CTT **ACT CAT ACA** TTG ATA ACT GCA TGC ATA ATC – 3') as reverse primer. DNA was purified using NucleoSpin Gel and PCR Clean-up kit from Macherey-Nagel according to manufacturer's instructions and then analysed by 3% agarose gel electrophoresis to control the expected size of the products.

Samples were then submitted to GeneWiz NGS services. Sequencing data was analysed using a python script built in collaboration with Dr. Tom McAllister. The data was checked (containing right adaptors, the right size and not containing sequencing errors), then a list is generated of each sequence, the number of reads they account for and the peptide they code for. This list is then further processed to give a list of peptides with the count of all DNA sequences coding for them, the amount of total sequence count they account for and finally their proportion of all reads. The data proportion of reads from the last round was then compared to the proportion of reads in the initial library to give an  $E$  (enrichment score) to each peptide like follows:

$$E_{peptide} = \frac{f_{final\ round}}{f_{initial\ library}}$$

Then each residue in P672 was assigned a score ( $e$ ) as sum of contribution from each peptide containing this residue such as:

$$e_{residue} = \sum_{\substack{\text{peptides} \\ \text{containing} \\ \text{residue}}} \frac{E_{peptide}}{length_{peptide}}$$

This final result was plotted as heat map to highlight P672 residues contributing the most to peptide enrichment.

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