School of Medicine
Institute of Infection and Immunity
Ysgol Meddygaeth
Adran Heintiau Imiwnedd

CXCL14 function and target cells in healthy tissues

Ariadni Kouzeli

Thesis presented for the Degree of Doctor of Philosophy

November 2019

Division of Infection and Immunity

School of Medicine

Cardiff University

Acknowledgements

I would first like to thank my primary supervisor Prof Bernhard Moser for his continued guidance and support throughout my PhD. His enthusiasm and passion for scientific research is admirable and inspiring. I would also like to thank my co-supervisor Prof Matthias Eberl, for the valuable conversations helping me overcome hurdles both scientifically, but mostly personally. Their supervision and encouragement have helped me push through the difficult times of my project.

I am also extremely grateful to my colleagues and friends Paul Collins and Michelle McCully for helping me during the start of my project. Without their encouragement and support I would never have changed my project. They taught me a lot in the lab during the start of my PhD, including chemotaxis assays, processing of tissues and flow cytometry. Apart from their guidance in the lab, their moral support during the hard times has been invaluable. Thank you to Chia-Te for the assistance in the *in vivo* mouse experiments and my third supervisor Prof Phil Taylor for the valuable discussions about my project. A huge thank you to my wonderful lab group, older and newer members; Hung-Chang, Anna Rita, Chris, Ann, Alex, Amy, Teja, Raya, Simone, Sarah, Loic, Nicola, Rebecca, Ross and Oliwia. You were always there to help, listen, give feedback and share many cakes with. I couldn't have wished for a nicer group of people to share the last four years with.

I must thank Prof Paul Proost and Prof Sofie Struyf for welcoming me into their lab at the Rega Institute in Leuven, Belgium. I was lucky enough to spend a total of three months in their lab to learn how to synthesise and purify chemokines and how to perform calcium mobilisation experiments. I am grateful for sharing their technical expertise, but also for taking care of me when I broke my arm... They supervise an amazing group of people who warmly welcomed me into their group and made my stay in Belgium one of the highlights of my PhD (apart from the broken arm!). I must give special mention to Mieke Metzemaekers for showing me the ropes and instilling some of her enthusiasm about chemokine biochemistry in me. Also, many thanks to Dr You Zhou and Dr Rob Andrews, for their help in analysing our RNA sequencing data.

Special thanks to the colleagues/friends without whom this journey wouldn't have been the same. To my close friend and fellow PhD student Dr Amy Brook for the laughs, cries,

distractions, rants and fun. I am so grateful that I had your help and support during this time. To Dr Amanda Thomson and Dr Katie Sime, for bringing my nice memories of Glasgow Uni to Cardiff, and always be there to help and support, both in and out of the lab. To my fellow office members (Owen, Abdullah, Marta, Teja and Amy), for the laughs, moans and advice. I would also like to thank the rest of the lab members for their advice, help, discussions and support during my PhD.

To my parents and my sister, you have believed in me when I didn't, in every step of the way. Even though you're far, you have been there to be happy for me when things are good and comfort me when things are bad. I could not be where I am today without your support.

And finally, to my husband-to-be, Neil. Moving to Cardiff has been the best thing for me because it was a new adventure with you. For all your patience when I came home angry or upset when experiments didn't work and your encouragement to just keep going. For everything you have done for me over the last four years, thank you. I could never have done this without you.

Thank you to everyone who helped me complete this PhD!

Summary

The chemokine CXCL14 stands out among other chemokines for its high sequence conservation across species. Its preferential expression in healthy peripheral tissues including skin, gut and kidney led to the hypothesis that CXCL14 is involved in immune surveillance at these sites. However, since its cognate receptor remains elusive, CXCL14 is one of the least understood chemokines in terms of function and target cell selectivity.

This PhD thesis examines the function of CXCL14 and adds to our knowledge of the strong synergism between CXCL14 and CXCL12. By combining evaluation of chemotactic and calcium mobilisation responses it demonstrates that CXCL14 can potently synergise with multiple homeostatic chemokines, including CCL19, CCL21 and CXCL13. Synergism with inflammatory chemokines was less apparent. To investigate the structure-function relationship of CXCL14, I synthesised CXCL14-CXCL12 hybrids by substituting the CXCL14 N-terminus with that of CXCL12. These hybrids were CXCR4 ligands able to induce chemotactic responses, but also maintained their ability to synergise with CXCL12. Finally, CXCL14 target cells were identified within the CD45-negative cell fraction of human and murine skin tissue, as well as among CD45-negative cells from multiple murine tissues. CXCL14 binding cells in murine skin were characterised by expression of markers such as EpCAM, CD34, CD31, sca-1, CX3CR1 and Ly6C. Comparison of the gene expression of CXCL14 binding cells in murine skin and colon with public gene expression data for multiple cell types revealed similarities with stromal cells.

The results of my research contribute substantially to our current model of CXCL14 playing a central role in tissue immune surveillance. CXCL14 may support tissue health along different pathways, including its synergistic action with other homeostatic chemokines as well as the proposed targeting of certain tissue cells in humans and mice.

Table of contents

CHAPTER 1: INTRODUCTION 1

1.1 LEUKOCYTE SUBSETS IN PERIPHERAL HUMAN BLOOD 1

1.1.1 The Mononuclear Phagocyte System 1

1.1.1.1 Monocytes 2

1.1.1.1.1 Identification of Monocyte Subsets 4

1.1.1.1.2 Functional Differences Between Monocyte Subsets 5

1.1.1.1.3 Monocyte Subsets in Mice 6

1.1.1.2 Dendritic cells 8

1.1.1.2.1 Classical Dendritic Cells 8

1.1.1.2.2 Plasmacytoid Dendritic Cells 9

1.1.2 T Lymphocytes 11

1.1.3 B Lymphocytes 12

1.1.4 Natural Killer Cells 13

1.1.5 Granulocytes 14

1.2 IMMUNOLOGY OF THE SKIN 15

1.2.1 Anatomy of the Skin 15

1.2.2 Mononuclear Phagocyte System of the Skin 19

1.2.2.1 Langerhans Cells 19

1.2.2.2 Dermal DCs 22

1.2.2.3 Dermal Macrophages 23

1.2.3 T Cells in Skin 25

1.2.4 Innate Lymphoid Cells in Skin 25

1.2.5 Mast Cells in Skin 26

1.3 THE CHEMOKINE SUPERFAMILY 26

1.3.1 Chemokines 26

1.3.2 Chemokine Receptors 32

1.3.3 Chemokine Receptor Signal Transduction and Functional Responses 34

1.3.4 Functional Responses Following Chemokine Binding 38

1.3.5 Chemokine-controlled Immune Cell Responses 40

1.3.5.1 Chemokines in homeostatic immune responses 41

1.3.6 Chemokine (C-X-C Motif) Ligand 14 45

1.3.6.1 Structural Properties 45

1.3.6.2 Expression in Tissues 49

1.3.6.3 Chemoattractant Activity and Target Cells for CXCL14 50

1.3.6.4 Antimicrobial Activity 54

1.3.6.5 CXCL14 in Disease 54

1.3.7 Synergism in the Chemokine Family 56

1.4 HYPOTHESES AND RESEARCH QUESTIONS 60

2 CHAPTER 2 – MATERIALS AND METHODS 61

2.1 CHEMOKINES 61

2.1.1 Chemokines Used in Functional Assays 61

2.1.2 Fluorochrome-labelled Chemokines 62

2.2 CELL CULTURE MEDIA AND BUFFERS 62

2.2.1 Media 62

2.2.1.1 Complete RPMI Medium 62

2.2.2 Buffers 62

2.2.2.1 Fluorescence-Activated Cell Sorting Buffer 62

2.2.2.2 Magnetic-Activated Cell Sorting Buffer 62

2.2.2.3 Chemotaxis Buffer 62

2.2.2.4 Calcium Buffer 63

2.2.2.5 Calcium Staining Buffer 63

2.3 BLOOD CELL ISOLATION 63

2.3.1 Isolation of Peripheral Blood Mononuclear Cells 63

2.3.2 Enrichment of Monocytes from Peripheral Blood Mononuclear Cells 63

2.3.3 Enrichment of T Cells from Peripheral Blood Mononuclear Cells 64

2.4 CELL CULTURE 64

2.4.1 Stimulations 64

2.4.2 T Cell Expansions 64

2.4.3 Culture of Immortalised Cell-Lines 65

2.4.3.1 THP-1 65

2.4.3.2 300-19 65

2.4.4 Recovery of Immune Cells from Human Split Skin 65

2.4.5 Recovery of Immune Cells from Murine Tissues 66

2.4.5.1 Animals 66

2.4.5.2 Mouse Ear and Back/Flank Skin 66

2.4.5.3 Mouse Intestine 67

2.4.5.4 Mouse Kidney 67

2.4.5.5 Mouse Lung 68

2.4.5.6 Mouse Liver/Heart 68

2.4.5.7 Mouse Spleen 68

2.5 PHENOTYPING AND FUNCTIONAL ASSAYS 68

2.5.1 Cell Staining and Flow Cytometry 68

2.5.2 Labelling with Alexa Fluor® 647-CXCL14 71

2.5.3 Transwell Chemotaxis Assay 71

2.5.4 Intracellular Ca^{2+} Rise 72

2.5.5 Chemokine Receptor Internalisation 73

2.5.6 Immunohistochemistry 73

2.5.7 In Vivo Synergy of CXCL14 and CXCL12 ..... *74*
2.6 RNA Sequencing (RNA seq) 74
2.6.1 Sample Preparation *74*
2.6.2 Sequencing *75*
2.7 Synthesis of Chemokine Variants 75
2.8 Statistical Analysis 79
2.9 Ethics 79
3 CHAPTER 3: CXCL14 SYNERGISM WITH OTHER CHEMOKINES 80
3.1 Introduction 80
3.2 Aims 83
3.3 CXCL14 Synergism with Homeostatic Chemokines 84
3.3.1 CXCL14 Synergises with Homeostatic Chemokines CCL19 and CCL21 in the Induction of Chemotactic Responses *84*
3.3.1.1 Primary Human Cells 84
3.3.1.2 CCR7-Transfected Mouse 300-19 Pre-B Cell Lines 87
3.3.2 CXCL14 Synergises with Homeostatic Chemokines CCL19 and CCL21 in Triggering Ca^{2+} Mobilisation *89*
3.3.3 CXCL14 does not Affect CCR7 Cell Surface Expression on CCR7-Transfectant 300-19 Cells 91
3.3.4 CXCL14 Synergises with Homeostatic Chemokine CXCL13 in the Induction of Chemotactic Responses *93*
3.3.5 CXCL14 Synergises with CXCL13 in the Induction of Ca^{2+} Release, but not in Receptor Cell Surface Expression *96*
3.4 Study of CXCL14 Synergism with Inflammatory Chemokines 98
3.4.1 CXCL14 does not Synergise with the Inflammatory Chemokine CCL5 ..... *98*
3.4.2 CXCL14 does not synergise with the Inflammatory Chemokines CXCL10 and CXCL11 ..... *104*
3.5 CXCL14 Synergism with CXCL12 in the Murine System 110
3.5.1 Analysis of In Vitro Synergism Between CXCL14 and CXCL12 ..... *110*
3.5.2 In Vivo Synergism Between CXCL14 and CXCL12 ..... *113*
3.6 Discussion 116
4 CHAPTER 4: STRUCTURE-FUNCTION ANALYSIS OF CXCL14 121
4.1 Introduction 121
4.2 Aims 125
4.3 Synthesis of Functional CXCL14 and CXCL14-12 Hybrids Using Solid-Phase Synthesis 126
4.4 Assessment of Functionality of Synthesised Proteins 131
4.5 Interaction of Synthetic Hybrids with CXCR4-Expressing Cells 133
4.5.1 Induction of Migratory Response with Primary Cells *133*
4.5.2 Induction of Migratory Responses by 300-19-CXCR4 Transfected Cells *136*

4.6 CXCL14 and CXCL14-12 Hybrids Synergise with CXCL12 in the Induction of Migratory Responses 138
4.6.1 300-19-CXCR4 Transfected Cells ... 138
4.6.2 Primary Cells ... 140
4.6.3 Blockade of CXCR4 Abolishes the Synergy Between CXCL14-12 Hybrids and CXCL12 on Primary Lymphocytes ... 142
4.7 CXCL14-12 Hybrids Induce Calcium Mobilisation Responses in 300-19-CXCR4 Transfected Cells 144
4.8 CXCL14-12 Hybrid Synergism with CXCL12 is not Evident in Calcium Mobilisation Responses ... 146
4.9 Discussion ... 148

5 CHAPTER 5: CXCL14 TARGET CELLS IN PERIPHERAL TISSUES ... 153

5.1 Introduction ... 153
5.2 Aims ... 157
5.3 CXCL14 Target Cells in Human Peripheral Blood ... 158
5.4 CXCL14 Target Cells in Mice ... 160
5.4.1 CXCL14 Target Cells in Murine Splenocytes ... 160
5.4.2 Identification of CXCL14 Target Cells in Murine Tissues ... 163
5.4.3 RNA Sequencing Analysis of TR14 Cells in Skin and Colon ... 165
5.4.4 Phenotypic and Morphological Characterisation of TR14 Cells ... 174
5.4.5 Enzymatic Digestion Affects Assessment of Functionality of TR14 Cells ... 177
5.5 Discussion ... 180

6 CHAPTER 6: GENERAL DISCUSSION ... 188

6.1 Summary ... 188
6.2 CXCL14 Synergism with Other Chemokines ... 189
6.3 CXCL14 Structure-Function Relationship ... 192
6.4 TR14 Cells ... 194
6.4.1 Human TR14 Cells ... 195
6.4.2 Mouse TR14 Cells ... 196
6.5 Conclusion ... 199

7 CHAPTER 7: REFERENCES ... 201

8 CHAPTER 8: APPENDIX ... 232

8.1 Supplementary Data ... 232
8.1.1 CXCL14 Target Cells ... 232
8.2 Publications During this PhD ... 235
8.3 Presentations During my PhD Studies ... 235
8.3.1 Poster Presentations ... 235

8.3.2 Oral Presentations 236

List of Figures

Figure 1.1. Phenotype of human and murine mononuclear phagocyte subsets. 10

Figure 1.2. Healthy human skin anatomy and resident immune cells. 18

Figure 1.3. General structure of CXC chemokines. 28

Figure 1.4. Atypical chemokine receptors: Ligand specificity, cell expression and biological functions in mouse and/or human. 33

Figure 1.5. Signalling cascade and molecule activation following chemokine binding to its cognate receptor. 37

Figure 1.6. Sequence alignment of CXCL14 and selected other CXC chemokines. 47

Figure 1.7. Structure, peptide sequence and conservation among species of CXCL14 and CXCL12. 48

Figure 1.8. Modes of action of chemokine synergy. 57

Figure 2.1. Sequence alignment of CXCL14 with CXCL12 and two CXCL14-CXCL12 hybrids. 61

Figure 2.2. Modified protocol of single-amino acid cycle during solid-phase synthesis of proteins. 77

Figure 3.1. CXCL14 is a positive allosteric modulator for CXCR4. 82

Figure 3.2. CXCL14 synergises with the CCR7 ligands CCL19 and CCL21 in the induction of chemotactic responses in primary human T cells. 85

Figure 3.3. CXCL14 synergises with the CCR7 ligands CCL19 and CCL21 in the induction of chemotactic responses in primary human B cells. 86

Figure 3.4. CXCL14 synergises with CCL21 and CCL19 in the induction of chemotactic responses in 300-19 cells stably transfected with CCR7. 88

Figure 3.5. CXCL14 synergises with CCL19 and CCL21 in the induction of rapid cellular responses. 90

Figure 3.6. CXCL14 does not decrease cell surface expression of CCR7. 92

Figure 3.7. CXCL14 synergises with the CXCR5 ligand CXCL13 in the induction of chemotactic responses in primary human B cells. 94

Figure 3.8. CXCL14 synergises with CXCL13 in the induction of chemotactic responses in 300-19 cells stably transfected with CXCR5..... 95

Figure 3.9. CXCL14 synergises with CXCL13 in the induction of rapid cellular responses. 97

Figure 3.10. CXCL14 does not decrease cell surface expression of CXCR5. 97

Figure 3.11. CXCL14 does not synergise with CCL5 in the induction of chemotactic responses in 300-19 cells stably transfected with CCR5..... 99

Figure 3.12. CXCL14 does not synergise with inflammatory chemokine CCL5 on primary human T cells. 101

Figure 3.13. CXCL14 synergises with CCL5 in the induction of rapid cellular responses. 103

Figure 3.14. CXCL14 does not decrease cell surface expression of CCR5. 103

Figure 3.15. CXCL14 synergises with the inflammatory chemokines CXCL10 and CXCL11 in the induction of chemotactic responses in 300-19 cells stably transfected with CXCR3. 105

Figure 3.16. CXCL14 does not synergise with inflammatory chemokine CXCL10 on primary human T cells. 106

Figure 3.17. CXCL14 synergises with CXCL10 and CXCL11 in the induction of rapid cellular responses. 107

Figure 3.18. CXCL14 effect on CXCR3 cell surface expression. 108

Figure 3.19. CXCL14 synergises with CXCL12 in the induction of chemotactic responses by murine cells *in vitro.* 112

Figure 3.20. Gating strategy for identification of major cell subsets in the peritoneal cavity of mice. 114

Figure 3.21. Cell recruitment in response to CXCL14 or CXCL12 *in vivo.* 115

Figure 4.1. Schematic diagram of the two-step mechanism for chemokine receptor-chemokine interaction. 122

Figure 4.2. Structural similarities of CXCL14 and CXCL12. 124

Figure 4.3. Structure and peptide sequence of CXCL12, CXCL14 and CXCL14-12 hybrids. 126

Figure 4.4. Mass spectrometry analysis of unfolded synthetic CXCL14 and CXCL14-12 hybrids. 128

Figure 4.5. Mass spectrometry of folded synthetic CXCL14 and CXCL14-12 hybrids... 130

Figure 4.6. Synthetic CXCL14 and CXCL14-12 hybrids show moderate activity on freshly isolated monocytes. 132

Figure 4.7. Synthetic CXCL14 and CXCL14-12 hybrids activity is not affected by AMD3100 treatment in primary peripheral blood cells. 135

Figure 4.8. Synthetic CXCL14 and CXCL14-12 hybrids do not show clear interaction with 300-19 cells stably transfected with CXCR4. 137

Figure 4.9. Synthetic CXCL14 and CXCL14-12 hybrids synergise with CXCL12 in the induction of chemotactic responses in 300-19 cells stably transfected with CXCR4. 139

Figure 4.10. Synthetic CXCL14 and CXCL14-12 hybrids synergise with CXCL12 in the induction of chemotactic responses by primary T and B cells. 141

Figure 4.11. CXCR4-dependent synergism between CXCL14-12 hybrids and CXCL12 in the induction of chemotactic responses by primary T and B cells. 143

Figure 4.12. CXCL14-12 hybrids can induce calcium mobilisation responses in 300-19-CXCR4 transfected cells. 145

Figure 4.13. CXCL14-12 hybrids do not synergise with CXCL12 in the induction of calcium mobilisation responses in 300-19-CXCR4 cells. 147

Figure 5.1. Identification of a novel subsets of CXCL14 target cells expressing myeloid cell markers in the $CD45^-$ fraction of human dermis. 156

Figure 5.2. Classical and intermediate monocytes are the major targets of CXCL14 while non-classical monocytes are not CXCL14 targets 159

Figure 5.3. Murine monocytes are the major targets for CXCL14 among splenocytes. 162

Figure 5.4. Tissue Resident CXCL14 binding cells (TR14 cells) are present in different murine tissues. 164

Figure 5.5. Gating strategy to identify immune cell populations isolated from murine skin and colon for RNA sequencing. 166

Figure 5.6. RNA sequencing analysis of sorted TR14 cells. 169

Figure 5.7. Chemokine and chemokine receptor analysis of sorted samples from murine skin and colon. 171

Figure 5.8. TR14 cells do not express F4/80 on their cell surface. 173

Figure 5.9. Phenotypic and morphological characterisation of TR14 cells. 176

Figure 5.10. Enzymatic digestion of tissues causes decrease in migration of immune cells. 179

Figure 8.1. Migratory responses of human peripheral blood monocytes towards CXCL14. 232

Figure 8.2. Migratory responses of murine splenocytes towards CXCL14. 233

Figure 8.3 Staining of skin TR14 cells and spleen cells with CCR3-PE antibodies. 234

List of Tables

Table 1.1. The human chemokine superfamily. 30

Table 1.2. CXCL14 target cells and reported functional effects of CXCL14 in murine and human immunity 52

Table 1.3. CXCL14 expression in murine and human tissues and cells. 53

Table 2.1. Fluorochrome-conjugated monoclonal antibodies used for flow cytometry 70

Table 4.1. Experimental M_r values of CXCL14 and CXCL14-12 hybrids determined by mass spectrometry, compared to theoretical values, and yield of synthetic proteins obtained. 131

Table 5.1. Summary of human skin cells examined during this work and their responsiveness to CXCL14 157

Table 5.2. Normalised gene counts from RNA seq analysis show expression of the marker F4/80. 172

Table 5.3. Cell surface and gene expression of selected markers by mouse skin TR14 cells. 185

List of Abbreviations

2-ME	2-mercaptoethanol
ACKR	Atypical Chemokine Receptor
ADCC	Antibody-Dependent Cell-Mediated Cytotoxicity
AF-CXCL14	Alexa Fluor 647-conjugated CXCL14
AF-muCCL1	Alexa Fluor 647-conjugated murine CCL1
AMP	Antimicrobial Peptide
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
BMAC	B Cell-And Monocyte-Activating Chemokine
BRAK	Breast and Kidney-Expressed Chemokine
BRET	Bioluminescence Resonance Energy Transfer
Ca^{2+}	Calcium ion
cAMP	Cyclic Adenosine Monophosphate
cCKR	Conventional Chemokine Receptor
CCL	Chemokine (C-C motif) Ligand
CCR	CC-Chemokine Receptor
CCRL	CC-Chemokine Receptor-Like
CD	Cluster of Differentiation
cDC	Conventional Dendritic Cell
CMP	Common Myeloid Progenitor
cRPMI	Complete RPMI Medium
CSF1	Colony-Stimulating Factor 1
CSR	Chemokine Recognition Site
CXCL	Chemokine (C-X-C motif) Ligand
CXCL14-KO	CXCL14-Knockout Mouse
CXCR	CXC-Chemokine Receptor
DAG	Diacylglycerol
DAMP	Danger-Associated Molecular Patterns
DC	Dendritic Cell
DIEA	N,N-Diisopropylethylamine
DMEM	Dulbecco's Modified Eagle Medium
DMF	N,N-Dimethylmethanamide
DTR	Diphtheria Toxin Receptor
EDTA	Ethylenediaminetetraacetic Acid

EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
EpCAM	Epithelial Cell Adhesion Molecule
FACS	Fluorescence - Activated Cell Sorting
Fc	Fragment Crystallisable
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
Fluo-4- AM	Fluo-4-Acetoxymethyl Ester
FMLF	Peptide *N*-Formyl-Methionyl-Leucyl-Phenylalanine
FMO	Fluorescence Minus One
Fmoc	Fluorenylmethyloxycarbonyl
FRC	Fibroblastic Reticular Cell
FRET	Förster Resonance Energy Transfer
FSC	Forward Scatter
Fura - 2AM	Fura-2-Acetoxymethyl Ester
FXIIIa	Factor XIIIa
GAG	Glycosaminoglycan
GALT	Gut-Associated Lymphoid Tissue
GDP	Guanosine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GM-CSF	Granulocyte/Macrophage Colony-stimulating Factor
GPCRs	G Protein-coupled Receptor
GRK	GPCR Kinase
GTP	Guanosine Triphosphate
HATU	Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium
HBSS	Hank's Balanced Salt Solution
HBTU	Hexafluorophosphate Benzotriazole Tetramethyl Uronium
HEV	High Endothelial Venule
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HMGB1	High Motility Group Box 1
HOBt	Hydroxy Benzotriazole
HPC	Haematopoietic Precursor Cell
HSC	Haematopoietic Stem cell
HSCT	Haematopoietic Stem Cell Transplantation

ICL	Intracellular Loop
iDC	Immature Dendritic Cell
IFNγ	Interferon Gamma
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate Lymphoid Cell
IP_3	Inositol Triphosphate
IPA	Ingenuity Pathway Analysis
KIR	Killer Cell Immunoglobulin-Like Receptor
LC	Langerhans Cell
LEC	Lymphatic Endothelium
LFA-1	Lymphocyte Function-Associated Antigen 1
LPS	Lipopolysaccharide
LTB_4	Lipid Mediator Leukotriene B_4
M-CSF	Macrophage Colony-Stimulating Factor 1
mAb	Monoclonal Antibody
MACS	Magnetic-Activated Cell Sorting
MAPK	Mitogen-Activated Protein Kinases
MFI	Mean Fluorescence Intensity
MGI	Mouse Genome Informatics
MHC	Major Histocompatibility Complex
MIF	Macrophage Migration Inhibitory Factor
MMR	Macrophage Mannose Receptor
MPS	Mononuclear Phagocyte System
M_r	Relative Molecular Mass
mRNA	messenger RNA
Na-But	Sodium Butyrate
NET	Neutrophil Extracellular Trap
NK cell	Natural Killer
NKT	Natural Killer T cell
NMP	*N*-Methylpyrrolidone
NMR	Nuclear Magnetic Resonance
NOS	Nitric Oxide Synthases
PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cells

PBS	Phosphate-Buffered Saline
pDC	Plasmacytoid Dendritic Cell
PE	Phycoerythrin
PGE_2	Prostaglandin E_2
PHA	Phytohaemagglutinin
PI3K	Phosphoinositide 3-Kinase
PIP_3	Phospatidylinositol-1,4,5-Triphosphate
PKC	Protein Kinase C
PLC-β	Phospholipase C-β
PRR	Pattern Recognition Receptor
RBC	Red Blood Cell
RNA seq	RNA Sequencing
ROS	Reactive Oxygen Species
RP-HPLC	Reverse-Phase High Performance Liquid Chromatography
RPKM	Reads Per Kilobase of Transcript per Million Mapped Reads
RPM	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute
SEM	Standard Error of the Mean
S1P	Sphingosine-1-Phosphate
SD	Standard Deviation of the Mean
SLO	Secondary Lymphoid Organ
SSC	Side Scatter
T_{CM}	Central Memory T Cell
TCR	T cell Receptor
T_{EM}	Effector Memory T Cell
TFA	Trifluoroacetic Acid
T_{FH}	Follicular Helper T Cell
T_{FR}	Follicular Regulatory T Cell
T_H	T helper
TLR	Toll-Like Receptor
TM	Transmembrane
TNFα	Tumour Necrosis Factor-Alpha
TR14	Tissue Resident CXCL14 Binding Cells
Treg	Regulatory T cell
T_{RM}	Tissue-Resident Memory T Cells

Chapter 1: Introduction

1.1 Leukocyte Subsets in Peripheral Human Blood

The term "leukocytes" refers to a diverse group of immune cells, derived from a common progenitor, the haematopoietic stem cells (HSCs). Their diverse repertoire of functions is responsible for maintaining the body's health, as well as mounting immune responses. Differentiation into distinct cell types with varying physical and functional characteristics depends on internal and external factors. Leukocytes comprise granulocytes and monocytes (myeloid cells) and lymphocytes. In particular, peripheral blood mononuclear cells (PBMCs) include lymphocytes (which are divided into T, B and natural killer (NK) cells), monocytes and dendritic cells (DCs). The frequencies of these populations vary across individuals, although lymphocytes generally comprise 65-90% of PBMCs, monocytes comprise 10-30% of PBMCs, while DCs are typically low in numbers comprising 1-2% of PBMCs (Kleiveland and Kleiveland, 2015). Other cells present in circulation include granulocytes, which mainly consist of neutrophils, the most abundant leukocyte subset in peripheral blood. Throughout the years, we have gained an increasing understanding of the functions and interactions of immune cells within the blood and the periphery. In this section, I will discuss the immune cell populations present in human blood.

1.1.1 The Mononuclear Phagocyte System

The mononuclear phagocyte system (MPS) refers to a group of cells that were first described as a population of bone marrow-derived cells circulating in the blood as monocytes that differentiate into macrophages in tissues during steady-state and inflammatory conditions (van Furth and Cohn, 1968). The MPS comprises monocytes, macrophages and DCs. These cells share a number of phenotypic features (e.g. a single nucleus) and functional properties, including 1) surveying the microenvironment by sensing stress signals and microbial products; 2) internalising or phagocytosing and digesting of microbes, dying cells and viruses; 3) presenting antigens to cells of the adaptive immune system; 4) displaying cytotoxic activity against tumour and senescent/damaged cells; and 5) secreting chemokines and cytokines leading to migration and activation of both immune and nonimmune cells, as well as inducing their

own migration (Lavoie and Levy, 2017). These cells are involved in inflammatory conditions, as well as fulfilling homeostatic functions throughout an organism's life, relating to tissue repair, remodelling, angiogenesis and the neural system (Hume, 2006). Macrophages reside in every organ throughout the body and are highly adapted to their tissue of residence, thus displaying substantial heterogeneity with respect to phenotype, function and turnover (Davies, Jenkins, *et al.*, 2013). Although once thought to be the result of a linear progression from progenitor to monocyte and monocyte to macrophage, there is increasing evidence of a much more complex system. Recent evidence show that many macrophage populations populate tissues during early embryogenesis, independent of the haematopoietic system. Although these populations are maintained throughout life by local proliferation during homeostasis, they can be reconstituted by circulating monocytes during inflammation (Epelman *et al.*, 2014). Soon after their discovery in the 1970s, DCs were included into the family of MPS (Steinman, Adams and Cohn, 1975). Significant progress has been made to understand the human and mouse mononuclear phagocyte biology, including important progress in understanding their ontogeny and development (reviewed in Liu and Nussenzweig, 2010; Epelman *et al.*, 2014; Ginhoux and Jung, 2014; Scott, Henri and Guilliams, 2014). A remarkably constructive strategy has been to comparatively analyse the findings between human and mouse, which identified homologous subsets and allowed a unified classification of mononuclear phagocytes across species (Robbins *et al.*, 2008; Guilliams *et al.*, 2014; McGovern *et al.*, 2014).

1.1.1.1 Monocytes

Monocytes represent a conserved population of leukocytes that is present in all vertebrates (Ginhoux and Jung, 2014). They were originally identified on the basis of their morphology and glass adherence (van Furth and Cohn, 1968). Human monocytes are derived from the bone marrow dividing monoblasts present in the bone marrow, which are bipotent cells that originate from HSCs and are released into the circulation as non-dividing cells. They are present in the blood and represent 4% of all nucleated cells in the blood of mice and 10% in the blood of humans (Van Furth and Sluiter, 1986). Within the blood, monocytes exhibit a short half-life of 20-22 hours (van Furth and Cohn, 1968; Ginhoux and Jung, 2014), thus it is plausible that as much as 50% of circulating

monocytes can leave the bloodstream under steady state conditions each day. It was thought that circulating monocytes act as a reservoir for tissue-resident mononuclear phagocytes under both steady-state and inflammatory conditions (Wiktor-Jedrzejczak and Gordon, 1996). However, experimental data revealed that differentiation of monocytes *in vivo* is limited and their contribution to this complex cellular network remains a highly active area of research (Auffray, Sieweke and Geissmann, 2009; Geissmann *et al.*, 2010; Mass *et al.*, 2016).

Monocytes were initially described based on their morphology, using histological and cytochemical techniques. Some typical morphological features include the irregular shape of the cell and its nucleus, in particular a kidney-shaped nucleus, a high cytoplasm-to-nucleus ratio and light blue cytoplasm (when staining with Giemsa stain) (Ziegler-Heitbrock, 2000). Monocytes are also the largest of the mononuclear leukocyte subsets, measuring 13-18 µm in diameter. Nevertheless, there is still variability in their size and shape, which makes it difficult to use morphology or light scatter analysis to unequivocally distinguish them from activated lymphocytes, blood DCs and NK cells. Advances in flow cytometry allowed the identification of monocyte subsets by staining for particular cell-surface markers, in combination with lineage markers to exclude T cells, B cells, NK cells and DCs.

In vitro, monocytes adhere to plastic, exhibit phagocytic activity and upon stimulation produce large amounts of reactive oxygen species (ROS), cytokines including tumour necrosis factor-alpha (TNFα), interleukin (IL)-1β, IL-6 and IL-10, and other inflammatory mediators including prostaglandins, complement factors and proteolytic enzymes (Hume, 2006; Haniffa, Bigley and Collin, 2015). Their ability to sense microbial products is mediated through pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), which enable recognition of various pathogens such as bacteria, viruses and fungi (Lavoie and Levy, 2017). Additionally, scavenger receptors allow recognition of lipids and dying cells (van Furth and Cohn, 1968; Blumenstein *et al.*, 1996). Although it is thought that DCs have the biggest role in adaptive immunity among all members of the MPS, monocytes have also been described to affect the polarisation and expansion of lymphocytes, with the ability to shape primary and memory T cell responses in humans and mice (Geissmann *et al.*, 2008). *In vitro* experiments have shown that cytokine

induction can lead to differentiation of monocytes to macrophages and DCs, thereby supporting the claim that monocytes give rise to these cells *in vivo* (Sallusto and Lanzavecchi, 1994; Zhou and Tedder, 1996).

1.1.1.1.1 Identification of Monocyte Subsets

Early studies established the existence of functional monocyte subsets in human blood (Passlick, Flieger and Loms Ziegler-Heitbrock, 1989). They were originally defined by their expression of CD14, the co-receptor for lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria. The introduction of CD16 (a low affinity receptor for the Fc portion of IgG antibodies) as an additional cell surface marker led to the observation that a small subset of monocytes expressed a combination of CD14 and CD16 (Passlick, Flieger and Loms Ziegler-Heitbrock, 1989). The $CD14^{++}CD16^{-}$ subset makes up the vast majority of monocytes, while the $CD14^{+}CD16^{+}$ makes up a smaller portion (Ziegler-Heitbrock *et al.*, 2010). $CD14^{+}CD16^{+}$ monocytes have higher major histocompatibility complex (MHC) class II expression and following stimulation by TLR ligand, they produced higher levels of TNFα (Belge *et al.*, 2002). Similarly, increased numbers of these monocytes were found in patients with sepsis, Human Immunodeficiency Virus (HIV) and tuberculosis, which led to these cells termed “pro-inflammatory monocytes” (Ziegler-Heitbrock, 1996). On the other hand, work in mice revealed similar subsets of monocytes, defined by a combination of Ly6C and chemokine receptor expression (Geissmann, Jung and Littman, 2003). However, based on adoptive transfer experiments, these subsets can be divided based on chemokine receptors expression and unique migratory properties. The “inflammatory subset” exhibits high CCR2 and low CX3CR1 expression and homes to inflamed tissues, where it could trigger immune responses (Gordon and Taylor, 2005). In contrast, the “resident subset” has a longer half-life in the blood, shows high expression of CX3CR1 but lacks expression of CCR2 and homes to noninflamed tissues (Geissmann, Jung and Littman, 2003). In the same study, the researchers reported this dichotomy in monocyte subsets in human as well. $CD14^{++}CD16^{-}$ monocytes in human express CCR2 but lack CX3CR1 expression, whereas the $CD14^{+}CD16^{+}$ monocytes express high levels of CX3CR1 and are negative for CCR2 (Geissmann, Jung and Littman, 2003). $CD14^{+}CD16^{+}$ monocytes also lack expression of the inflammatory chemokine receptors CCR1, CXCR1 and CXCR2, which suggests that

similar to their CX3CR1^{hi}CCR2^{-} murine counterparts, they are likely excluded from inflamed tissues (Geissmann, Jung and Littman, 2003). These findings contradicted the existing view of CD16^{+} monocytes as inflammatory, which led to confusion regarding the functional differences between monocyte subsets. Gene expression analysis later confirmed the functional similarities between mouse and human monocyte subsets (Ingersoll *et al.*, 2014). Monocyte subset nomenclature has since changed, with CD14^{++}CD16^{-} monocytes termed "classical monocytes" and CD14^{+}CD16^{+} monocytes termed "non-classical monocytes". More recently, a third subset of monocytes was identified with an intermediate phenotype between classical and non-classical monocytes. Specifically, these cells express high levels of CD14 and are positive for CD16 (CD14^{++}CD16^{+}) and are less abundant in adult blood (<5% of all monocytes) (Zawada *et al.*, 2011). This subset shows distinct functional properties and is increased in inflammatory settings (Skrzeczyńska-Moncznik *et al.*, 2008; Moniuszko *et al.*, 2009). The division of the human blood monocytes into three subsets: classical, intermediate and non-classical, is now widely accepted by the wider research community (Ziegler-Heitbrock, 2014).

1.1.1.1.2 Functional Differences Between Monocyte Subsets

The majority of research regarding the functional differences between the three monocyte subsets has been performed *ex vivo*, with most studies focusing on the function of classical and non-classical monocytes. When culturing human monocytes, both subsets differentiated into DCs in the presence of differentiating factors (granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4)) (Sallusto and Lanzavecchi, 1994; Sánchez-Torres *et al.*, 2001). Furthermore, using an *in vitro* model of transendothelial migration, it was shown that monocytes can migrate across an endothelial barrier and differentiate into macrophages or DCs (Randolph *et al.*, 2002). In this mouse model, CD16^{+} monocytes were found to more likely differentiate into DCs and transmigrate through a layer of resting endothelial cells than classical monocytes, thereby indicating that non-classical monocytes might be precursors of DCs in the steady state. Since these earlier studies focused on the two subsets of monocytes, it is unclear whether these properties are attributed to non-classical or intermediate monocytes. It was later observed that human non-classical

monocytes intravenously injected into mice "crawled" along the endothelium, in contrast to classical monocytes (Cros *et al.*, 2010). That report suggested that the main function of non-classical monocytes is to patrol blood vessels, potentially acting as a blood-resident population that does not so readily exit into peripheral tissues, in agreement with their observed longer half-life in circulation (Geissmann, Jung and Littman, 2003; Hanna *et al.*, 2015). Extensive gene expression and phenotypic analysis of the human monocyte subsets in healthy conditions revealed that the intermediate subset is much more related to the non-classical subset than the classical subset (Wong *et al.*, 2011). In clinical settings, similar to the non-classical monocytes, the intermediate subset has been shown to increase in number in several diseases, including asthma (Moniuszko *et al.*, 2009), rheumatoid arthritis (Cooper *et al.*, 2012) and HIV infection (Kim *et al.*, 2010).

Various ontogenetic studies have started revealing whether these subsets represent distinct cell types or rather a single lineage at various differentiation states. Certain studies proposed that the intermediate monocyte subset is an intermediary stage of differentiation between classical and non-classical monocytes (Ziegler-Heitbrock *et al.*, 2010). This developmental relationship is supported by studies showing that during the course of an infection, or treatment with colony-stimulating factor 1 (CSF1; also known as M-CSF), intermediate monocytes first increase, followed by an increase in non-classical monocytes (Bukowski *et al.*, 1994; Ziegler-Heitbrock *et al.*, 2010). Fate-mapping studies in mice have shown that classical monocytes constitute obligatory precursors of blood-resident non-classical monocytes under steady state conditions (Yona *et al.*, 2013). Monocyte repopulation kinetics in patients following haematopoietic stem cell transplantation (HSCT) showed that classical monocytes appear in the blood first, followed by the intermediate and non-classical subset (Haniffa, Ginhoux, *et al.*, 2009), therefore supporting this concept.

1.1.1.1.3 Monocyte Subsets in Mice

In mice, the definition of monocyte subsets is based on different phenotypic markers than their human counterparts. Monocyte development and survival in mice is completely dependent on CSF1, and mice deficient in this growth factor or its receptor CSF1R exhibit severe monocytopenia (Cecchini *et al.*, 1994; Wiktor-Jedrzejczak and

Gordon, 1996; Dai *et al.*, 2002). Monocytes arise from common myeloid precursor cells in the foetal liver and bone marrow during both embryogenesis and adult haematopoiesis (Ginhoux and Jung, 2014). In mice, differentiation of $CSFR1^{+}$ monocytes begins with their distinction based on expression of CCR2, CD62L (L-selectin) and CX3CR1 (Palframan *et al.*, 2001). In addition, Ly6C was also identified as a marker of $CCR2^{+}$ monocytes (Geissmann, Jung and Littman, 2003). These studies showed that $CCR2^{+}CD62L^{+}CX3CR1^{low}Ly6C^{hi}$ mouse monocytes corresponded to $CD14^{++}CD16^{-}$ (classical) human monocytes, whereas $CCR2^{-}CD62L^{-}CX3CR1^{hi}Ly6C^{lo}$ mouse monocytes corresponded to non-classical monocytes. CD43 was identified as a differentiation marker for monocyte subsets in rats (Ahuja, Miller and Howell, 1995), but has also been used in mice to divide monocytes into three subsets. Classical monocytes show high Ly6C expression and low CD43 ($Ly6C^{hi}CD43^{+}$), intermediate monocytes have intermediate Ly6C expression of both markers ($Ly6C^{int}CD43^{++}$), whereas non-classical monocytes show low Ly6C expression and high CD43 expression ($Ly6C^{lo}CD43^{++}$) (Ziegler-Heitbrock *et al.*, 2010). The identification of mouse monocyte subsets opened the possibility to study their trafficking and differentiation pattern *in vivo*, using different techniques (Tacke and Randolph, 2006). $Ly6C^{hi}$ monocytes were recently shown to derive from a newly identified precursor, termed “common monocyte progenitor” (Hettinger *et al.*, 2013) and have been shown to be the precursor of $Ly6C^{lo}$ cells (Yona *et al.*, 2013). Notably, apart from the blood vasculature, undifferentiated $Ly6C^{hi}$ monocytes can also be found in peripheral tissues, including the spleen, skin, intestine and lungs in steady state conditions (Scott, Henri and Guilliams, 2014). Upon inflammation, $Ly6C^{hi}$ monocytes can differentiate into monocyte-derived cells and fulfil functions similar to those of DCs and macrophages, including antigen presentation, pathogen elimination and angiogenesis (Guilliams *et al.*, 2014). Advances in experimental tools continuously shed light to the contribution of monocytes to the maintenance of peripheral tissue macrophage and DC populations primarily under inflammatory conditions, while studies in the steady state have been scarce.

1.1.1.2 Dendritic cells

1.1.1.2.1 Classical Dendritic Cells

DCs in human blood represent a rare population (<1% of mononuclear cells in the blood), as opposed to the more numerous monocytes, thus they were initially identified and studied in more detail in mice. However, human DC development and function has also been studied in detail. Peripheral blood DCs are divided into conventional (cDC; also known as classical, conventional or myeloid DCs) and plasmacytoid (pDC) subsets. Classical DCs make up approximately 50% of circulating DCs in humans and are identified by the expression of MHC Class II and the integrin CD11c, as well as lacking expression of the monocyte markers CD14 and CD16 (Haniffa, Bigley and Collin, 2015). Classical DCs are further divided into two subsets, differentiated based on the expression of CD1c and CD141 on their cell surface (MacDonald *et al.*, 2002). Both subtypes express the myeloid markers CD13 and CD33, indicating their direct derivation from the myeloid lineage. The $CD141^+$ DC subset represent a very minor subset of peripheral blood leukocytes, with $CD1c^+$ DCs outnumbering them by approximately 10-fold (Ziegler-Heitbrock *et al.*, 2010).

Both subsets of myeloid DCs have the ability to induce immunity to foreign antigens and enforce tolerance to self-antigens owing to a few key attributes (Merad *et al.*, 2013). Firstly, their primary location in nonlymphoid tissues and in the spleen marginal zone in the steady state allows them to constantly survey tissue and blood antigens through PRRs. Additionally, they possess antigen processing and presentation machinery (Villadangos and Schnorrer, 2007; Segura and Villadangos, 2009; Joffre *et al.*, 2012). Following acquisition of antigens in the tissue, DCs have the ability to migrate to the T cell zone of lymph nodes, both in the steady and inflamed state (Förster, Braun and Worbs, 2012). Finally, they have the ability to interact with and activate naïve T cells (Banchereau and Steinman, 1998). However, they most likely do not present antigen to T cells in the blood, as the close cell-to-cell interactions required are not permitted under flow conditions. Instead, blood DCs are thought to be in transit, maturing into fully functional DCs that can present antigen to and prime T cells in secondary lymphoid tissues such as lymph nodes and spleen (Haniffa, Collin and Ginhoux, 2013).

1.1.1.2.2 Plasmacytoid Dendritic Cells

Plasmacytoid dendritic cells (pDCs) in tissues were originally described as T-associated plasma cells, plasmacytoid T cells, or plasmacytoid monocytes (Facchetti *et al.*, 1988). Subsequently, it was shown that they are present in circulation in equivalent numbers to classical DCs. They express low levels of MHC Class II and CD4 and have the capacity to express high levels of interferon α in response to viruses (Perussia, Fanning and Trinchieri, 1985; Facchetti *et al.*, 1988; Hettinger *et al.*, 2013). Following contact with viruses, mediated through PRRs like toll like receptors (TLRs), pDCs can enter lymph nodes through high endothelial venules (HEV) to prime T cells (Cella *et al.*, 1999). Phenotypic analysis of these cells showed that they express low levels of CD11c and lack expression of myeloid markers CD14 and CD33, distinguishing them from cDCs and monocytes. Instead, pDCs can be identified by positive expression of CD123 (IL-3 receptor), CD303 (BDCA-2) and CD304 (BDCA-4) (Haniffa, Bigley and Collin, 2015). The cell types constituting the MPS in human and mouse, as well as the markers that define them are summarised in **Figure 1.1.**

Cell	Human	Both	Mouse
CD1c⁺ DC (cDC1)  CD11b⁺ DC	CD1c CD1a (skin) CD1b CLEC7A CLEC6A	CD11c FLT3 CD11b CX3CR1 CD172/SIRPα	CD24 CD4
CD141⁺ DC (cDC2)  CD8⁺/CD103⁺ DC	CD141 CD4+	BTLA FLT3 XCR1 CLEC9A TLR3 CD26	Langerin CD8 CD103 CD205
Plasmacytoid DC (pDC)  pDC	CD303 CD304	CD4 CD45RA CD123	SiglecH BST2 Ly6C
Macrophage 	CD206 (Lung) FXIIIA CD209	CD11b CD14 CD68 CD163 LYVE-1 CD64	F4/80 MerTK
Classical monocyte 	CD14^{hi}	CCR2^{hi} CX3CR1^{lo} CD11b	Ly6C^{hi} Gr1^{hi} CD43^{+}
Intermediate monocyte 	CD14^{hi} CD16	CCR2^{hi} CX3CR1^{int}	Ly6C^{int} Gr1^{int} CD43^{++}
Non-classical monocyte 	CD16 CD14^{lo}	CD11c CCR2^{lo} CX3CR1^{hi} CD11b	Ly6C^{lo} Gr1^{lo} CD43^{++}

Figure 1.1. Phenotype of human and murine mononuclear phagocyte subsets.
Expression markers commonly used to identify these populations in humans and mice are shown. cDC; conventional DC, pDC; plasmacytoid DC. Figure adapted from Haniffa, Bigley and Collin, 2015.

1.1.2 T Lymphocytes

T lymphocytes in the blood mainly consist of $CD4^+$ and $CD8^+$ αβ T cells. $CD4^+$ T cells outnumber $CD8^+$ T cells by roughly 2-fold, as $CD4^+$ T cells comprise 25-60% of PBMCs, while $CD8^+$ T cells make up 5-30% of PBMCs. Based on their functionality, both types of αβ T cells can be further sub-divided into naïve, effector, central memory and effector memory subsets that exist during steady state or inflammatory conditions and can be distinguished based on expression of specific markers (Snook, Kim and Williams, 2018). In order to generate an appropriate immune response for a particular pathogen or threat, $CD4^+$ T cells are polarised into different effector states. In particular, $CD4^+$ T cells give rise to T helper (T_H) cells, which play an important role during immune responses by inducing maturation of B cells into plasma cells and memory B cells, as well as activation of cytotoxic T cells and macrophage killing activity (Raphael *et al.*, 2015). Polarisation of $CD4^+$ T cells can give rise to various functional subsets that are distinguished based on their expression of characteristic cytokines, surface markers, transcription factors and chemokine receptors (Kaliński *et al.*, 1999; Oestreich and Weinmann, 2012). These specialised subsets include regulatory T cells (Tregs), T_H1, T_H2, T_H9, T_H17 and T_H22 cells, as well as follicular B helper T cells (T_{FH}) and fulfil distinct roles in immune responses to pathogens. T_H1 cells are vital against intracellular bacteria and protozoa. They are characterised by secretion of interferon γ (IFN-γ) and expression of the master transcription factor *TBX21* (Thieu *et al.*, 2008). In contrast, T_H2 cells are most effective against extracellular pathogens such as helminths, and are characterised by production of IL-4 and expression of the transcription factor *GATA3* (Ouyang *et al.*, 2000; Seki *et al.*, 2004). T_H17 cells contribute to pathogen clearance at mucosal sites and are characterised by production of IL-17 and express the transcription factor *RORC*. The expression of chemokines and chemokine receptors by $CD4^+$ T cells is discussed in more detail in a later section (Section 1.3.5). Comprehensive reviews of the functional subsets of $CD4^+$ T cells can be found in elsewhere (Nakayamada *et al.*, 2012; Schmitt and Ueno, 2015; Vinuesa *et al.*, 2016; Taniuchi, 2018).

$CD8^+$ T cells are characterised by their ability to kill infected, transformed and damaged cells, giving them the name cytotoxic T cells. These cells can recognise short self-derived or foreign peptides that are presented by cell surface bound to MHC Class I molecules

(Taniuchi, 2018). They carry out their killing function through the release of two types of cytotoxic proteins (granzymes and perforin), or via cell surface receptors, including Fas ligand-mediated cell killing, which leads to activation of the intracellular caspase cascade and apoptosis of the infected cell. $CD8^+$ T cells also produce IFNγ, thereby inhibiting viral replication and mediating macrophage activation (Taniuchi, 2018). Like $CD4^+$ T cells, $CD8^+$ T cells assume polarised states, although the full extent of $CD8^+$ T cell subsets in infectious disease settings are only beginning to be appreciated.

In addition to αβ T cells γδ T cells are the alternative T lymphocyte lineage present in all jawed vertebrates. They are composed of distinct pairs of Vγ and Vδ genes, giving rise to different functional subsets that differ between humans and mice. In human peripheral blood, γδ T cells constitute a minor subset of $CD3^+$ T cells (1-5%), whereas they are the majority in epithelial tissues (10-20% of total T cells in intestinal epithelium) (Borst *et al.*, 1991; Bonneville, O'Brien and Born, 2010; Vantourout and Hayday, 2013). They have been portrayed as a bridge between innate and adaptive immunity. Additionally, they mediate αβ T cell activation and target cell lysis (characteristic of adaptive immunity), as well as recognition of non-peptide antigens in a TCR-dependent, MHC independent manner (characteristic of innate immunity) (Vermijlen *et al.*, 2018). Finally, in addition to recognising a variety of antigens on stressed or neoplastic cells, they can act as antigen-presenting cells and stimulate conventional (αβ) T cells (Brandes, Willimann and Moser, 2005; Tyler *et al.*, 2015).

1.1.3 B Lymphocytes

B lymphocytes comprise 5-20% of PBMCs and are widely identified based on the expression of CD19 or CD20 in humans, whereas in mice they are identified based on B220 expression (Lebien and Tedder, 2008). In fact, CD19 constitutes part of the B cell receptor complex, with which B cells recognise antigens, which can lead to activation, proliferation and differentiation into antibody secreting plasma cells. Like T cells, circulating B cells consist of naïve and memory subsets, but they also comprise antibody-secreting plasmablasts and plasma cells. Plasmablasts are short-lived, proliferating cells that specialise in antibody secretion, while plasma cells are long-lived, non-proliferating cells that also secrete antibodies upon re-stimulation. Similar to T cells, the presence of regulatory B cells has been reported (Mizoguchi *et al.*, 2002). Additionally, different

subsets based on developmental stages have been identified, differing on their location within primary and secondary lymphoid organs. Antibodies contribute to the main effector functions of B cells and mediate the removal of extracellular pathogens through various means, including opsonisation, initiation of the complement cascade and induction of the direct killing by cytotoxic T cells and NK cells (Kurosaki, Shinohara and Baba, 2010). These functions of humoral immunity have been termed antibody-dependent cell-mediated cytotoxicity (ADCC). Moreover, B cells also mediate other functions essential for immune responses and homeostasis. For instance, B cells can process and present antigens to T_{FH} cells in an MHC-restricted manner during the germinal centre reaction and are able to produce a range of cytokines, thus regulating the activity of other cell types.

1.1.4 Natural Killer Cells

NK cells constitute approximately 5-15% of circulating lymphocytes in humans. NK cells are distinguished by their expression of neural cell adhesion molecule, also known as CD56, by which they can be identified using flow cytometry (Abel *et al.*, 2018). Although relatively small in number, NK cells are generally considered key mediators of early innate immune defence as they do not require prior activation to kill target cells. This function is achieved via three distinct mechanisms: ADCC, cytokine production and lytic cell surface receptors including natural cytotoxicity receptors (Vivier *et al.*, 2008). They can lyse virally infected cells and tumour cells without prior sensitisation. NK cells differ from other lymphocytes as they do not express an antigen-specific receptor. Instead, they express two distinct types of NK cell receptors, whose balance of positive and negative signals control NK cell activity, NK cell inhibitory and activating receptors (Vivier *et al.*, 2008). NK cell inhibitory receptors maintain an inactive state within NK cells through the recognition of self-antigens on target cells. These include killer cell immunoglobulin-like receptors (KIRs), which recognise both classical (HLA-A, B, C) and non-classical (HLA-E) MHC Class I ligands (Di Santo, 2006). Normal cells express MHC Class I, therefore recognition of healthy tissues with normal levels of MHC expression by KIRs inhibits NK cell killing activity (Moretta and Moretta, 2004). Activating receptors include CD16, which recognises the Fc portion of the IgG antibody and mediates ADCC, as well as NKG2D which binds to ligands structurally homologous to MHC Class I and

expressed on stressed cells (Abel *et al.*, 2018). NK cells therefore employ a sophisticated repertoire of receptors that recognise a vast array of antigens and regulate their activity. This ensures the host is protected against pathogens and the development of tumours, while preventing autoimmune responses.

1.1.5 Granulocytes

Granulocytes (also known as polymorphonuclear cells) are a subset of leukocytes with a characteristic morphology, having large cytoplasmic granules and a bi-lobed or multi-lobed nucleus. They have a role both in innate and adaptive immune responses against bacterial, viral and parasitic infections. They consist of four types of cells: basophils, eosinophils, neutrophils and mast cells. Neutrophils are the most abundant granulocytes, constituting 50-60% of total leukocytes in peripheral blood (Nathan, 2006). Neutrophils are the first white blood cells recruited to sites of acute inflammation in response to chemotactic cues, reaching the site of an infection within 30-60 minutes. At the site of infection, they provide first line of defence against invading pathogens by phagocytosis of pathogens and/or release of antimicrobial factors contained in specialised granules (Lawrence, Corriden and Nizet, 2018). They can directly recognise pathogens through recognition of pathogen-associated molecular patterns (PAMPs) by PRRs, or indirectly, through recognition of opsonised microbes by Fc receptors or complement receptors. Neutrophils are short-lived, as they undergo programmed cell death at the site of infection (Nathan, 2006). Neutrophils also produce neutrophil extracellular traps (NETs), comprising of a web of fibres made of chromatin and serine proteases that trap and kill microbes extracellularly (Brinkmann *et al.*, 2004). Additionally, they produce an array of pro-inflammatory and immunomodulatory cytokines and chemokines capable of enhancing the recruitment and effector functions of other cells (Nathan, 2006). These functions place them at the interface between innate and adaptive immunity.

Eosinophils constitute 0.5-1% of circulating leukocytes and are important in immunity against extracellular pathogens such as parasites and helminths (Flier, Underhill and Weller, 1991). Basophils constitute the least abundant immune cell type in peripheral blood, comprising 0.1-0.3% of circulating leukocytes. Eosinophils, along with mast cells and basophils also mediate allergic responses through histamine release. Following

activation, these cells rapidly release mediators within their granules, including histamine (mast cells also release heparin), as well as generating and releasing lipid mediators of inflammation (prostaglandins and leukotrienes). These cells also release a range of cytokines that activate other arms of the immune response, such as IL-4 for induction of T_H2 T cell differentiation (Sokol and Medzhitov, 2010). It was initially thought that mast cells represented tissue resident basophils, due to their similarities in morphology and function. However, it has been shown that the two cell subsets develop from different haematopoietic precursors (Franco *et al.*, 2010).

1.2 Immunology of the Skin

As well as being the largest organ of the body, the skin acts as an active immune organ, where microbiome, chemical, physical and immune barriers form an interactive network. Disruption of that barrier contributes to pathogenic skin conditions including infections, inflammation, allergy and cancer. The vast number of immune cells present in this tissue are diverse in terms of origin and function and can sense danger signals, protect against pathogens and mount memory responses, thereby providing an immunological barrier to infection. The skin fulfils numerous functions including, but not limited to, physical sensing, balancing of body temperature and moisture, barrier function and immunity.

1.2.1 Anatomy of the Skin

Human skin is made up of two main compartments, the outer epidermis and the underlying dermis. The epidermis functions as a physical barrier between the body and the outside environment. It consists of four stratified layers (stratum basale, stratum spinosum, stratum granulosum and the outermost layer stratum corneum) and is made up of specialised epithelial cells called keratinocytes, which make up more than 90% of epidermal cells (**Figure 1.2**) (Kabashima *et al.*, 2019). The outermost layer of the epidermis, called the stratum corneum, consists of layers of dead keratinocytes (known as corneocytes) and intracellular lipids. This layer performs the main barrier functions, including physical protection against potentially harmful invaders. This layer coexists with numerous microorganisms (known as the microbiota) that live on the surface of the skin, in a commensal relationship (Byrd, Belkaid and Segre, 2018). Keratinocytes in

the basal layer of the epidermis, known as the stratum basale are responsible for establishing and maintaining the upper layer of corneocytes through cell division. Basal keratinocytes migrate upwards towards the stratum corneum as they differentiate and eventually die, forming the corneocytes. Apart from the structural support, owing to their strategic positioning at the interface between the body and the environment, keratinocytes receive signals from the outside and transmit them to immune cells in the skin (Eyerich *et al.*, 2018). This communication is achieved through sensing of microorganisms through PRRs, including TLRs and production of inflammatory mediators such as cytokines, chemokines and antimicrobial proteins in response to pathogenic stimuli (Nestle *et al.*, 2009). Keratinocytes sense danger signal and immune triggers through PRRs, such as TLRs (Eyerich *et al.*, 2018), which leads to the production of cytokines and inflammatory chemokines, including CXCL8, CCL2 and CXCL10. This leads to the recruitment of neutrophils, monocytes and effector T cells, respectively. Cytokines produced by keratinocytes include members of the IL-1, IL-10 and TNF cytokine families (Nestle *et al.*, 2009). Another specialised cell present in the epidermis is a melanocyte, which produces the skin pigment melanin. Other non-epithelial immune cells also reside in the epidermis, including Langerhans cells (LCs), a specialised type of DC and the main epidermis-resident immune cell in human skin (Kabashima *et al.*, 2019). In contrast to humans, mouse skin also contains γδ T cell receptor-expressing dendritic epidermal T cells, which play a role in epidermal stress surveillance (Hayday, 2000).

The dermis layer of the skin is abundant in collagen-rich extracellular matrix produced by stromal cells such as fibroblasts. Other stromal cells present include fibrocytes and structural cells of the blood and lymph vessels (Roozendaal and Mebius, 2011; Nowarski, Jackson and Flavell, 2017). Dermis is separated from the epidermis by a continuous basement membrane and contains many different populations of myeloid and lymphoid immune cells that either reside or traffic through the dermis. Resident immune cells include DCs (of which there are various subsets), $CD4^+$ and $CD8^+$ T cells, innate lymphoid cells such as γδ T cells and NK cells, as well as macrophages and mast cells. A network of blood and lymphatic vessels present throughout the dermis facilitate the entry of immune cells from the blood and exit to the lymph node, respectively. Hair follicles

originate in the dermal layer of the skin but come into contact with the environment, and have been characterised as a site of immune privilege (Paus *et al.*, 2003). Disruption of this immune privilege state at this site leads to the development of responses against skin microbiota and can potentially result in autoimmune responses against hair follicles (Kang *et al.*, 2010). Hair follicles also serve as a niche for keratinocyte, melanocyte and mast cell progenitors (Kumamoto *et al.*, 2003). The anatomy of the human skin and resident immune cells under steady state conditions are shown in **Figure 1.2.**

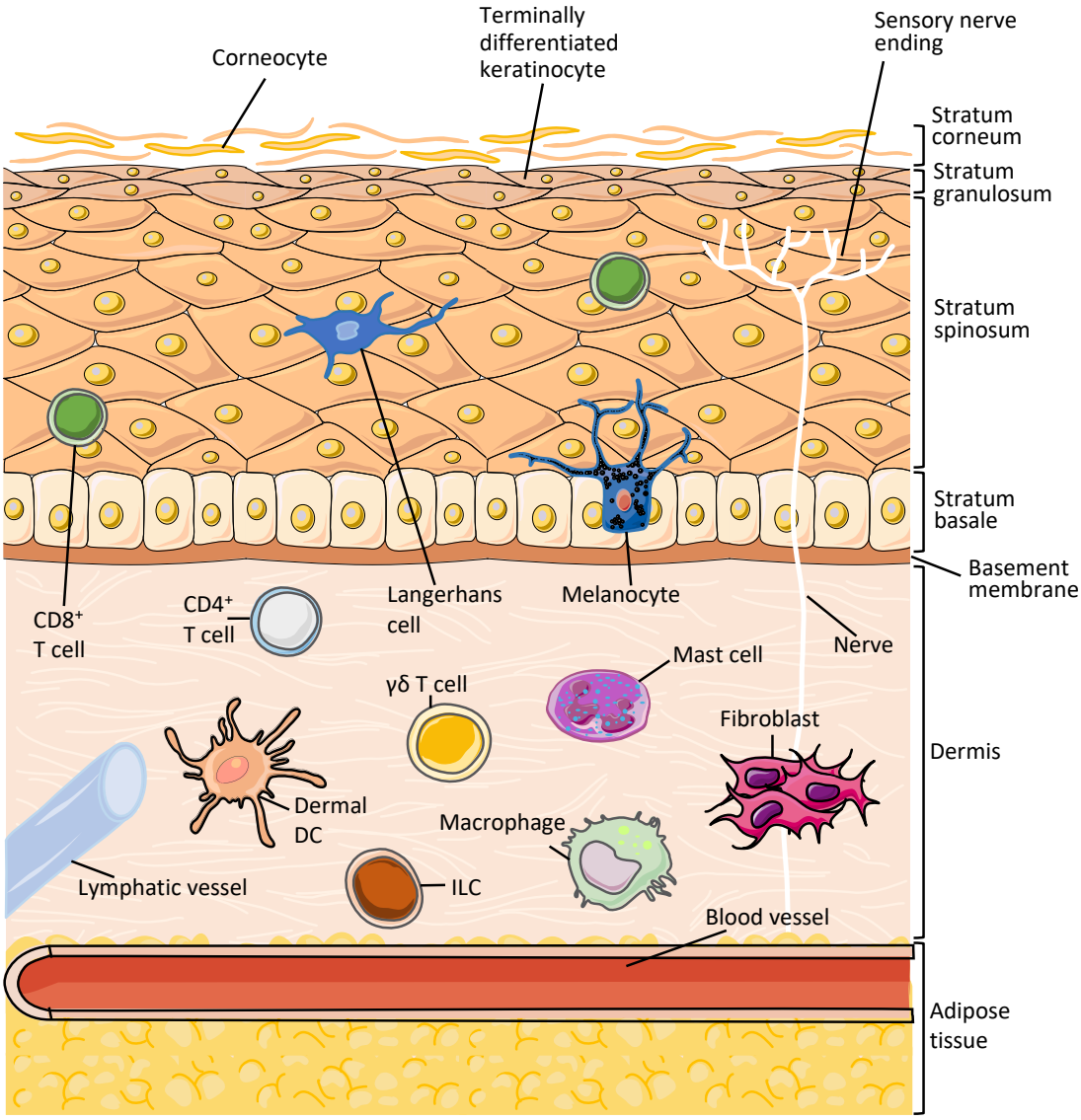


Figure 1.2. Healthy human skin anatomy and resident immune cells.

The structure of the skin indicates the complexity of its functions, such as acting as a protective barrier, maintenance of body temperature, gathering sensing information from the environment and playing a vital role in the immune system. The epidermis contains different layers, including the stratum basale, the stratum spinosum, the stratum granulosum and the outermost layer, the stratum corneum, which consists of corneocytes and is responsible for the barrier function of the skin. The epidermis also includes specialised cells, including Langerhans cells and melanocytes which produce the pigment melanin. Rare T cells, mainly $CD8^+$ T cells (also known as resident memory T cells) reside between keratinocytes in the stratum basale and stratum spinosum. The dermis composes of collagen, elastic tissue and reticular fibres. Most immune cells within skin are found in this layer, including DC subsets, T cell subsets, macrophages, fibroblasts, innate lymphoid cells (ILCs) and mast cells. Blood and lymphatic vessels are present throughout the dermis, where immune cells can enter and exit the skin. Sensory nerves span the layers of the skin. Below the dermis there is a layer of adipose tissue. Figure adapted from Kabashima *et al.*, 2019.

1.2.2 Mononuclear Phagocyte System of the Skin

The MPS of the skin consists of two main families of cells, namely the migratory DCs and tissue-resident macrophages. Skin DCs can be further divided into LCs and dermal DCs. These cells differ in origin, but share overlapping functions, including phagocytosis, cytokine production and antigen presentation. Countless studies have been performed both in mice and humans, which have provided valuable information regarding their origin and function. Due to difficulties associated with human *in vivo* studies, DCs have mainly been studied through the use of monocyte-derived or HSC-derived DCs as a surrogate *in vitro* model of DCs. It is now established that DC subsets share an intimate link between their ontogeny in the bone marrow and blood and their function, in contrast to macrophages whose function is determined by development, as well as the microenvironment of their tissue of residence (Kashem, Haniffa and Kaplan, 2017). The sections below summarise the existing literature regarding the MPS in skin.

1.2.2.1 Langerhans Cells

Human epidermal LCs were the first tissue DCs to be described. They reside in the suprabasal layers and are regularly spaced between keratinocytes (Valladeau and Saeland, 2005). Under homeostatic conditions, LCs are the sole resident antigen presenting cells (APCs) in the epidermis and account for 3-5% of epidermal cells (Merad, Ginhoux and Collin, 2008). Their location is key as they can extend their dendrites to sample and acquire antigens through tight junctions from the stratum corneum (Kubo *et al.*, 2009). Following uptake of exogenous antigens, LCs stop sampling the environment, upregulate and redistribute MHC Class II molecules, costimulatory molecules such as CD40 and the chemokine receptor CCR7 on their cell surface (Larsen *et al.*, 1990). In mice, upregulation of CCR7 has been shown to facilitate LC migration to skin draining lymph nodes via dermal lymphatic vessels (Ohl *et al.*, 2004), where they come into contact with and present antigen to T cells within T cell areas (Randolph, Ochando and Partida-Sánchez, 2008). LCs have been shown to constitutively migrate to skin draining lymph nodes under steady state conditions (Hemmi *et al.*, 2001). However, their rate of migration increases during inflammation (Stoitzner *et al.*, 2005).

LCs can be identified by their dendritic morphology and the presence of a special type of intracytoplasmic organelle, the tennis racket-shaped Birbeck granules (Merad *et al.*, 2013). These structures are formed upon capture of antigen by langerin (CD207), a type II C-type lectin receptor that binds mannose and related sugars, leading to a proposed role in antigen processing and presentation (Valladeau *et al.*, 2000). Both human and murine LCs express CD45, MHC Class II and langerin (Valladeau *et al.*, 2000). Additionally, they express the epithelial cell adhesion molecule (EpCAM; CD326), and E-cadherin (CD324), which promotes LC migration by decreasing adhesion to keratinocytes as well as CD11b, CX3CR1 and SIRPα (CD172α). Other surface markers highly expressed by human LCs include CD1a, which presents microbial lipid antigens to unconventional αβ T cells.

Although LCs share many vital properties with other DC populations, they have some distinctive qualities. The majority of lymphoid organ or peripheral DC populations are eliminated by local irradiation and subsequently replaced by circulating donor-derived precursor cells following congenic haematopoietic stem cell transplant (HSCT) (Iijima *et al.*, 2007). In contrast, LCs are partially resistant to radiotherapy, indicating a distinct origin for these cells. Human studies showed that LCs remain of donor origin for up to 10 years after limb transplantation, but are replaced by donor-derived cells following nonmyeloablative HSCT, which may be due to chemotherapy and conditioning prior to transplantation (Kanitakis, Petruzzo and Dubernard, 2004; Chorro *et al.*, 2009; Kanitakis *et al.*, 2011). Therefore, in the absence of inflammation, LC populations are thought to be maintained through self-renewal. Studies in mice showed that LC are seeded in the epidermis from embryonic (yolk-sac derived) precursors during embryonic development, independent of haematopoiesis (Merad, Ginhoux and Collin, 2008). More recently, it is thought that Langerhans cells are a specialised subset of tissue-resident macrophages, mainly originating from the foetal liver (Doebel, Voisin and Nagao, 2017; Bassler *et al.*, 2019). *In vivo* imaging of LCs in mice showed that in the absence of inflammation, the turnover of LCs is slow, with an estimated half-life of 53-78 days (Vishwanath *et al.*, 2006). Indeed, studies using langerin-DTR (diphtheria toxin receptor) mice, where LCs can conditionally be ablated by administration of diphtheria toxin, showed that repopulation of LCs is slow and requires several weeks for complete

reconstitution (Bennett *et al.*, 2005; Kissenpfennig *et al.*, 2005). This is in contrast to the few days required for reconstitution of conventional DC populations in the spleen or lymph nodes (Jung *et al.*, 2002). Although studies examining LC ontogeny are more difficult to carry out in humans, experimental results from transplantation studies show similar LC replacement dynamics.

Approximately 2-3% of LCs actively proliferate at any time point, as evidenced by fluorescence microscopy (Merad, Ginhoux and Collin, 2008). Given the slow rate of turnover of LCs, this proliferating rate is likely sufficient to maintain the LC population during steady state. Renewal of LCs during inflammation has been studied in mice, where exposure of the skin to ultra-violet B radiation, leads to inflammation in the epidermis and severe LC loss (Ginhoux *et al.*, 2006). In this model, LC replenishment requires recruitment of circulating monocytes, a mechanism dependent on the expression of chemokine receptors CCR2 and CCR6 by monocytes. The infiltrating monocytes proliferate locally and repopulate the LC pool within 10-15 days (Merad *et al.*, 2002). However, in the absence of inflammation, CCR2- and CCR6-deficient mice have a normal LC network in the epidermis (Sato *et al.*, 2000; Chorro *et al.*, 2009). *In vitro* studies of monocyte differentiation to LCs show a role for GM-CSF, IL-4 and transforming growth factor-beta 1 (TGF-β1), as well as sequential expression of CCR2 and CCR6 (Geissmann *et al.*, 1998). Expression of TGF-β1 by keratinocytes, as well as LCs themselves, is required for the development of LCs *in vivo* (Borkowski *et al.*, 1996). A study from our group using a skin artificial epidermal equivalents model showed that $CD14^+$ monocytes can differentiate into LCs in the epithelium. These experiments also proposed that chemokine (C-X-C motif) ligand 14 (CXCL14) is important in guiding monocytes to the epidermal niches, where they can differentiate into LCs (Schaerli *et al.*, 2005).

An additional subset of epidermal DC has been described, which differs from LCs in the expression of macrophage mannose receptor (MMR; CD206) and is found in the inflamed epidermis of patients with atopic dermatitis (Wollenberg *et al.*, 1996). These cells have many confusing synonyms and have been referred to as inflammatory monocytes, inflammatory macrophages and inflammatory DCs. They have been identified in hair follicles, and when entering the epidermis, they are called

inflammatory dendritic epidermal cells. These cells overexpress high-affinity Fc receptor for IgE (FcεR1), enabling their reactivity to IgE-coated allergens and resulting in the pro-inflammatory allergen-specific response observed in these patients (Bieber, 2007).

1.2.2.2 Dermal DCs

There are three cell subsets classified in the DC family within the dermis: conventional DCs, pDCs and monocyte derived DCs. Under steady state conditions, pDCs are absent from the skin, only being recruited in the skin during inflammation, when they promote wound repair through the production of type I interferons (Gregorio *et al.*, 2010). Therefore, they will not be discussed in detail in this section. The specific contribution of circulating blood DCs and monocytes to skin DC subsets has been the subject of many studies, both in mice and humans. The notion that circulating human monocytes contribute to tissue DC pools was a central dogma of the MPS as originally conceived, although evidence supporting this notion in the steady state in mice and humans was limited until recently. In mice, it has been shown that Ly6C^{hi} (classical monocytes) continuously extravasate from the blood into tissues including skin and lung during the steady state, where they acquire APC functions (Jakubzick *et al.*, 2013; Tamoutounour *et al.*, 2013). However, these studies did not show differentiation of monocytes into DCs upon entry to the tissues. Experiments on human skin explants showed that CD14^{+}, non-autofluorescent cells in the dermis align more closely with blood monocytes and dermal macrophages than DCs as well as being distinct from cDCs, at both a phenotypic and transcriptional level (McGovern *et al.*, 2014). This study also showed that in patients undergoing HSCT, reconstitution of CD14^{+} dermal cells coincides with the recovery of blood monocytes (Haniffa, Ginhoux, *et al.*, 2009; McGovern *et al.*, 2014). In addition to the short half-life of these cells in blood during the steady state (< 6 days), these studies add further weight to the argument that CD14^{+} dermal cells are derived from monocytes, which upon entry into tissues, differentiate into a resident population.

In contrast to LCs, cDC populations in human dermis have a short half-life, while it appears that they are also dependent on circulating precursors for their continual replenishment (McGovern *et al.*, 2014). This was shown in patients lacking circulating monocytes and DCs, who also lack dermal DC populations (Bigley *et al.*, 2011). Under steady state conditions, human dermis is populated by monocyte-derived CD14^{+} “DCs”

(discussed above), as well as two conventional DC subsets; $CD141^+$ DCs (also known as cDC1 DCs) (Haniffa *et al.*, 2012) and $CD1a^+$ DCs (also known as cDC2 DCs) (Angel *et al.*, 2006). $CD141^+$ DCs produce the regulatory cytokine IL-10 and have potent immunoregulatory functions *in vitro* and *in vivo* (Chu *et al.*, 2012). In contrast, $CD1a^+$ DCs are potent activators of $CD8^+$ T cells, due to their ability to cross-present antigens (Haniffa *et al.*, 2012). $CD141^+$ DCs have also been identified in the blood, where they possess a skin-homing profile. In contrast, their skin counterparts have a lymph node-homing profile, as discussed on (Haniffa *et al.*, 2012).

1.2.2.3 Dermal Macrophages

Macrophages are found in almost every tissue throughout the body. Their origin has been part of the long-standing debate regarding the relationship between blood monocytes and tissue macrophages, which has been the centre of one of the most rapidly evolving fields of research within immunology. There is now compelling evidence for the non-monocytic origin of most tissue macrophages in adults, while these populations show remarkable functional heterogeneity depending on the anatomical location. For instance, alveolar macrophages in the lung specialise in the clearance of surfactant, microglia in the brain specialise in synaptic pruning and immune surveillance, whereas intestinal macrophages play a role in the regulation of the host-microbe balance (Davies, Jenkins, *et al.*, 2013). On the basis of their function during inflammatory responses, macrophages can be divided into classically activated (pro-inflammatory) M1 macrophages, regulatory M2 macrophages and wound-healing macrophages (Mantovani, Sica and Locati, 2005; Mosser and Edwards, 2008). Advancements in experimental tools allowed the delineation of multiple distinct embryonically derived macrophage lineages (Davies, Jenkins, *et al.*, 2013; Sieweke and Allen, 2013; Wynn, Chawla and Pollard, 2013). Additionally, numerous studies in mice have demonstrated that macrophages in most adult tissues are entirely of embryonic origin, with no contribution from circulating monocytes. In particular, at least two different types of embryonic precursors have been proposed: yolk sac macrophages and foetal liver monocytes (Scott, Henri and Guilliams, 2014). Although it is difficult to unravel the contribution of foetal and adult derived cells to tissue macrophage populations in humans, a 10-week old patient suffering from a genetic mutation causing the absence

of circulating DCs and monocytes showed preservation of dermal macrophages, indicating that these cells are seeded *in utero* (Haniffa, Bigley and Collin, 2015).

Human dermal macrophages are large cells with a foamy cytoplasm, which contain melanin granules (Haniffa, Ginhoux, *et al.*, 2009). They express MHC Class II and CD14, but lack expression of CD1a. Additionally, they express CD163 (a scavenger receptor expressed by most tissue macrophages) and factor XIIIa (FXIIIa; a component of the coagulation cascade with a potential function in wound healing) (Zaba *et al.*, 2007). Dermal macrophages are highly autofluorescent, partly owing to their cytoplasmic content including melanin granules. This property facilitates their detection by flow cytometry (Haniffa *et al.*, 2009). Dermal macrophages are predominantly sessile, i.e. they are not detected among emigrant cells during skin tissue culture, although they migrate to draining lymph nodes under certain inflammatory conditions (Van Furth *et al.*, 1985). In contrast to dermal cDCs and monocyte-derived DCs, they have a poor antigen-presenting capacity (Malissen, Tamoutounour and Henri, 2014). They express a unique set of genes that support specific roles in scavenging cell debris and killing microorganisms. This is in addition to the high expression of the anti-inflammatory cytokine IL-10 (Tamoutounour *et al.*, 2013), indicating they might have a more anti-inflammatory, wound healing, M2-like function. Some dermal macrophages located in close proximity to the blood vasculature produce chemokines that play a role in the recruitment of neutrophils into the dermis during infection, therefore showing M1-like functions (Abtin *et al.*, 2014). Consequently, in addition to their role in homeostasis and tissue repair, dermal macrophages appear to play a role in the early response to invading microorganisms.

Dermal macrophages in mice and humans have a slower rate of turnover and a longer half-life than their neighbouring dermal DCs (Haniffa, Ginhoux, *et al.*, 2009; McGovern *et al.*, 2014). It is now clear that dermal macrophages are seeded in tissues during embryonic development. Moreover, they appear to maintain their population during adult life through local proliferation, independently from circulating precursors (Schulz *et al.*, 2012; Perdiguero and Geissmann, 2016). However, it has been shown that dermal macrophages in adult mice consist of a subset that is established prenatally, as well as a subset that develops postnatally from Ly6C^{hi} monocytes (Jakubzick *et al.*, 2013;

Tamoutounour *et al.*, 2013). The contribution of Ly6C^{hi} monocytes to the dermal macrophage pool increases through repeated episodes of inflammation (Ginhoux and Guilliams, 2016), as shown for cardiac macrophages in mice (Epelman *et al.*, 2014). Therefore, the origin and reconstitution of the dermal macrophage niche under homeostasis is still not well understood.

1.2.3 T Cells in Skin

T cells are present in vast quantities in healthy skin, with an estimated 20 billion T cells in adult human skin (Clark *et al.*, 2006). These cells are composed of 1-10% γδ T cells, with αβ T cells making up the remaining population (Holtmeier and Kabelitz, 2005; Clark *et al.*, 2006). CD4$^+$ T cells are found in large numbers in the dermis, with only a small fraction of skin CD4$^+$ T cells found in the epidermis. In contrast, CD8$^+$ T cells are mainly found in the epidermis, where they reside for long periods (**Figure 1.2**). CD4$^+$ and CD8$^+$ T cells in the dermis that do not recirculate are commonly referred to as tissue-resident memory T cells (T_{RM}) (Schenkel and Masopust, 2014). T cell compartments in peripheral tissues, including the skin, play a vital role in immune surveillance. Their localisation and therefore maintenance of immune surveillance with the ultimate goal of tissue health is partly maintained by chemokines (McCully, Kouzeli and Moser, 2018).

1.2.4 Innate Lymphoid Cells in Skin

Innate lymphoid cells are a diverse family of immune cells that produce cytokines and coordinate immunity and inflammation in body surface tissues, including the intestine, lungs and skin (Spits *et al.*, 2013). They are subdivided into three subsets, based on their cytokine and transcription factor expression profile: group 1 ILCs (ILC1s and NK cells), group 2 ILCs (ILC2s) and group 3 ILCs (ILC3s and lymphoid tissue inducer cells) (Pasparakis, Haase and Nestle, 2014). Although all ILC subsets have been found in the skin, we currently have a better understanding of the function of ILC2s and ILC3s in skin tissue. For instance, the presence of NKp46$^+$ ILC1 NK cells and IL-13 producting ILC2 cells have been described in mouse skin, while there is evidence indicating that equivalent populations exist in healthy human skin (Mueller, Zaid and Carbone, 2014). Finally, NKp44$^+$ ILC3s are present in high numbers in healthy human skin, and their numbers are further increased in psoriatic arthritis patients (Villanova *et al.*, 2014).

1.2.5 Mast Cells in Skin

Mast cells are derived from haematopoietic progenitor cells and undergo maturation and differentiation in peripheral tissues. Mast cells in peripheral tissues can be identified by the expression of stem cell growth factor receptor (also known as c-kit) as well as IgE receptors (Valent and Bettelheim, 1992). Mast cells perform specialised first-line surveillance functions in the skin, due to the presence of a variety of preformed proinflammatory mediators stored within their cytoplasmic granules. They specialise in IgE receptor-mediated release of histamine during allergic reactions and therefore play an important role in the pathology of allergic conditions, including atopic dermatitis (Liu, Goodarzi and Chen, 2011).

1.3 The Chemokine Superfamily

Chemokines are key orchestrators of the immune system by controlling immune cell traffic (Bachelerie *et al.*, 2013; Griffith, Sokol and Luster, 2014; Hughes and Nibbs, 2018). They play a role in immune cell development, maturation in the primary lymphoid organs (bone marrow and thymus) and egress into the bloodstream. Chemokines also fine-tune immune cell extravasation into peripheral tissues, where they can populate distinct locations within the tissue for differentiation to effector cells to take place. Many chemokines play a major role in inflammatory conditions, where they orchestrate the traffic of effector immune cells. However, aside from cell migration, chemokines also induce cellular responses that are related to survival and proliferation, can contribute to virus-host interactions and play a role in tumour growth, metastasis and angiogenesis (Zlotnik, 2006). Apart from the multitude of effects they have on their target cells, chemokines have alternative functions, such as direct antimicrobial activity against many types of pathogenic microorganisms (Wolf and Moser, 2012).

This extraordinary range in functions of chemokines is possibly the reason why chemokine biology has become such an active area of research since the emergence of the field in 1987, following the cloning of the human gene encoding CXCL8.

1.3.1 Chemokines

More than 30 years ago, the first chemoattractants were identified, including the bacterial peptide *N*-formyl-methionyl-leucyl-phenylalanine (FMLF), the C5a fragment of

serum complement and the lipid mediator leukotriene B_4 (LTB_4) (Yoshimura, 2015). However, the major starting point for the field was the purification of CXCL8 from natural sources (secreted by activated monocytes), followed by isolation of the corresponding gene (Walz *et al.*, 1987; Yoshimura *et al.*, 1987; Gregory *et al.*, 1988; Van Damme *et al.*, 1988). CXCL8 triggered functional responses in neutrophils, similar to other chemoattractants (Thelen *et al.*, 1988). The identification of the three-dimensional structure of CXCL8 indicated that chemokine-like proteins had already been identified, including CXCL10 (formerly known as IP10), CCL1 (formerly known as I309 and TCA3) and CCL3 (formerly known as MIP-1α) (Luster, Unkeless and Ravetch, 1985; Obaru *et al.*, 1986; Burd *et al.*, 1987; Miller *et al.*, 1990). This led to the establishment of the protein family referred to as chemokines, an abbreviated version of "chemotactic cytokines".

All the approximately 50 members of the chemokine family identified to date are structurally very similar, highly basic proteins of 70-125 amino acids with molecular masses ranging from 6 to 14 kDa. From a structural point of view, chemokines can be classified based on the position of two highly conserved N-terminal cysteine residues, into CC, CXC, XC and CX3C chemokines. Although the sequence identity between chemokines is often quite low, their secondary and tertiary structure show striking similarities (Clark-Lewis *et al.*, 1995). Most chemokines contain four cysteine residues in highly conserved positions (**Figure 1.3**). All chemokines contain a flexible N-terminus, preceding the first cysteine residue, followed by a rigid loop leading to three anti-parallel β-sheets. The C-terminus is helical and also unfolded at its end. The cysteine residues form two disulphide bonds, one between the first and third cysteines and one between the second and fourth cysteines, which connect the N-terminus of the rigid loop with the β-strands. These disulfide bonds determine the three-dimensional folding and maintain the structure of the chemokine monomer. The relative position of the first two NH_2-terminal cysteine residues distinguishes the two major structural subfamilies of chemokines. First, CXC chemokines (also known as α-chemokines), where the cysteines are separated by a single amino acid residue and second, the CC chemokines (also known as β-chemokines), where the cysteines are in adjacent positions. There are three homologous molecules also regarded as chemokines. These are CX3CL1 (fractalkine), in

which three amino acid residues separate the two NH_2-terminal cysteines, and XCL1 and XCL2, which lack two out of the four canonical cysteines. The human chemokine superfamily is summarised in **Table 1.1.**

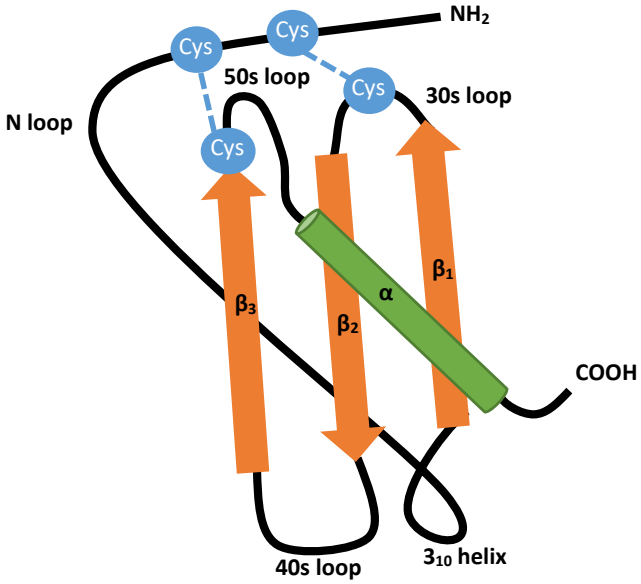


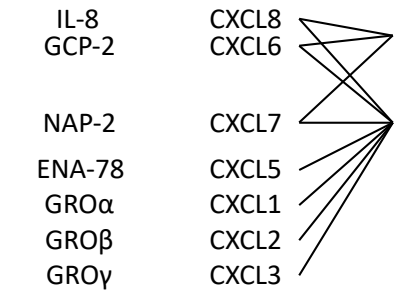
Figure 1.3. General structure of CXC chemokines.
A flexible N-terminal region precedes the first cysteine residue. The region between the second cysteine residue (blue) and the 3_{10} helix is known as the N loop and its flexibility is thought to play an important role in chemokine receptor binding and/or activation. The single-turn 3_{10} helix is followed by three anti-parallel β strands (orange) forming a β-pleated sheet. Each structural element is connected by 30s, 40s and 50s loops, which reflects the numbering of residues in the mature protein. The 30s and 50s loops contain two of the four cysteine residues. The 50s loop connects the third β strand with the COOH-terminal α helix (green). The 3D structure of the mature secreted chemokine is stabilised by two disulfide bridges formed between the four conserved cysteine residues. Figure adapted from Metzemaekers *et al.*, 2016.

The vast majority of chemokines are secreted, with the exception of two chemokines, namely CXCL16 and CX3CL1, which are membrane-bound due to the presence of a transmembrane domain that is connected to a mucin-like stalk followed by a functional chemokine domain (Bazan *et al.*, 1997; Moser *et al.*, 2004). In order to exert their migratory functions, chemokines need to form a gradient by becoming immobilised on cell surfaces and extracellular matrices (Hamel *et al.*, 2009). Since chemokines are highly basic proteins containing many positively charged amino acids, they interact with negatively charged glycosaminoglycans (GAGs), which can limit their dissemination

(Handel et al., 2005). This large family of unbranched proteoglycans can be divided into five main groups: heparin, heparan sulphate, chondroitin sulphate, dermatan sulphate and hyaluronic acid. They are present in the extracellular matrix and on the surface of cells. The low affinity interaction between GAGs and chemokines is responsible for immobilising them on cell surfaces, thus preventing them from being washed away in the bloodstream (Handel *et al.*, 2005). This in turn induces the creation of a high local concentration gradient and so allowing them to facilitate leukocyte arrest and extravasation (Hughes and Nibbs, 2018). GAGs interact with the C-terminal portion of chemokines, whereas the N-terminus interacts with the receptor, thereby allowing presentation of the chemokine by proteoglycans in an active conformation (Monneau, Arenzana-Seisdedos and Lortat-Jacob, 2016).

Table 1.1. The human chemokine superfamily.

Chemokine		Receptor	Leukocyte distribution	Key immune functions
Common name	Systematic name			
HCC-1	CCL14	CCR1	Monocyte, macrophage, neutrophil, T_H1, basophil, DC	Innate and adaptive immunity
MCP-4	CCL13			
MPIF-1	CCL23			
MCP-1	CCL2	CCR2	Classical monocyte, macrophage, T_H1, NK, iDC, basophil	Monocyte trafficking, T_H1-type adaptive immunity
RANTES	CCL5			
HCC-2	CCL15			
HCC-4	CCL16	CCR3	Eosinophil > basophil, mast cell	T_H2-type adaptive immunity, eosinophil distribution and trafficking
MCP-3	CCL7			
Eotaxin	CCL11			
Eotaxin-2	CCL24	CCR4	T_H2, T_H17, skin-and lung-homing, Treg, $CD8^+$ T, monocyte, B cell, iDC	Homing of T cell to skin and lung, T_H2-type immune responses
Eotaxin-3	CCL26			
MIP-1α	CCL3	CCR5	Monocyte, macrophage, T_H1, $CD8^+$ T cell, NK, Treg, DC, neutrophil	T_H1-type adaptive immunity
TARC	CCL17			
MCP-2	CCL8			
MDC	CCL22			
MIP-1β	CCL4			
MIP-3α	CCL20	CCR6	T_H17 > iDC, γδ T, NKT, NK, Treg, T_{FH},	iDC trafficking, T_H17 adaptive immune responses
ELC	CCL19	CCR7	Naive T, T_{CM}, mature DC, B cell	Mature DC, B and T cell trafficking to T cell zone of lymph nodes, egress of DC and T cells from tissue
SLC	CCL21			
I-309	CCL1	CCR8	T_H2, Treg, skin T_{RM}, γδ T	Immune surveillance in skin, T_H2-type adaptive immunity
PARC	CCL18			
TECK	CCL25	CCR9	Gut-homing, T, thymocytes, B cell, DC, pDC	Homing of T cells to gut, GALT development and function
CTACK	CCL27	CCR10	Skin-homing T cell, IgA^+ plasma cells	Humoral immunity at mucosal sites including skin
MEC	CCL28			



IL-8 GCP-2	CXCL8 CXCL6	CXCR1	Neutrophil > monocyte, NK, mast cell, basophil, $CD8^+$ T cell	Neutrophil trafficking
NAP-2	CXCL7	CXCR2	Neutrophil > monocyte, NK, mast cell, basophil, $CD8^+$ T cell	Neutrophil egress from bone marrow, neutrophil trafficking
ENA-78	CXCL5			
GROα	CXCL1			
GROβ	CXCL2			
GROγ	CXCL3			
PF4	CXCL4	CXCR3	T_H1, $CD8^+$ T_{CM} and T_{EM}, Treg, T_{FH}, NK, NKT, pDC, B cell	T_H1-type adaptive immunity
IP-10	CXCL10			
MIG	CXCL9			
I-TAC	CXCL11			
SDF-1	CXCL12	CXCR4	Most (if not all) leukocytes	Haematopoiesis, organogenesis, bone marrow homing
BCA-1	CXCL13	CXCR5	$CD8^+$ T_{EM}, T_{FR}, T_{FH}, B cell	B and T cell trafficking to B cell zone of lymph nodes
SR-PSOX	CXCL16	CXCR6	T_H1, T_H17, γδ T, NK, NKT, plasma cell	Innate lymphoid cell function, adaptive immunity
DMC	CXCL17	GPR35/ CXCR8	Mast cells, basophils, eosinophils, monocytes	Macrophage homing to mucosal sites, in particular lung
BRAK	CXCL14	Unknown		Monocyte and macrophage homing to mucosal sites
Lymphotactin	XCL1	XCR1	Cross-presenting $CD8^+$ DC, thymic DC	Antigen cross-presentation by $CD8^+$ T cells
SCM-1β	XCL2			
Fractalkine	CX3CL1	CX3CR1	Non-classical monocyte, macrophage, T_H1, $CD8^+$ T_{EM}, NK, γδ T cell, DC	Patrolling monocytes in innate immunity, T_H1-type adaptive immunity

Abbreviations: DC, dendritic cell; GALT, gut-associated lymphoid tissue; iDC, immature dendritic cell; ILC, innate lymphoid cell; NK, natural killer; NKT, natural killer T; TCM, central memory T cell; TEM, effector memory T cell; TFH, T follicular helper cell; TFR, follicular regulatory T cell; TH, T helper; Treg, regulatory T cell; TRM, resident memory T cell. > indicates higher level of receptor expression relative to other cell types. Figure adapted by Griffith, Sokol and Luster, 2014; Xu *et al.*, 2015.

1.3.2 Chemokine Receptors

Chemokines exert their function by binding to G protein-coupled receptors (GPCRs), which are typically 340-370 amino acids in length and are characterised by seven transmembrane domains spanning the lipid bilayer of the cell membrane. Chemokine receptors constitute the largest branch of the γ subfamily of rhodopsin-like receptors. They can be divided into two categories based on their signalling abilities following ligand binding, conventional and atypical chemokine receptors (ACKRs). Conventional chemokine receptors (cCKRs) signal by activating $G_{\alpha i}$-type G proteins, as first revealed in experiments showing that treatment of neutrophils with *Bordetella pertussis* toxin inhibited stimulation with CXCL8 (Thelen *et al.*, 1988). Activation of an intricate chain of molecular mediators leads to cell migration, adhesion and/or a variety of other biological responses. The human chemokine receptor system contains 19 conventional GPCRs, at present. These include CCR1-10, CXCR1-6, XCR1, CX3CR1 and the recently added GPR35 (CXCR8) **(Table 1.1)**. ACKRs, of which five have been identified to date, structurally resemble cCKRs but do not trigger prototypical chemokine receptor signalling pathways (Bachelerie *et al.*, 2013; Nibbs and Graham, 2013). ACKRs include DARC (ACKR1), D6 (ACKR2), CXCR7 (ACKR3), CCRL1 (ACKR4) and CCRL2 (**Figure 1.4**). These receptors are involved in regulating chemokine localisation, distribution and abundance by different mechanisms, such as acting as scavengers, transporters or decoy receptors.

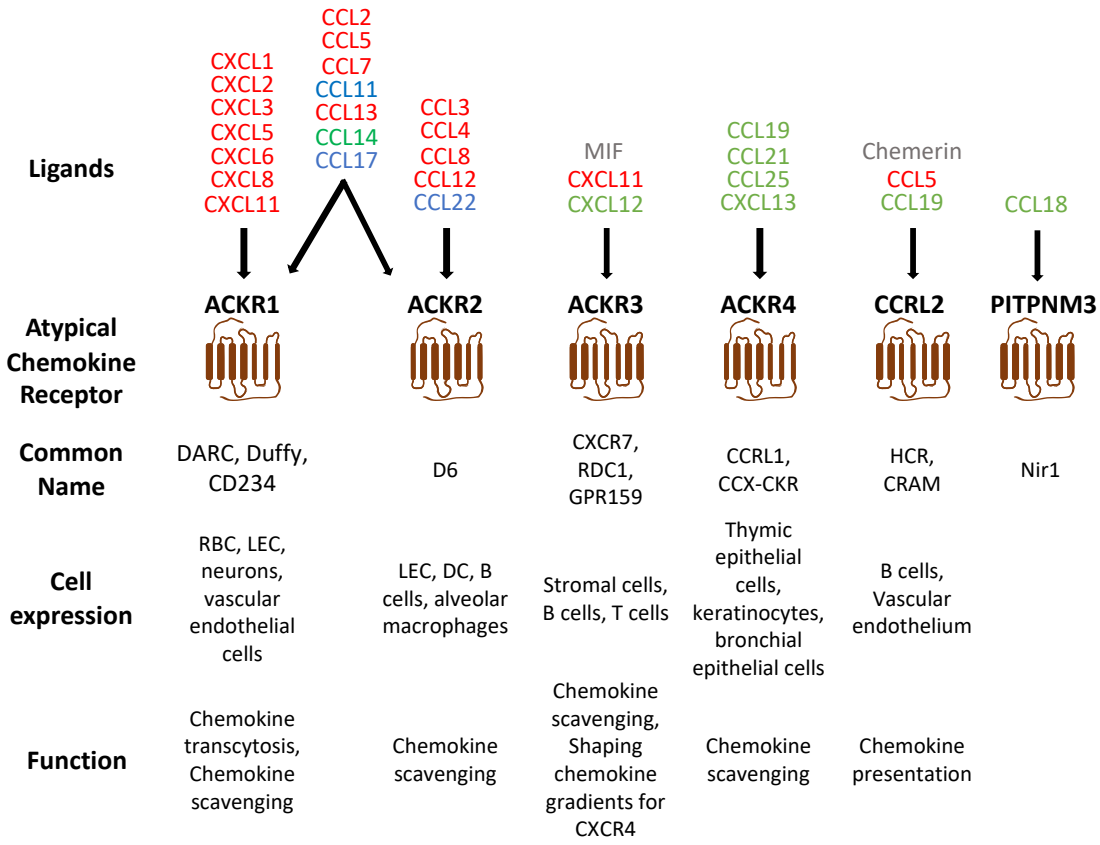


Figure 1.4. Atypical chemokine receptors: Ligand specificity, cell expression and biological functions in mouse and/or human.

ACKRs recognise chemokines and regulate their distribution in tissues, thereby acting as a regulatory mechanism of the chemokine system.

Chemokines are colour coded as inflammatory (red), homeostatic (green) and dual function (blue). Non-chemokine ligands are reported in grey. Figure is adapted from Borroni *et al.*, 2018.

Abbreviations: RBC, red blood cell; LEC, lymphatic endothelium; DC, dendritic cell; MIF, macrophage migration inhibitory factor.

A key structural factor that distinguishes cCKRs and ACKRs is the presence of shared peptide motifs, which are also conserved throughout vertebrate evolution. The amino acid sequence DRYLAIV (Asp-Arg-Tyr-Leu-Ala-Ile-Val), or slight variations of that sequence, is found at the end of the third transmembrane domain (TM-III) and that is part of the second intracellular loop (ICL-2). It plays a vital role in coupling cCKRs to intracellular signal transduction pathways following chemokine engagement. The absence of signal transduction after chemokine engagement to ACKRs has been attributed to marked alterations of the DRYLAIV motif in these receptors (Nibbs and

Graham, 2013). However, substitution of the DRYLAIV motif in ACKRs does not restore chemokine responsiveness, suggesting that additional chemokine receptor residues are required for canonical chemokine receptor signalling.

1.3.3 Chemokine Receptor Signal Transduction and Functional Responses

Considering the number of chemokines, compared to the number of receptors identified to date, it is easy to presume that there is a great amount of promiscuity within this system. Chemokine receptors are responsible for translating activation due to chemokine binding to cellular responses. It will take considerable time before the molecular interactions responsible for matching 19 chemokine receptors and 50 chemokines are fully explained, because these interactions likely differ between each receptor-ligand pair. Nevertheless, a key response, namely chemotaxis, is a common outcome of the interaction of all chemokines with all chemokine receptors. Research over the past years has identified many of the molecular events that can lead from chemokine-receptor interaction to cell movement.

The proposed model of interaction between chemokines and their receptors is established in the context of a 1:1 stoichiometric complex. A two-step model of chemokine receptor activation was initially proposed (Monteclaro and Charo, 1996; Crump, 1997). According to this model, firstly the globular domain of the chemokine binds to the extracellular amino-terminal extension and second extracellular loop of its receptor with high affinity (Monteclaro and Charo, 1996). Secondly, once the chemokine is tethered to its cognate receptor, its unstructured N-terminus enters the receptor's heptacelical domain to induce a conformational change. Specifically, the N-terminal portion of the chemokine prior to the first cysteine interacts with certain residues in the ligand-binding pocket, which is buried within the extracellular loops within the transmembrane domains of the receptor (Nygaard *et al.*, 2009). However, accumulating evidence suggests that both interaction sites can be physically and allosterically linked and that additional interactions between the receptor and chemokine are possibly required to ensure full receptor activation (Kleist *et al.*, 2016). Regardless of the complexity of this interaction, it is established that it causes an overall conformational change in the receptor, demonstrated by the reorganisation of the seven α-heptacelices–containing transmembrane bundle (Govaerts *et al.*, 2003). This structural

change of the receptor represents the switch from an inactive to an active state, based on its ability to couple to downstream signalling molecules.

Upon activation, GPCRs act as guanine nucleotide exchange factors (GEFs) by catalysing the exchange of GDP for GTP on the Gα subunit. This leads to the dissociation of the GTP-bound Gα subunit and the Gβγ subunits from the chemokine-activated receptor. The diverse signalling cascades and effector molecules involved in chemokine receptor signalling are comprehensively reviewed elsewhere (Mellado, Rodríguez-Frade, Mañes, *et al.*, 2001; Ritter Hall, 2009; and Patel, Channon and McNeill, 2013)**.** The dissociated GTP-bound α subunits can result in the activation of calcium channels and modulation of adenyl cyclases and cyclic adenosine monophosphate (cAMP) (Kehrl, 1998). The Gα-GTP subunit is then hydrolysed and promotes the reassembly and regeneration of the inactive heterotrimeric G-protein. Both the Gα and Gβγ subunit also trigger calcium mobilisation through the activation of phospholipase C-β (PLC-β), which catalyses the conversion of phospatidylinositol-1,4,5-triphosphate (PIP_3) into inositol triphosphate (IP_3) and diacylglycerol (DAG), which then triggers activation of protein kinase C (PKC). Both Gα and Gβγ subunits can also activate phosphoinositide 3-kinase (PI3K), which results in the activation of the kinases Akt and the mitogen-activated protein kinases (MAPKs). The Gβγ complex appears to be responsible for regulating cell migration (Neptune and Bourne, 1997). Activated chemokine receptors also recruit GPCR kinases (GRKs), which phosphorylate serine and threonine residues mainly in the C-terminus of the receptor (Legler *et al.*, 2017). Subsequently, β-arrestin proteins bind with high affinity to phosphorylated GPCRs and function in desensitisation of the receptor by two processes. Firstly, arrestin binding to the phosphorylated receptor blocks G-protein and receptor interaction, therefore leading to termination of signalling by G protein effectors (desensitisation) (Ritter and Hall, 2009). Secondly, it targets the GPCR to clathrin coated pits, within minutes of chemokine binding and subsequent internalisation of chemokine-receptor complexes. Chemokines are mostly degraded in the endosomal/lysosomal compartment whereas many of the chemokine-free receptor recycle back to the cell surface to be ready for the next round of activation (Luttrell and Lefkowitz, 2002). A summary of the signalling cascades and molecules involved in activation of chemokine receptors by chemokines is shown in **Figure 1.5**. Differences in

the internalisation process are also seen with various chemokines. For instance, both CCL19 and CCL21 are able to induce β-arrestin recruitment to CCR7 and stimulate extracellular signal-regulated kinase 1/2 activation, but only CCL19 promotes efficient CCR7 internalisation (Otero, Groettrup and Legler, 2006; Otero *et al.*, 2008; Zidar *et al.*, 2009).

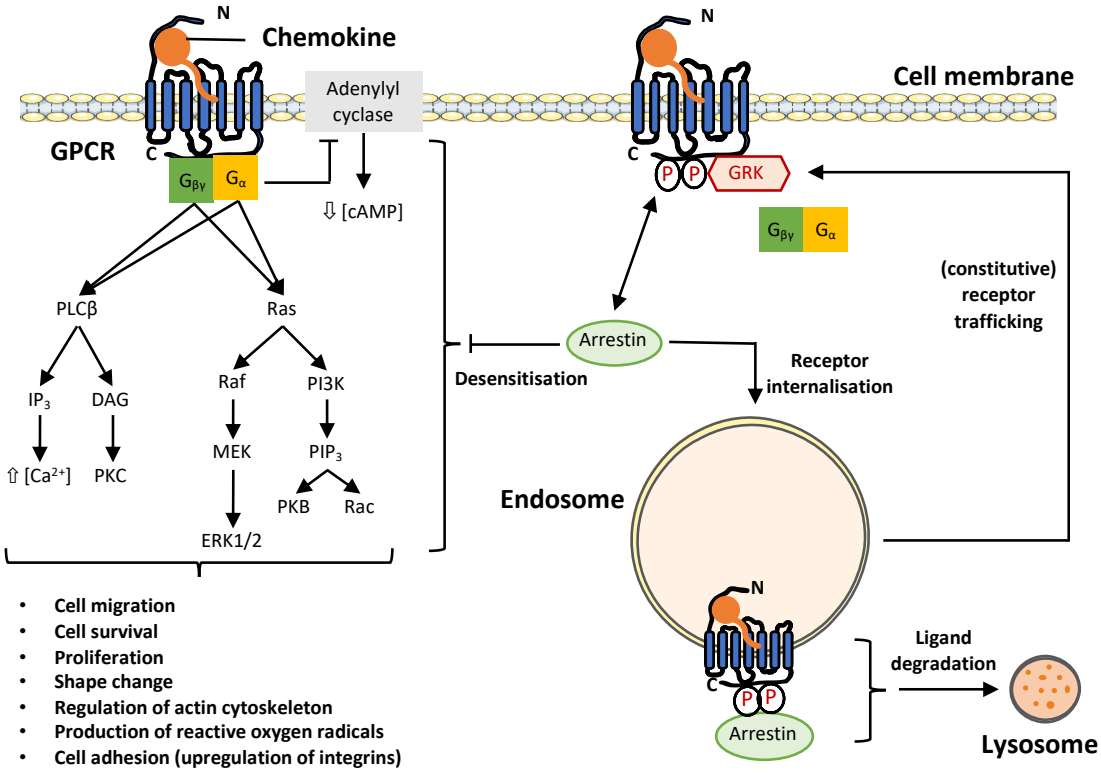


Figure 1.5. Signalling cascade and molecule activation following chemokine binding to its cognate receptor.

Chemokine receptors are G-protein coupled receptors; therefore chemokine-induced signalling is G-protein dependent. Binding of a chemokine to its cognate receptor induces a conformational change of the receptor's transmembrane domain, thereby facilitating its binding to a heterotrimeric G-protein. GDP to GTP exchange at the nucleotide binding site of the G_α subunit mediates inhibition of adenylyl cyclase, resulting in decreasing cyclic adenosine monophosphate (cAMP) concentrations. The $G_{\beta\gamma}$ subunit of the G-protein activates phospholipase Cβ (PLCβ), resulting in initiation of downstream pathway activation, leading to calcium release from the endoplasmic reticulum. Additionally, $G_{\beta\gamma}$ interacts with Ras, activating additional downstream pathways. Ultimately, modulation of actin-dependent processes regulates various leukocyte functions and initiates chemotaxis. In addition to G-protein-dependent chemokine signalling, some chemokine receptors are phosphorylated by G protein-coupled receptor kinases (GRK), which leads to arrestin recruitment. This uncouples the receptor from its G-protein, thereby attenuating further receptor signalling (desensitisation). Furthermore, arrestin interaction leads promotes internalisation of the receptor to endosomes and ligand degradation, leading to initiation of an additional round of cell signalling, or receptor recycling to the cell membrane. Figure adapted from Metzemaekers *et al.*, 2016.

Ongoing research on the chemokine/receptor interaction shows that many variables can influence the functional outcome. It was initially described that the length and amino acid composition of the chemokine N-terminus determines to which receptor(s) it will bind, the affinity with which it will bind, and the functional outcome of the interaction (Clark-Lewis *et al.*, 1995). Ligands are therefore classified into inverse, partial, full agonists or antagonists (Christopoulos, 2014). Furthermore, CXC chemokines can be further classified according to the presence of the tripeptide motif ELR (glutamic acid-leucine-arginine) in the N-terminal region immediately preceding the first cysteine residue. ELR-containing chemokines are specific for CXCR1 and/or CXCR2, which are expressed on myeloid cells. In contrast, ELR-negative chemokines attract a variety of leukocytes by interacting with multiple chemokine receptors, but not CXCR1/2. Posttranslational modifications, including proteolysis can affect both the NH_2 and the COOH terminal region of chemokines and result in either decreased or increased activity of chemokines, or even altered receptor specificity (Mortier, Van Damme and Proost, 2008). Additional modifications, such as citrullination, nitration and glycosylation that modify their activity have been detected on natural chemokines (Struyf, Proost and Van Damme, 2003; Metzemaekers *et al.*, 2016). It has recently been shown that lipid composition proximal to the receptor and the membrane environment of downstream signalling molecules play a role in its function (Legler *et al.*, 2017). Adding to the complexity of this system, biased signalling of GPCRs has been described, where signalling bias can depend on the ligand, the receptor, or the cellular context of the receptor (Hauser *et al.*, 2016; Karin, Wildbaum and Thelen, 2016).

1.3.4 Functional Responses Following Chemokine Binding

The biological outcomes following chemokine binding their receptors are multiple and diverse and have been substantially studied *in vitro* and *in vivo.* By far the most studied function of chemokines is, as the name implies, their ability to induce migration of cells expressing the corresponding receptor towards areas of higher chemokine concentration. Apart from chemotaxis, different types of cell movement are also regulated by chemokine/receptor interaction, including haptotaxis, chemokinesis, haptokinesis and transcellular migration (Hughes and Nibbs, 2018). Cells stimulated by chemokines assume a polarised morphology by creating a wide pseudopod at the

leading edge and a tail-like projection at the trailing end (uropod) (Stossel, 1994). Re-structuring of cytoskeletal fibres and actin polymerisation at the pseudopod, together with retraction of the uropod leads to cell locomotion. Other processes can cause these responses, but chemokine-induced movement is gradient-imposed and unidirectional. Other short-term effects of chemokine activation include induction of integrin-mediated adhesion. This process facilitates adhesion and ultimately arrest of cells on the endothelium, leading to transendothelial migration from vessels to tissues (extravasation) during homeostatic and inflammatory conditions.

A wide variety of other biological processes can be induced in activation of chemokine receptors by their corresponding ligand. Early studies on CXCL8 showed that chemokines can induce a variety of functions in human neutrophils including respiratory burst, degranulation and bactericidal protease release (Baggiolini, Walz and Kunkel, 1989; Proost *et al.*, 1993). More recently, chemokines were shown to induce NET formation (Pang *et al.*, 2013; Hazeldine *et al.*, 2014). In addition to short-term effects, chemokine/receptor interaction leads to the activation of long-term responses, including changes in gene expression controlling leukocyte differentiation, proliferation, cytokine expression and survival (López-Cotarelo *et al.*, 2017). Chemokine responses are also implicated in cancer, through induction of angiogenesis, tumour growth and metastasis (Strieter *et al.*, 1995; Zlotnik, 2006; O'Hayre *et al.*, 2008). Which signalling cascade(s) and ultimately which cellular responses are triggered, depends on the chemokine/receptor pair engagement. Recent research into the inflammatory receptors CCR1/2/3/5 showed redundancy in resting cell recruitment to the skin but specificity of receptor use in recruitment of myelomonocytic cells to acutely inflamed sites (Dyer *et al.*, 2019). Similar evidence is clear with CCR5, which is missing in some individuals. However, other receptors such as CXCR4 are essential for survival (Murphy *et al.*, 2000).

A variety of techniques is routinely used to assess chemokine function, based on various signalling mediators activated downstream of GPCR activation. Initially, radiolabelled chemokines were used to assess chemokine binding to cells expressing their corresponding receptor. However, that method is now more difficult to use due to the safety hazards associated with it, as well as the availability and high costs of disposing of the starting material. Alternatively, fluorescently labelled chemokines have been used

to asses binding (Purvanov *et al.*, 2018). Other signalling mediators downstream of GPCRs that can be measured include calcium mobilisation, reactive oxygen release, PI3K, cAMP and many others. Cell shape change, by measuring actin polymerisation, can also be assessed following chemokine activation. Detection of β-arrestin recruitment is used to asses activation of GPCRs, but also for the discovery of novel ligands for orphan GPCRs (Southern *et al.*, 2013). β-arrestin-mediated receptor internalisation can be measured using fluorescently labelled antibodies against the receptor, as well as tracking of endosomes or lysosomes following chemokine binding to the receptor. Although these techniques are reliable means of assessing chemokine/receptor interaction, the most fundamental function induced by chemokines is cell migration. Cell migration is a result of the activation of a multitude of signalling pathways downstream of GPCRs. Chemotaxis is most commonly measured using transwell migration assay systems or Boyden chambers. To mimic physiological conditions, pores in transwell plates can be coated with extracellular matrix, such as collagen.

It is therefore clear that, although all conventional chemokine receptor on the surface of cells are able to induce cell migration, the signalling mechanisms downstream of the receptors are not shared. Instead, a complex network of signalling mediators controls biased signalling, signal specificity and promiscuous signalling. This is paired with chemokine scavenging and presentation, to ultimately promote chemokine-induced cell migration.

1.3.5 Chemokine-controlled Immune Cell Responses

Immune cell localisation is of critical immunological importance. Specifically, leukocytes need to be at the right place at the right time, for their immunological functions to be suitably localised and directed. Chemokines are the primary controllers of leukocyte migration and localisation during immune cell development and homeostasis, as well as generation of humoral and cellular immune responses and recruitment of effector cells in disease (Griffith, Sokol and Luster, 2014). Based on the function they exert and the context in which they function, chemokines are divided into two functional subsets: inflammatory and homeostatic chemokines. **Inflammatory chemokines** are normally not expressed under steady state conditions and are upregulated in response to proinflammatory stimuli such as cytokines (e.g. TNFα and IL-1β) and microbial products

(e.g. LPS and alarmins). These chemokines regulate recruitment of a host of effector cells in an effort to ultimately restore homeostasis. The inflammatory profiles of diseased tissues typically include chemokines that bind to the promiscuous cCKRs (**Table 1.1**), including CXCL1 and CXCL8 which bind to CXCR1 and CXCR2, respectively. CXCL1 and CXCL8 play a role in the initiation of inflammatory responses by controlling the recruitment of neutrophils. Inflammatory chemokines are also involved in the later stages of inflammatory immune responses, including the initiation of adaptive immune responses. For example, CXCR3 ligands CXCL9, CXCL10 and CXCL11 are involved in the differentiation and recruitment of effector T cells (Griffith, Sokol and Luster, 2014). The apparent receptor/ligand promiscuity in the inflammatory group of chemokines has most likely evolved to inhibit microbial subversion by establishing robust leukocyte responses during infection. Such mechanisms are used by HIV, which has evolved to exploit chemokine receptors, namely CXCR4 and CCR5 as coreceptors mediating viral entry into cells (Mellado, Rodríguez-Frade, Vila-Coro, *et al.*, 2001). Receptor antagonism by Maraviroc or genetic variation of the Δ32-*CCR5* allele results in slower progression to AIDS (Arenzana-Seisdedos and Parmentier, 2006; Woollard and Kanmogne, 2015). On the other hand, homeostatic chemokines exhibit much less redundancy, with each ligand usually binding a single receptor. **Homeostatic chemokines** control cell migration during steady state, which requires continuous trafficking of leukocytes out of the bone marrow and circulation into and out of other tissues of the body. This occurs in concert with orchestration of immune responses by inflammatory chemokines. In the absence of chemokine-controlled leukocyte migration, immune surveillance fails, and protective immune responses are weakened. Nevertheless, chemokine-driven migration can also lead to autoimmunity, cancer cell metastasis, allergy, chronic inflammatory diseases and many others. In that aspect, targeting chemokines can have therapeutic potential.

1.3.5.1 Chemokines in homeostatic immune responses

Primary Lymphoid Organs

Immune cell precursors develop and differentiate within the primary lymphoid organs, which include the bone marrow and thymus, under the precise control of chemokines. Within the thymus, T cell development is controlled by the homeostatic chemokine receptors CCR4, CCR9 and particularly CCR7, which regulate the entry, distribution and

exit of T cells within the thymus (Hughes and Nibbs, 2018). Within the bone marrow, HSCs are retained within the tissue under the control of CXCL12, which is produced by bone marrow stromal cells (Ara *et al.*, 2003). CXCL12 is one of the most primitive chemokines and has been strongly conserved through evolution. It is critical for the development of multiple organs and immune systems, shown by deletion of either *Cxcl12* or *Cxcr4* resulting in a variety of developmental abnormalities and death *in utero* (Nagasawa *et al.*, 1996; Zou *et al.*, 1998). Blockade of CXCR4 function by AMD3100 (Plerixafor), which is used clinically to mobilise HSCs into peripheral blood for collection prior to autologous stem cell transplantation (Bilgin and De Greef, 2016). The CXCL12/CXCR4 axis is also important for development of a range of immune cell lineages, including B cells, monocytes, neutrophils, macrophages, NK cells and pDCs (Mercier, Ragu and Scadden, 2012). During maturation, neutrophils downregulate CXCR4 from the surface, thereby allowing their egress from the bone marrow into blood and peripheral tissues, although other chemokines may also be involved (Suratt *et al.*, 2004). For instance, CCR2 expression on monocytes is required for their mobilisation from the bone marrow into blood, as well as recruitment to inflammatory sites (Boring *et al.*, 1997; Tsou *et al.*, 2007; Shi and Pamer, 2011).

Secondary Lymphoid Organs

Secondary lymphoid organs (SLOs) include the lymph nodes, spleen and Peyer's patches in the gut. During embryonic life, lymphoid tissue inducer cells migrate out of the blood into sites where SLOs will form. This process, as well as maintenance of SLO architecture and recruitment of cells to these sites is regulated by homeostatic chemokine binding to CXCR5 and CCR7 (Griffith, Sokol and Luster, 2014). In mature SLOs, follicular DCs in the B cell follicles produce CXCL13, which maintains the homeostatic localisation of B cells expressing CXCR5 (Legler *et al.*, 1998). Within the spleen, marginal zone B cell localisation and retention is controlled by ACKR3 (Wang *et al.*, 2012). In the T cell area, fibroblastic reticular cells (FRCs) produce CCL19, CCL21 and CXCL12, which promote entry and localisation of T cells and DCs through CCR7 and CXCR4 (Wang *et al.*, 2012). In order to mount successful immune responses, naïve T cells must meet with APCs, which will present them with a cognate antigen. Following antigen capture, DCs undergo maturation. During maturation, they downregulate most of their initial chemokine

receptors and upregulate CCR7 in order to migrate to SLOs through afferent lymphatics, which present CCL21 and CCL19 (Martín-Fontecha *et al.*, 2003). These chemokines are also responsible for positioning DCs within the T cell areas in the cortex (Gunn *et al.*, 1999). Most naïve T cells express several essential traffic molecules, including the adhesion molecules L-selectin, LFA-1 and α4β7 integrin, as well as the chemokine receptors CCR7 and CXCR4 (Griffith, Sokol and Luster, 2014). These molecules are responsible for their trafficking only within lymphoid tissues and no other regions of the body. Naïve T cells enter lymph nodes via HEVs, which present CCL19 and CCL21 on the luminal endothelium. CXCL12, which is produced by FRCs and is transcytosed across the HEV to be presented on the luminal surface, also plays a role in this process. Once in the lymph node, T cells follow CCL19 and CCL21 gradients into the T cell area (Masopust and Schenkel, 2013). Here, naïve T cells scan DCs for antigen via their T cell receptor (TCR). The amount of time T cells spend within lymph nodes is controlled by two factors. Sphingosine-1-phosphate (S1P) attracts T cells and is expressed in the blood and lymph but is absent from the T cell zone of lymph nodes and splenic white pulp. As T cells enter the lymph node, S1P induces desensitisation of the S1P receptor 1, leading to accumulation of T cells (Cyster, 1999). At the same time, prolonged exposure to CCL19 eventually leads to downregulation of the receptor and therefore loss of CCR7-mediated retention signals. Sensitivity to S1P originating in efferent lymph is concurrently increased, gradually leading to T cell egress via the lymphatics. T cells eventually return to lymph and blood, where CCR7 expression is restored in order to continue recirculation within SLOs in search for a cognate antigen (Masopust and Schenkel, 2013).

Peripheral Tissues

Cellular immunity occurring under steady state conditions in the periphery is thought to be controlled by a complex network of migratory cues elicited by chemokines. During immune surveillance immune cells patrol the body's tissues scanning for and destroying invading microorganisms that could cause an infection, as well as eliminating transformed cells to prevent cancer. Effective immune surveillance requires localisation of immune cells throughout the body, not just in lymphoid organs. Constitutive and tissue specific expression of chemokines is thought to play an important role in this process. Much of the research regarding peripheral immunity under homeostasis has

focused on resident T cell populations. Our group has identified CCR8 as a marker for skin-resident T cell populations, with approximately half of all human skin $CD4^+$ and $CD8^+$ T cells expressing CCR8 on their surface (McCully *et al.*, 2018). $CCR8^+$ T cells are rare in the blood and completely absent from the gut, indicating that $CCR8^+$ T cells are specific for healthy skin (Schaerli *et al.*, 2004; McCully *et al.*, 2012). Making this notion stronger is the fact that CCL1, the ligand for CCR8, is produced by human skin LCs in the epidermis and dermal perivascular cells (Schaerli *et al.*, 2004). More recently, it was shown that CCR8 distinguishes a subset of T cells within the skin with a "long-lived memory" that have been termed T_{RM} (Gebhardt *et al.*, 2009; McCully *et al.*, 2012). These cells have been identified both in human and murine healthy skin, suggesting that they have an important role in immune surveillance. Our group has shown that skin-specific factors produced by keratinocytes under steady-state conditions, including prostaglandin E2 (PGE_2) and the active vitamin D metabolite 1,25 -dihydroxy vitamin D3 ($1,25(OH)_2D3$), can induce CCR8 upregulation on activated naïve T cells (Islam *et al.*, 2011; McCully *et al.*, 2012). Further research into $CCR8^+$ skin T cells showed that they are clonotypically distinct, stable *in vitro* and show similar levels of telomere erosion as $CCR8^-$ cells, suggesting that there is a nonlinear differentiation pathway for skin T_{RM} cells. Other chemokine receptors have been implicated in the recruitment of effector T cells to the skin during inflammatory conditions, including CCR4, CCR6 and CCR10. The CCR10-CCL27 axis is important in recruiting effector T cells to the inflamed skin, but there is also evidence of its involvement in T cell traffic control under steady state conditions (McCully, Kouzeli and Moser, 2018). Similarly, CCR6 ligand CCL20 is present at low levels in healthy skin but is massively upregulated during inflammation, in addition to contributing to the maintenance of memory γδT17 cells in healthy skin and local lymph nodes (Hartwig *et al.*, 2015; Ramírez-Valle, Gray and Cyster, 2015; Zhang *et al.*, 2016). Other chemokine receptor-ligand pairs that play a role in homing in peripheral tissues include CXCR6, CCR6 and CCR9 and their ligands, which are implicated in recruitment of cells in the skin and small intestine under inflammatory conditions (McCully, Kouzeli and Moser, 2018). Of note, the sole ligand of CCR9, CCL25, is prominently expressed in intestinal epithelia in the steady state but is further upregulated under inflammatory conditions. Although in conjunction with the α4β7 integrin it is involved in T cell homing

to the gut, it is still not fully clear how CCR9 contributes to the immune surveillance traffic in the intestine (Iwata *et al.*, 2004; Marsal and Agace, 2012).

In addition to CCL1, CXCL12 and CXCL14 are the only other homeostatic chemokines that are highly expressed in human skin (Pablos *et al.*, 1999; Meuter and Moser, 2008; McCully and Moser, 2011). CXCL12 primarily controls the positioning of bone marrow and thymic progenitor cells during immune cell development, but is also involved in embryonic tissue development and wound healing (Pablos *et al.*, 1999; Nagasawa, 2014; Luo *et al.*, 2016). Recent findings revealed that the atypical chemokine receptor ACKR3 can internalise and degrade extracellular CXCL12, thereby controlling homeostatic cell traffic (Graham *et al.*, 2012). Similar to CXCL12, CXCL14 expressed in developing organs of embryos from multiple species (Gordon *et al.*, 2011; Ojeda, Munjaal and Lwigale, 2013). Its targets include blood monocytes, while lymphocytes fail to exhibit a response (Kurth *et al.*, 2001). CXCL14 is unique among other homeostatic chemokines as it is highly expressed in the periphery but absent from primary and secondary lymphoid organs. Its expression profile thus suggests that it may play a role in maintaining tissue resident populations contributing to immune surveillance in healthy peripheral tissues. In depth characterisation of the target cells in peripheral tissues has been problematic because its receptor has remained elusive.

Below, I will summarise what is currently known about CXCL14, including its structural properties, patterns of expression and activity on immune cells. I will then outline the aims of this project and the questions regarding CXCL14 that I have sought to answer during my PhD.

1.3.6 Chemokine (C-X-C Motif) Ligand 14

1.3.6.1 Structural Properties

CXCL14 is also known as breast and kidney-expressed chemokine (BRAK), B cell-and monocyte-activating chemokine (BMAC) and macrophage inflammatory protein 2-gamma (MIP-2γ). It was one of the last chemokines to be discovered (Hromas *et al.*, 1999; Frederick *et al.*, 2000; Sleeman *et al.*, 2000). Initially, CXCL14 is expressed as a 99-amino acid pro-peptide, of which 22 amino acids are cleaved from the NH_2-terminus to produce a full-length protein consisting of 77 amino acids **(Figure 1.6).** In comparison to

other members of the α-chemokine family and specifically the CXC chemokines, CXCL14 does not possess the ELR motif in its N-terminal region. In fact, compared to other chemokines, CXCL14 has an unusually short N-terminal amino acid sequence of only two amino acid residues (Ser-Lys) prior to the first cysteine residue. N-terminal residues have been shown to be important for receptor activation and most other chemokines contain five or more amino acid residues in their N-terminal region (Clark-Lewis *et al.*, 1994). Comparison of CXCL14 with other family members reveals another unique characteristic of CXCL14. Namely, CXCL14 contains a unique sequence of five amino acids (^{41}VSRYR^{45}) within the 40s loop not seen in any other CXC chemokines. This amino acid insertion does not alter its canonical chemokine fold, but instead is essential for proteasomal degradation of CXCL14 in cancer cells (Peterson *et al.*, 2006). Despite these atypical structural characteristics, the amino acid sequence of CXCL14 is highly conserved across different species, including mammals, birds and fish (**Figure 1.7**). In particular, human and mouse CXCL14 differ by only two conserved amino acid substitutions (Ile_{36} -> Val_{36} and Val_{41} -> Met_{41}) (Wolf and Moser, 2012). CXCL12 is the only other chemokine that exhibits such conservation throughout evolution (Nagasawa, 2014). Both CXCL14 and CXCL12 are considered as evolutionarily ancient chemokines because of their sequence conservation throughout different vertebrate classes (**Figure 1.7).** Interestingly, there is evidence that CXCL14 and CXCL12 can affect each other's activity and our group has specifically shown that CXCL14 can synergise with CXCL12 (Sadler, 2008; Tanegashima, Suzuki, *et al.*, 2013; Tanegashima, Tsuji, *et al.*, 2013; Collins *et al.*, 2017).

Figure 1.6. Sequence alignment of CXCL14 and selected other CXC chemokines.

Comparison of CXCL14 sequence with other chemokines by alignment reveals unique features of CXCL14. Evident is the lack of the ELR motif, but most importantly, the short N-terminal region of CXCL14 consisting of only two amino acids prior to the first cysteine residue. The five-residue sequence unique to CXCL14 (VSRYR) is highlighted in orange. The four conserved cysteine residues that are characteristic of all CC and CXC chemokines are highlighted in green. Connecting brackets indicate the disulfide bridge formed between the highlighted cysteine residues. Other residues shared between CXCL14 and most or all the other chemokines are highlighted by an arrow. Sequence alignments were performed using the Clustal Omega multiple alignment tool. Figure adapted from Collins, 2016.

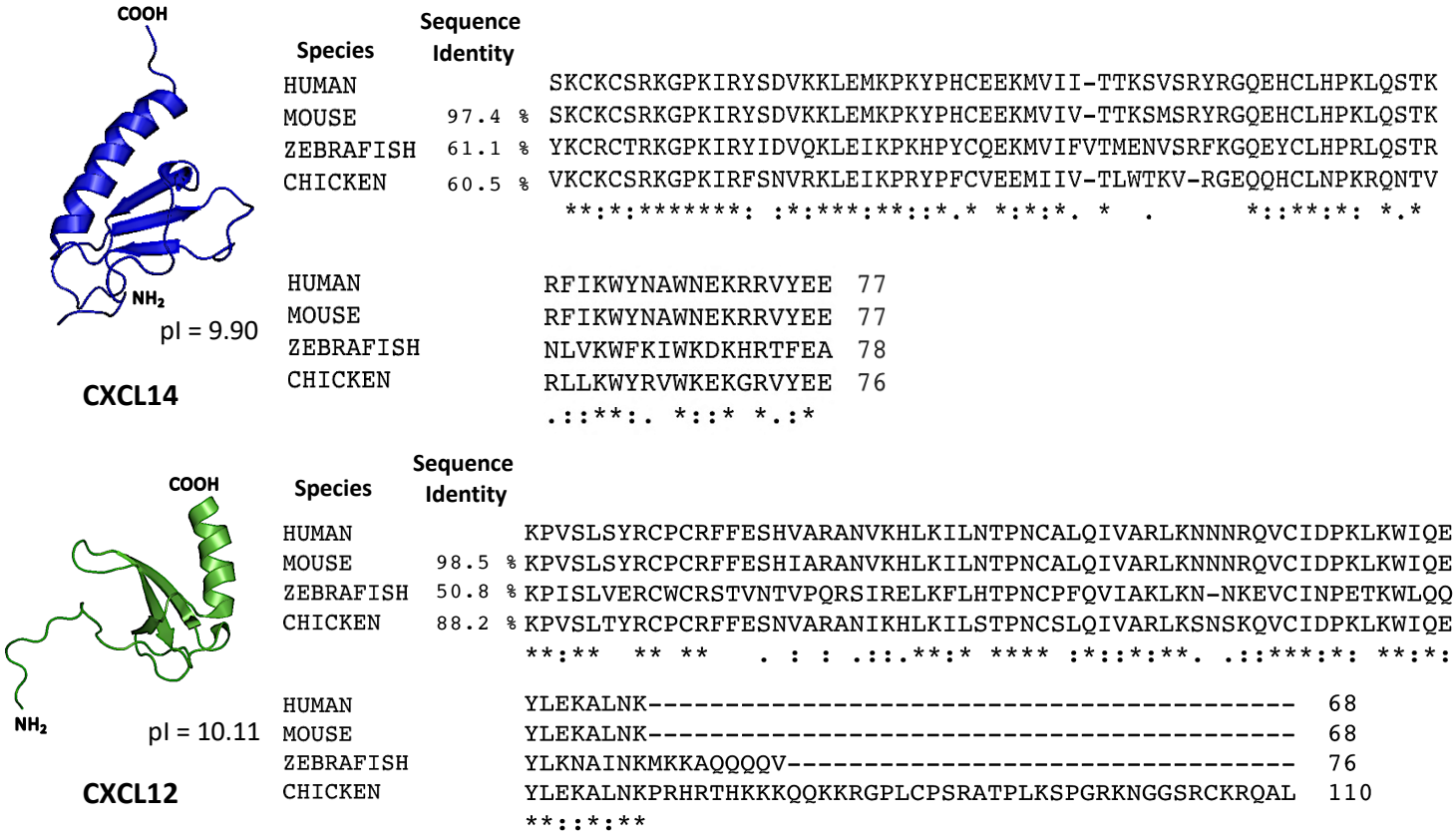


Figure 1.7. Structure, peptide sequence and conservation among species of CXCL14 and CXCL12.

(a) Mature human CXCL14 is a 77 amino acid peptide consisting of all the hallmark structures of a chemokine, including three anti-parallel β-strands and a C-terminal α-helix. Notably, it has a high isoelectric point (pI = 9.90), which is consistent with its function as an antimicrobial peptide. **(b)** Human CXCL12 is a 68 amino acid peptide that has similar structure to CXCL14. Both CXCL14 and CXCL12 show a remarkable degree of sequence similarity throughout evolution. Sequence alignments were performed using the Clustal Omega multiple sequence alignment tool. Amino acids conserved between all species are shown by an asterisk. Figure adapted from Wolf and Moser, 2012.

1.3.6.2 Expression in Tissues

The literature investigating the functions of CXCL14 in physiological and pathological conditions is limited, and in many cases contradictory, potentially because no receptor has been unequivocally identified. CXCL14 was first isolated from human breast and kidney cells, which is where the name BRAK originated (Hromas *et al.*, 1999). Early studies revealed that CXCL14 transcripts were abundant in various human tissues under steady state conditions, including the skin, intestine, pancreas, heart, brain, placenta, liver, skeletal muscle and breast (Hromas *et al.*, 1999; Cao *et al.*, 2000; Frederick *et al.*, 2000; Kurth *et al.*, 2001). This expression profile of CXCL14, along with its absence from secondary lymphoid organs (Meuter and Moser, 2008) suggests that CXCL14 plays a role in cell trafficking under homeostatic conditions. In skin, CXCL14 protein is highly expressed in healthy human epidermis and scattered cells of the dermis. In the epidermis, its expression is much higher than any other chemokine that is constitutively expressed at these sites, including CCL1 and CXCL12 (Schaerli *et al.*, 2005). It has been demonstrated that the sources of CXCL14 within the epidermis include basal keratinocytes as well as more differentiated keratinocytes in a suprabasal location (Frederick *et al.*, 2000; Schaerli *et al.*, 2005). Within the dermis, its expression is more scattered, and it is mostly associated with blood vessels in the superficial dermal plexus, a site defining leukocyte extravasation. Additionally, it was shown that macrophages, mast cells and potentially fibroblasts are sources of CXCL14 within human dermis, under steady state conditions (Schaerli *et al.*, 2005; Meuter and Moser, 2008). Another study showed increased *cxcl14* gene expression in taste buds of human tongue (Hevezi *et al.*, 2009).

Despite the striking structural homology between mouse and human CXCL14, their sites of expression differ slightly. Similar to human CXCL14, murine CXCL14 is highly expressed in tissues of epithelial origin, including the skin and gastrointestinal tract, while remaining absent from lymphoid tissues (Meuter and Moser, 2008). In contrast to humans, murine CXCL14 is strongly expressed in the lung. Additionally, expression by mast cells within the dermis is not detectable in murine skin. Another study demonstrated murine CXCL14 expression in brain and muscle (Sleeman *et al.*, 2000).

1.3.6.3 Chemoattractant Activity and Target Cells for CXCL14

Numerous published studies have investigated the target cells of CXCL14, yet some of the findings were contradictory. Studies performed by our group demonstrated that CXCL14 is a low potency chemoattractant for human blood monocytes, as well as the human monocytic cell line THP-1 (Kurth *et al.*, 2001; Schaerli *et al.*, 2005). In previous studies from our group, Schaerli et al. used a coculture model employing CXCL14-producing artificial epidermal equivalents attracted $CD14^+$ monocytes to the suprabasal layer. There, they underwent differentiation into Langerhans-like cells, acquiring DC-like morphology and DC and LC markers. Studies of immune pathology in skin show that in mice, monocytes are the precursors for differentiation into LCs (Ginhoux *et al.*, 2006; Ferrer *et al.*, 2019) and these data suggests that the same may be true in human skin. Our group has also shown a role of CXCL14 in macrophage development as CXCL14-producing fibroblasts in the skin co-localised with macrophages (Kurth *et al.*, 2001). Recently, Cereijo et al showed that brown adipocytes are a source of CXCL14, which in turn recruits and polarises local macrophage populations (Cereijo *et al.*, 2018). Other studies have demonstrated that CXCL14 has additional target cells. For instance, it is involved in chemotaxis of immature DCs (Shellenberger *et al.*, 2004; Sadler, 2008), neutrophils (Cao *et al.*, 2000) as well as activated NK cells (Starnes *et al.*, 2006). Contradiction between studies is seen regarding migration of B cells (Sleeman *et al.*, 2000), which were regarded as targets of CXCL14 by these studies, whereas later studies were unable to confirm these initial findings (Kurth *et al.*, 2001). Most other non-ELR CXC chemokines are chemotactic for activated T cells (Bleul *et al.*, 1996; Mokhtar *et al.*, 2009), yet CXCL14 fails to induce chemotaxis of naïve or activated T cells (Cao *et al.*, 2000; Sleeman *et al.*, 2000; Kurth *et al.*, 2001). CXCL14 has also been implicated in trophoblast and NK cell recruitment to the uterus during pregnancy (Kuang, Chen, Fan, *et al.*, 2009; Kuang, Chen, Zhang, *et al.*, 2009; Mokhtar *et al.*, 2009). The target cells and functions of CXCL14 reported in the literature to this date are summarised in **Table 1.2**, while CXCL14 expression in mouse and human is summarised in **Table 1.3.** Despite some efforts, the *in vivo* relevance of these data is yet to be established.

Some studies investigating CXCL14 function have employed the CXCL14 knockout (CXCL14-KO) mouse, which has failed to give further insights into its physiological

functions. According to the Mouse Genome Informatics database, at least eight successful targeting attempts at knocking out *Cxcl14* have been made, four of which have been published. These independent genetically modified mouse cell strains show replicable lower genotype ratios at weaning age, compared to the expected Mendelian ratios (Meuter *et al.*, 2007; Tanegashima *et al.*, 2010; Yajima, Izukuri and Hata, 2010; Dai *et al.*, 2015). Although it remains unclear whether the mortality defect is due to embryonic or perinatal mortality, it may be due to trophoblast attachment inhibition in the early stages of pregnancy (Kuang, Chen, Fan, *et al.*, 2009). Another cause of perinatal mortality of knockout animals could be due to the reduced food intake and impaired ability to adapt to a new environment as observed in adult *Cxcl14* $^{-/-}$ mice (Tanegashima *et al.*, 2010). Data from our group has shown that in viable CXCL14-KO mice no immune phenotype was detected (Meuter *et al.*, 2007). Specifically, macrophage and DC populations in healthy peripheral tissues were not impaired, as well as recruitment of immune cells to inflamed peritoneum and skin wound healing following tissue injury. The survival of some of the animals indicates that there may be functional redundancy, with other chemokine(s) able to compensate for the absence of CXCL14, as often seen in the chemokine system.

Table 1.2. CXCL14 target cells and reported functional effects of CXCL14 in murine and human immunity.

Target cells	Cellular functions	Sources of CXCL14	References
CESS, THP-1	Chemotaxis; Induction of inflammation (Nude mice)	Synthetic murine CXCL14	(Sleeman *et al.*, 2000)
Human neutrophils and DCs	Chemotaxis	Recombinant human CXCL14	(Cao *et al.*, 2000)
Human monocytes (fresh, PGE_2 or forskolin)	Chemotaxis, Ca^{2+} mobilisation	Synthetic human CXCL14	(Kurth *et al.*, 2001)
Human endothelial cells, iDCs	Chemotaxis (iDCs), Inhibition of chemotaxis (endothelial cells)	Recombinant human CXCL14	(Shellenberger *et al.*, 2004)
Human iDCs	Chemotaxis	Recombinant human CXCL14	(Shurin *et al.*, 2006)
Human $CD14^+$ DC precursors	Chemotaxis, Langerhans cell differentiation (*in vitro* skin model	Synthetic and natural human CXCL14	(Schaerli *et al.*, 2005)
Human NK cells, monocyte derived iDCs	Chemotaxis	Synthetic and recombinant human CXCL14	(Starnes *et al.*, 2006)
Human and murine iDCs	Chemotaxis (synergy with activin A)	Recombinant and synthetic human CXCL14	(Salogni *et al.*, 2009)
Human and mouse trophoblasts	Inhibition of cell adhesion and growth	Recombinant human and mouse CXCL14	(Kuang, Chen, Fan, *et al.*, 2009; Kuang, Chen, Zhang, *et al.*, 2009)
Human uterine NK cells	Chemotaxis	Recombinant human CXCL14	(Mokhtar *et al.*, 2009)
Human THP-1 and $CD34^+$ HPCs	Inhibition of chemotaxis of CXCL12	Recombinant human CXCL14	(Tanegashima, Suzuki, *et al.*, 2013)
THP-1 cells (PGE_2)	Chemotaxis	Recombinant human CXCL14	(Dai *et al.*, 2015)

Abbreviations: DC, dendritic cell; iDC, immature dendritic cell; NK, natural killer; HPC, haematopoietic precursor cell; PGE_2, prostaglandin E2; CESS, human B cell line; THP-1, human monocytic leukaemia cell line.

Table 1.3. CXCL14 expression in murine and human tissues and cells.

CXCL14 expression	References
Human	
mRNA (Northern blot): intestine, colon, kidney, liver, spleen, thymus, placenta, brain, pancreas, skeletal muscle, heart, cervix, uterus and breast	(Sleeman *et al.*, 2000)
mRNA (Northern plot): kidney, intestine, brain, placenta, skeletal muscle, liver, spleen, thymus, pancreas testis, ovary, heart, lung	(Cao *et al.*, 2000)
mRNA (Northern blot, in situ hybridisation): skin, kidney, intestine, spleen, colon, muscle, liver, brain, placenta, thymus, breast, exocervix, ovary, heart, squamous epithelium, oral epithelial cells, epidermal keratinocytes, LPS activated B cells/monocytes, carcinoma-adjacent stromal cells	(Frederick *et al.*, 2000)
mRNA (In situ hybridisation, Northern blot): intestinal epithelial layer, kidney, stomach, colon, appendix, trachea, skin keratinocytes, dermal fibroblasts, lamina propria cells in intestine; HaCaT (human keratinocyte cell line)	(Kurth *et al.*, 2001)
Protein (immunohistochemistry): suprabasal layers of tongue mucosa, carcinoma-adjacent stromal cells	(Shellenberger *et al.*, 2004)
Protein (Immunohistochemistry): oral squamous epithelium	(Shurin *et al.*, 2006)
Protein (Immunohistochemistry): blood vessels in dermal plexus and epidermal keratinocytes	(Schaerli *et al.*, 2005)
mRNA (In situ hybridisation): skin macrophages and mast cells	(Meuter and Moser, 2008)
mRNA (Microarray): DCs stimulated with activin A	(Salogni *et al.*, 2009)
Protein (Immunohistochemistry): villous cytotrophoblasts and blood vessels of villous stroma	(Kuang, Chen, Zhang, *et al.*, 2009)
Protein (Immunohistochemistry) and mRNA (In situ hybridisation): glandular epithelial cells in endometrium in secretory phase of menstrual cycle	(Mokhtar *et al.*, 2009)
Mouse	
mRNA (Northern blot): mouse brain, ovary, lung and muscle;	(Sleeman *et al.*, 2000)
mRNA (in situ hybridisation): Epithelium of kidney tubules, liver hepatocytes	(Cao *et al.*, 2000)
mRNA (In situ hybridisation): macrophages in skin, lung, lamina propria of intestine	(Meuter and Moser, 2008)
mRNA (Low-density microarray): DCs stimulated with activin A	(Salogni *et al.*, 2009)
mRNA (In situ hybridisation): embryo implantation sites and uterus epithelium	(Kuang, Chen, Fan, *et al.*, 2009)

Abbreviations: DC, dendritic cell; LPS, Lipopolysaccharide

1.3.6.4 Antimicrobial Activity

Due to certain structural characteristics, chemokines share many properties with antimicrobial peptides (AMPs) that may in part explain their antimicrobial activity. The overall tertiary structure of chemokines including disulfide bonds, anti-parallel β-strands and a C-terminal α-helix are structural elements that are also seen in the defensin family and cathelicidins (Wolf and Moser, 2012). Furthermore, AMPs comprise of high-density positive charges at physiological pH, similar to CXCL14 (**Figure 1.7**). *In vitro* experiments demonstrated that CXCL14 has direct killing activity against both Gram-positive and Gram-negative bacteria, including the skin commensal *Candida albicans* as well as the gut microbe *E.coli* (Maerki *et al.*, 2009). CXCL14 also contributes to killing lung bacterial pathogens both *in vivo* and *in vitro* and CXCL14-deficient mice showed defective clearance of *Streptococcus pneumoniae* pulmonary infection (Dai *et al.*, 2015). Interestingly, a short N-terminal fragment of CXCL14 demonstrated concentration-dependent bacterial killing that was indistinguishable from full-length CXCL14 (Dai *et al.*, 2015). This study, along with CXCL14 expression in the taste buds of the tongue (Hevezi *et al.*, 2009) and the epidermis, gives strong evidence of a role for CXCL14 in antimicrobial immunity. Of note, reduced antimicrobial activity against opportunistic microbes seen in CXCL14-KO mice, which obviously lack CXCL14 protein in epithelial tissues, may be another cause of the observed perinatal mortality.

1.3.6.5 CXCL14 in Disease

Although it is well established that CXCL14 protein is highly expressed in healthy human skin, markedly lower expression levels are observed in psoriatic and atopic dermatitis lesions (Maerki *et al.*, 2009). Moreover, Schaerli *et al* showed that treatment of freshly isolated keratinocytes and cultured dermal adherent cells with the proinflammatory cytokines TNFα and IL-1β resulted in substantial reduction of CXCL14 expression. In contrast, expression of the inflammatory chemokine CCL20 was dramatically increased (Schaerli *et al.*, 2005). These findings provide additional evidence of a role for CXCL14 in tissue homeostasis, as opposed to inflammatory diseases characterised by inflammatory chemokines. However, certain studies have shown that CXCL14 is significantly upregulated in inflamed joints of collagen-induced arthritis and its overexpression exacerbates arthritis in a mouse model (Chen *et al.*, 2010). This evidence is consistent

with studies carried out in individuals suffering from rheumatoid arthritis, showing that CXCL14 is upregulated in the synovial membrane of rheumatoid arthritis patients (Lindberg *et al.*, 2006).

Although numerous studies have implicated CXCL14 in cancer, its exact role remains unknown as its expression is increased in some forms of cancer and decreased in others (Frederick *et al.*, 2000; Schwarze *et al.*, 2005; Ozawa *et al.*, 2006; Shurin *et al.*, 2006; Wente *et al.*, 2008; Augsten *et al.*, 2009). In prostate cancer, CXCL14 has been shown to inhibit angiogenesis, thus dampening tumour growth and metastasis (Schwarze *et al.*, 2005). Furthermore, in head and neck cancer, the rate of tumour formation *in vivo* was significantly lower in CXCL14-transfected tumour cells (Ozawa *et al.*, 2006). In clear contrast, in other studies of prostate cancer, it was shown to be an autocrine growth factor for cancer-associated fibroblasts, promoted the growth of prostate cancer xenografts, increased tumour angiogenesis and macrophage infiltration (Augsten *et al.*, 2009). In breast cancer, CXCL14 expression is induced through the mobilisation of the transcription factor of activator protein-1 (AP-1), which leads to promotion of cancer metastasis through elevation of cytosolic Ca^{2+} release from the endoplasmic reticulum (Pelicano *et al.*, 2009). It has been postulated that loss of CXCL14 expression from tumours may facilitate neovascularisation as CXCL14 has been shown to interrupt *in vivo* angiogenesis by inhibiting endothelial cell migration (Shellenberger *et al.*, 2004). Therefore, loss of CXCL14 would allow a tumour to increase its own blood supply, providing an essential nutrient supply for further growth. Loss of CXCL14 expression by tumour cells may also play a role in the evasion from immune recognition mechanism, by reducing DC attraction and homing to the site of the tumour, since we know that CXCL14 is a chemoattractant for immature DC. Additionally, NK cells were shown to migrate in response to CXCL14, therefore another mechanism of immune evasion by tumour cells could be a reduction of NK cell recruitment in the absence of CXCL14. Therefore, CXCL14 has shown both pro- and anti-tumour functions with sometimes conflicting roles reported for the same type of cancer.

1.3.7 Synergism in the Chemokine Family

During an effort to understand which chemokines are produced in specific circumstances, researchers performed *in situ* experiments. This led to the realisation that a variety of chemokines are simultaneously produced at discrete tissue locations where subsets of immune cells are known to home to (Mazzucchelli *et al.*, 1999; Uguccioni *et al.*, 1999; Agace *et al.*, 2000; Smith *et al.*, 2003; Manzo *et al.*, 2005). How these chemokines affect local immune cells and whether the pattern of immune cell traffic is the result of diverse chemokines working with each other is still a subject of current investigations. Original studies on the interaction of different chemokines revealed that they can antagonise chemokine receptors or synergise with other chemokines (Fulkerson *et al.*, 2004; Petkovic *et al.*, 2004a, 2004b). Following these initial studies, there have been several publications describing functional synergism in the chemokine system both under physiological and pathological conditions. Various mechanisms of synergism occurring at different levels have been proposed and the corresponding models are summarised in **Figure 1.8**.

Although it was once thought that chemokine biology was composed of simple ligand-receptor interaction, it is now evident that the situation is far more complex. When chemokines reach high concentration levels, they can form homodimers or heterodimers and induce different functions to their corresponding receptors (Proudfoot and Uguccioni, 2016). It has been shown that certain chemokines can function as monomers, such as CXCL8, but monomer variants of CCL2, CCL4 and CCL5 were unable to function *in vivo* (Rajarathnam *et al.*, 1994; Proudfoot *et al.*, 2003). Oligomer formation is facilitated by binding to GAGs *in vivo*, as exemplified by CCL5 oligomer formation (Proudfoot *et al.*, 2003). In addition to homo-oligomer formation, there is plenty of evidence of heterocomplex formation of chemokines. The first evidence of such synergism was given by Struyf *et al.*, who showed that Regakine-1 synergises with CCL7 and CXCL8 in the induction of neutrophil migration (Struyf *et al.*, 2001). The same group later showed that CXCL8 can enhance CXCL12 responses, which was CXCR4 mediated (Gouwy *et al.*, 2004).

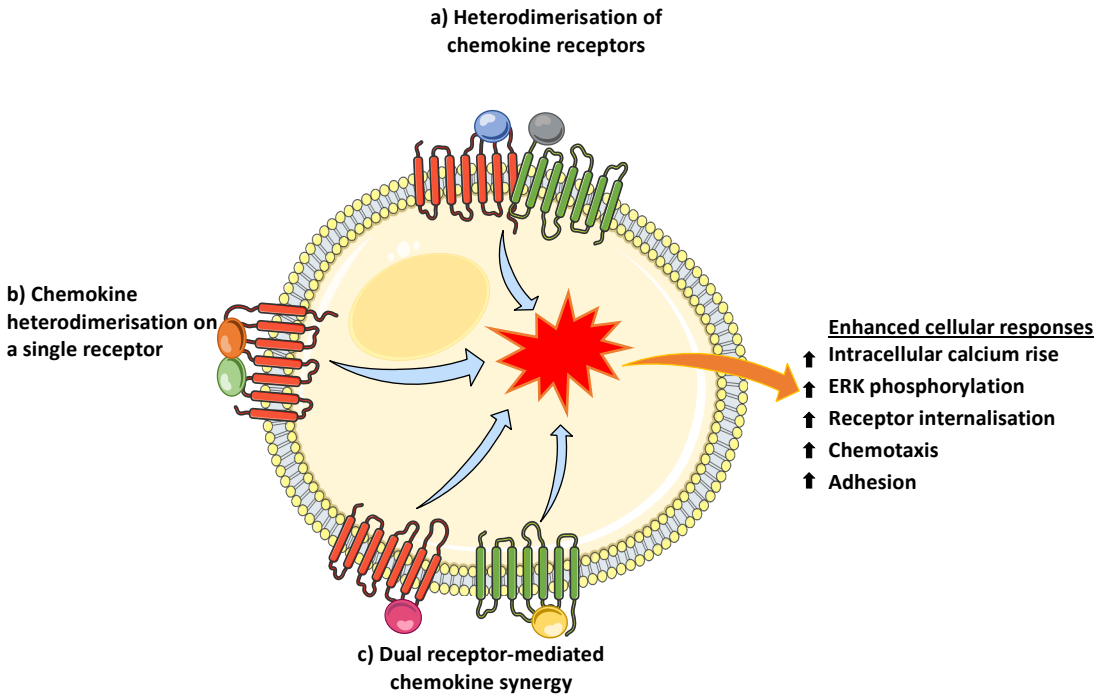


Figure 1.8. Modes of action of chemokine synergy.
There are several mechanisms proposed regarding the modes of chemokine synergism which leads to enhanced leukocyte responses. Responses include intracellular calcium rise, ERK phosphorylation, receptor internalisation, chemotaxis and adhesion. **(a)** Heterodimerisation of chemokine receptors which are activated by their respective ligands simultaneously or sequentially. **(b)** Chemokine heterocomplex formation activating a single receptor. **(c)** Two chemokine receptors are activated by their respective ligands synergise at the level of downstream signalling. This figure has been adapted from Gouwy et al., 2012.

More evidence of chemokine heterocomplex formation followed soon after. Heterodimers of CXCL13 with CCR7 agonists CCL19 or CCL21 activated CCR7 at lower agonist concentrations (Paoletti *et al.*, 2005). Further research into the CCR7 ligands CCL19 and CCL21 showed that they can form heterocomplexes with CCL7 and CCL2, resulting in amplified monocyte responses via CCR2, and preventing CCL7 and CCL2 uptake by ACKR2 (Kuscher *et al.*, 2009). The inflammatory chemokine CXCL10 was found to enhance CCL22-mediated triggering of CCR4 on lymphocytes found in inflamed skin, which was independent from CXCR3 or GAG binding (Sebastiani *et al.*, 2005). Additional studies showed that CCL5-mediated arrest of monocytes on activated endothelium is boosted by interactions with CXCL4. CXCL4 was also shown to synergise with CXCL8, thus increasing the chemotactic capacity of CXCL8 on CXCR1 and CXCR2 transfected cell lines

and enhancing the anti-proliferative effect of CXCL4 on endothelial cells (Nesmelova *et al.*, 2005). The relevance of chemokine heterodimer formation in cancer *in vivo* has also been studied in the tumour vasculature, in lymphomas of the primary central nervous system (Venetz *et al.*, 2010). CXCL9 and CXCL12 form heterodimers, leading to enhanced CXCR4-mediated recruitment of tumour-infiltrating $CD8^+$ T cells and malignant B cells to the perivascular cuffs. Finally, chemokines can also form complexes with non-chemokine mediators. The inflammatory molecule high motility group box 1 (HMGB1) interacts with CXCL12 to enhance CXCR4-mediated signalling, both *in vitro* and *in vivo* (Cecchinato *et al.*, 2016).

Chemokine biology becomes even more complex, as chemokine receptors themselves can form and function as oligomers as well. There is some evidence that some GPCR can function as monomers *in vitro* (Ernst *et al.*, 2007; Kuszak *et al.*, 2009). However, it is assumed that the proper function of GPCR, including chemokine receptors, fully depends on dynamic homo- and oligomerisation on cell surfaces (Thelen *et al.*, 2010). Biophysical techniques such as bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET), detect protein-protein interactions at the cell surface, which facilitates research into the effect of receptor heterocomplexes on their functionality (Issafras *et al.*, 2002). Using BRET analysis, Issafras et al. showed that complexes are formed during synthesis and maturation of the receptors (Issafras *et al.*, 2002). Additionally, CCR5 and CXCR4 heterocomplexes can be found in small trans-Golgi vesicles of macrophages and T cells (Singer *et al.*, 2001), providing more evidence of heterocomplex formation being ligand independent. However, it was later shown that subsequent ligand binding can induce conformational changes to the receptor complex (El-Asmar *et al.*, 2004; Isik, Hereld and Jin, 2008). Evidence of functionality of receptor complexes came from CXCR4 and CCR5 receptor co-internalisation studies in cultured Jurkat T cells (Contento *et al.*, 2008). Additionally, CXCR1 and CXCR2 form heterocomplexes when co-expressed, which are modulated by the presence of CXCL8 (Martínez Muñoz *et al.*, 2009). The functional relevance of receptor heterodimer formation remains controversial. Simultaneous presence of CCL2 and CCL5 triggers formation of homodimers and heterodimers of their cognate receptors CCR2 and CCR5 on PBMCs (Mellado, Rodríguez-Frade, Mañes, *et al.*, 2001). Nevertheless, chemokine

stimulation induces both increased and decreased responses in heterodimers, compared to homodimers (Mellado, Rodríguez-Frade, Vila-Coro, *et al.*, 2001; El-Asmar *et al.*, 2004). Additionally, CCR2-CCR5 heterodimers are only capable of binding a single chemokine molecule with high affinity (El-Asmar *et al.*, 2004). These reports demonstrate that cell surface chemokine receptors are in a homo/heterodimer equilibrium that can be regulated by both ligand and receptor expression levels. Nevertheless, unlike chemokine heterodimerisation, the role played by chemokine receptor heterocomplexes in chemokine synergism has not been fully explained yet.

Chemokine synergism can also occur due to the activation of various signalling pathways downstream of multiple chemokine receptors on the surface of cells. Chemokine receptors on the same cell can be activated simultaneously or even sequentially, following binding to their cognate ligands (Gouwy *et al.*, 2012). Notably, most research on this type of synergy involves the homeostatic chemokine CXCL12 and its receptor CXCR4. Chemokines CXCL8 and CXCL12 significantly enhanced migration of monocytes expressing their respective receptors CXCR2 and CXCR4 towards suboptimal concentrations of CCL2 and CCL7 (Gouwy *et al.*, 2008). Further evidence of synergism between CCR5 ligands and CXCL12 were described for primary cells including the chemotaxis of monocytes, T cells, PHA-activated T lymphoblasts and cord blood cells (Gouwy *et al.*, 2008; Basu and Broxmeyer, 2009). The inflammatory CXCR3 ligands CXCL9 and CXCL11 were also shown to induce enhanced migration of pDCs toward CXCL12, which was CXCR4-mediated (Vanbervliet *et al.*, 2003).

1.4 Hypotheses and Research Questions

CXCL14 remains one of the least understood chemokines despite being known for over 18 years. Our group and others have shown that it is highly expressed in peripheral tissues and a number of cell types have been identified as its target cells. Nevertheless, its function and targets are not fully understood, mainly due to the fact that the cognate receptor remains elusive. Novel developments have shown that CXCL14 also strongly synergises with the homeostatic chemokine CXCL12. The overall goal of this thesis was to better understand the overall functions and targets of CXCL14.

In particular, the research aims I have addressed in my PhD thesis project are:

- To investigate the synergistic function of CXCL14 with respect to other homeostatic and inflammatory chemokines
- To investigate the function of synthetic CXCL14-CXCL12 hybrid molecules
- To identify and characterise CXCL14-binding cells in tissues of mice and humans

Chapter 2 – Materials and Methods

2.1 Chemokines

2.1.1 Chemokines Used in Functional Assays

Human CXC chemokines used in this study were CXCL10, CXCL11, CXCL12, CXCL13 and CXCL14. Human CC chemokines used were CCL2, CCL5, CCL19 and CCL21. All had been chemically synthesised previously according to established protocols (Clark-Lewis, Moser, *et al.*, 1991). The murine chemokine CCL1 and CXCL14 were chemically synthesised by Almac (Craigavon, UK).

Additional human CXCL14 as well as CXCL14-CXCL12 hybrids were chemically synthesised based on fluorenyl methoxycarbonyl (Fmoc) chemistry using an Activo-P11 automated synthesiser (Activotec, Cambridge, UK), as previously described (Loos, Mortier and Proost, 2009)). For this study, full length CXCL14, CXCL14 with the first two amino acids of the CXCL12 N-terminus or all eight amino acids of the CXCL12 N-terminus (**Figure 2.1**) were synthesised.

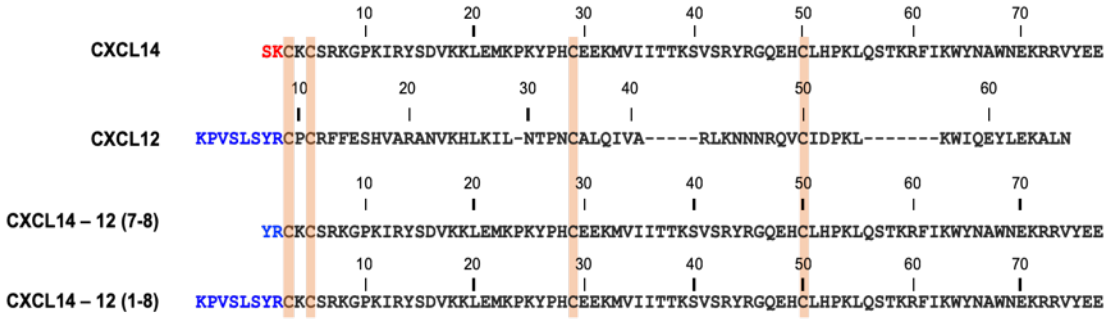


Figure 2.1. Sequence alignment of CXCL14 with CXCL12 and two CXCL14-CXCL12 hybrids.
Full-length CXCL14 was synthesised, as well as two N-terminus variants. The short N-terminal region of CXCL14 was replaced with either two or all eight amino acids from the CXCL12 N-terminus. Proteins were synthesised based on solid-phase synthesis using Fmoc chemistry (**section 2.7**). Structural variants were purified and folded to the correct tertiary structure to test their functionality *in vitro.*

2.1.2 Fluorochrome-labelled Chemokines

A synthetic custom-made version of human CXCL14 conjugated to the fluorochrome Alexa Fluor® 647 (AF-CXCL14) was synthesised by Almac. The AF647 was attached to a C-terminal Lysine (added to the natural Glu at the C-terminal end of CXCL14) via an 8-amino-3,6-dioxaoctanoic acid-Cysteine linker. A custom-made Alexa Fluor® 647-labelled murine CCL1 (AF-muCCL1) was also synthesised by Almac and used in this study.

2.2 Cell Culture Media and Buffers

2.2.1 Media

2.2.1.1 Complete RPMI Medium

The cell culture medium used throughout, unless otherwise stated, was RPMI-1640 medium (Gibco; Paisley; Scotland) supplemented with 10% heat-inactivated foetal calf serum (FCS; Gibco), 50 mg/ml penicillin/streptomycin, 2 mm l-glutamine, 1 mm sodium pyruvate and 1% minimum essential medium non-essential amino acids (MEM-NEAA; all purchased from ThermoFisher Scientific; MA USA).

2.2.2 Buffers

2.2.2.1 Fluorescence-Activated Cell Sorting Buffer

Fluorescence-activated cell sorting (FACS) buffer consisted of sterile phosphate-buffered saline (PBS) supplemented with 2% FCS and 0.02% sodium azide, passed through a 0.22 µm filter prior to use.

2.2.2.2 Magnetic-Activated Cell Sorting Buffer

Magnetic-activated cell sorting (MACS) buffer consisted of PBS supplemented with 2% FCS and 5 mM ethylenediaminetetraacetic acid (EDTA), passed through a 0.22 µm filter prior to use.

2.2.2.3 Chemotaxis Buffer

Plain RPMI-1640 medium was supplemented with 1% human serum albumin (CSL Behring, Bern, Switzerland) and 20 mM HEPES (Gibco).

2.2.2.4 Calcium Buffer

Calcium buffer comprised of 1x Hank's balanced salt solution (HBSS) containing $CaCl_2$ and $MgCl_2$ but no phenol red (Gibco), 10 mM HEPES, and 0.1% FCS, pH 7 and passed through a 0.22 µm filter prior to use.

2.2.2.5 Calcium Staining Buffer

HBSS buffer (no $CaCl_2$, $MgCl_2$ or phenol red) (Gibco), was supplemented with 0.5% human serum albumin and passed through a 0.22 µm filter prior to use.

2.3 Blood Cell Isolation

2.3.1 Isolation of Peripheral Blood Mononuclear Cells

All research requiring healthy human blood and tissue samples was approved by the local Research Ethics Committee and informed consent was obtained from each volunteer.

Healthy peripheral blood mononuclear cells (PBMC's) were collected from heparinised venous blood from local volunteers. Blood was subsequently separated using Lymphoprep (Lymphoprep density gradient separation media; Axis-Shield; Dundee, Scotland). Blood was layered on top of 15 ml Lymphoprep to a total volume of 40 ml and centrifuged at 687 x g at 18°C for 20 minutes with no brake. Mononuclear cells within the buffy coat layer were collected and washed three times with PBS to remove platelets and residual Lymphoprep solution. Cells were resuspended in PBS and counted using a haemocytometer, with trypan blue (Sigma-Aldrich; Gillingham, UK) staining to assess cell viability.

Alternatively, healthy PBMCs were isolated from blood bags supplied by the Welsh Blood Service (Velindre NHS Trust). Blood bags were diluted at a 1:2 ratio using sterile PBS, and PBMCs were isolated similarly using Lymphoprep.

2.3.2 Enrichment of Monocytes from Peripheral Blood Mononuclear Cells

Total monocytes were isolated from PBMC using the pan-monocyte isolation kit (negative selection) (Miltenyi Biotec; Bisley, UK), according to manufacturer's instructions. This kit does not deplete $CD16^+$ cells, as opposed to previous versions. It therefore allows simultaneous enrichment of classical ($CD14^{++}CD16^-$), intermediate

($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$) monocytes. Briefly, cells were incubated with biotin-conjugated monoclonal antibodies (mAbs) (specific antibodies used in this kit are not disclosed by Miltenyi Biotec), followed by incubation with anti-biotin microbeads. Cells were washed with MACS buffer before magnetically labelled non-monocytes were depleted over an LS column using a midi-MACS system. Negatively selected cells were passed through a second LS column to achieve higher purity. Purity of monocytes was assessed using flow cytometry prior to use in assays and ranged from 95-99% of live cells

2.3.3 Enrichment of T Cells from Peripheral Blood Mononuclear Cells

Total $CD3^{+}$ T cells were isolated from PBMC using the Pan-T cell Isolation Kit (negative selection; Miltenyi Biotec), according to manufacturer's instructions. Briefly, PBMC were labelled using a cocktail of biotin-conjugated mAbs against CD14, CD15, CD16, CD19, CD34, CD36, CD56, CD123 and CD235a (Glycophorin A). Labelled cells were then magnetically depleted using anti-biotin microbeads over two consecutive LS columns, as described above for monocyte isolation. Resulting purity ranged from 96-99%.

2.4 Cell Culture

2.4.1 Stimulations

PBMC, purified monocytes or THP-1 cells were stimulated for 1-2 days with Prostaglandin E_2 (PGE_2; Sigma-Aldrich) alone or in combination with Sodium Butyrate (Na-But) (Sigma-Aldrich). PGE_2 was used at a concentration of 1 µM and Sodium Butyrate at a concentration of 1 mM. Cells were cultured in 24- or 48- well plates (ThermoFisher Scientific) in a humidified incubator maintained at 37°C and a mixture of 95% air, 5% CO_2.

2.4.2 T Cell Expansions

T cells were isolated from PBMCs and blasts were generated to express CXCR3, CCR5 and CCR2, according to the protocol described in (Qin *et al.*, 1998). In short, $CD3^{+}$ T cells were magnetically isolated, as described above, and resuspended at 2 x 10^6 cells/ml in cRPMI in 24-well plates. Human T Activator CD3/CD28 Dynabeads® (Invitrogen) were added at a bead/cell ratio of 1:4, in addition to 100 U/ml IL-2 (Proleukin, Chiron). Media and IL-2 was replenished every 3-4 days, up to day 22, as previously described (Loetscher

et al., 1996). Expression of chemokine receptors was monitored regularly using flow cytometry.

2.4.3 Culture of Immortalised Cell-Lines

2.4.3.1 THP-1

THP-1 is a human acute myeloid leukaemia cell-line, purchased from the American Type Culture Collection (ATCC; LGC Standards, Teddington, UK). THP-1 cells cultured in cRPMI supplemented with 50 µM 2beta-mercaptoethanol (2-ME; Sigma-Aldrich). Culture was maintained at a cell density of between 2 x 10^5 and 8 x 10^5 cells/ml.

2.4.3.2 300-19

300-19 cells, a murine pre-B cell line, is established for stable transfection with chemokine receptors in our group and others (Loetscher *et al.*, 1996; Petkovic *et al.*, 2004b). Parental (non-transfected) and stable transfectants were cultured in cRPMI supplemented with 50 µM 2-ME (Sigma-Aldrich). Cultures were maintained at a density of up to 2 x 10^6 cells/ml. Clones of 300-19 cells stably transfected with the human chemokine receptors CCR2, CCR5, CCR7, CXCR3, CXCR4, CXCR5 were used in functional assays throughout this work.

2.4.4 Recovery of Immune Cells from Human Split Skin

Human skin tissue samples (approx. 10 cm^2, 0.4 mm thick) were obtained using a dermatome by a collaborating clinician. Samples were taken from the breast region of breast cancer patients undergoing mastectomy, or from excess skin graft tissue. The skin was cut into 1 cm^2 pieces and partially digested in 50 ml cRPMI (no serum) containing Dispase II (2.5 mg/ml), Collagenase D (1 mg/ml) and DNase I (20 U/ml; all purchased from Roche diagnostics, Burgess Hill, UK) in a sterile Erlenmeyer flask (Corning; Sigma-Aldrich). Tissue was incubated for 15-18 minutes in a shaking water bath maintained at 37°C. Digestion was stopped by transferring the skin fragments to a petri dish containing cold PBS/2 mM EDTA. The epidermis was carefully separated from the dermis using forceps. The dermis and epidermis were cultured separately for 48-72 hours at 37°C in six-well plates, in cRPMI supplemented with 10% human AB serum (Welsh Blood Service). Immune cells that had spontaneously migrated out of the tissue during the 48-72-hour incubation were collected by aspiration. In some experiments, the remaining

tissue fragments were further digested by overnight incubation in medium containing 10% human AB serum and 1 mg/ml collagenase D, causing total disruption of the tissue. This isolated tissue resident cells that do not migrate out of the tissue, including macrophages. Following 48-72-hour culture, single-cell suspensions were obtained by passing the supernatant through a 40 µm cell strainer. Further digested skin fragments were mechanically disrupted using a 5 ml syringe plunger and thoroughly washed with PBS to obtain single-cell suspension. Cells were then washed twice in PBS, counted and resuspended in MACS buffer before use in functional assays and/or phenotyping.

2.4.5 Recovery of Immune Cells from Murine Tissues

2.4.5.1 Animals

Animals in this study were housed and maintained at Cardiff University. All experiments were age and sex matched (mostly 6-10 week old females). C57BL/6 mice were obtained from Charles River (Massachusetts, US) or Envigo (Huntingdon, UK). All experiments were performed in accordance with institutional and United Kingdom Home Office guidelines under the project licence P05D6A456 and personal licence I76E17181. Mice were humanely culled and perfused with PBS, followed by isolation of tissues.

2.4.5.2 Mouse Ear and Back/Flank Skin

Ears were cut from the base and back/flank skin was shaved using an electric shaver, followed by hair removal cream (Nair; Church & Dwight Co., Inc, USA). The excised tissue was placed in cold PBS – 5 mM EDTA over ice for transport from the animal facility to the lab.

Back/flank skin was placed dermal side up on a petri dish and subcutaneous fatty tissue was scraped off using curved forceps and was then cut into thin strips. The thin strips were digested in 10 ml cRPMI (no serum) containing 5 mg/ml Dispase II in a petri dish at 37^{o}C for two hours. Extracted ears were separated into dorsal and ventral sides using forceps. Both parts were digested in cRPMI (no serum) supplemented with 2.5 mg/ml Dispase II for two hours. Digestion was stopped by placing the skin fragments in a petri dish containing cold PBS – 5 mM EDTA. The epidermis was carefully separated from the dermis using forceps and were diced into small parts. Tissue fragments were placed in separate Erlenmeyer flasks and were further digested in 15 ml of cRPMI (no serum)

supplemented with 25 µg/ml liberase (Sigma-Aldrich), 20 U/ml DNase I and 1x DNase buffer (10 mM Tris-HCl pH 7.5 at 25°C, 2.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$) in a shaking waterbath (37°C) for one hour. The digestion medium was filtered through a 40 µm cell strainer, tissue remnants were mechanically disrupted using a syringe plunger and washed with PBS. Remaining single cell suspension was washed twice with PBS, counted and resuspended in MACS buffer for use in functional assays and/or phenotyping.

2.4.5.3 Mouse Intestine

Small intestine was separated from the large intestine at the ileocecal valve, following removal of the mesentery, and placed in cold HBSS supplemented with 15 mM HEPES (ThermoFisher), on ice. Large intestine was separated from the anus at the anal verge and placed in HBSS, 15 mM HEPES, on ice. Fat and Peyer's patches were carefully dissected away from the small intestine. The remaining tissue was washed in HBSS/15 mM HEPES, cut into small fragments and placed in pre-warmed 30 ml HBSS supplemented with 5% FCS, 2 mM EDTA and 10 µg/ml gentamycin (ThermoFisher). Faeces were removed from the colon and tissue was washed thoroughly with HBSS/15 mM HEPES. Colon tissue was cut into small pieces and placed in HBSS/FCS/EDTA, like the small intestine. Both small intestine and colon were incubated in a shaking waterbath kept at 37°C for 20 minutes. This process was repeated four times, until the supernatant appeared clear. Tissue fragments were diced and placed in pre-warmed digestion media containing 25 µg/ml liberase, 20 U/ml DNase I and 10 µg/ml gentamycin diluted in cRPMI in an orbital shaker for 10-20 minutes at 37°C. Following digestion, remaining intestinal tissue was filtered through a 100 µm cell strainer to remove residual tissue fragments, and washed twice with cold HBSS supplemented with 5% FCS. The resulting single-cell suspension was counted and resuspended in MACS buffer for use in phenotyping and/or functional assays.

2.4.5.4 Mouse Kidney

Mice were perfused, kidneys were removed and kept in PBS on ice. Excised kidneys were homogenised and placed in a 24-well plate in 1 ml of digestion solution (0.2mg/ml Liberase TL, 0.2 mg/ml DNase I in DMEM-F12) for 30 minutes at 37°C. Tissue fragments were gently mashed with a syringe plunger, passed through a 40 µm cell strainer and thoroughly washed with cold DMEM-F12 medium (ThermoFisher). Cells suspension was

spun down, resuspended in cold PBS and counted using a haemocytometer. Cells were resuspended in MACS buffer for phenotyping.

2.4.5.5 Mouse Lung

Lungs were excised from perfused mice. Lungs were cut up into four-or five small pieces and washed with cold HBSS buffer. Lung fragments were further cut into fine pieces and digested in cRPMI (no serum) containing 20 U/ml DNase I and 1 mg/ml Collagenase for 30 minutes in a shaking water bath kept at 37^{o}C. Remaining cell suspension was filtered through a 40 µm cell strainer and washed twice. Cells were counted and resuspended in MACS buffer for further use.

2.4.5.6 Mouse Liver/Heart

Liver and heart were excised from perfused mice and diced into small fragments. Tissue fragments were filtered through a 40 µm cell strainer and cells obtained were washed twice in PBS, counted and resuspended in MACS buffer for use in phenotyping.

2.4.5.7 Mouse Spleen

Spleen was excised from mice and was mechanically disrupted using a syringe plunger. Cell suspension was filtered through a 40 µm cell strainer, washed and red blood cells were lysed using Red Blood Cell Lysis Buffer (Sigma-Aldrich), according to manufacturer's instructions. Cells were washed with PBS, counted and resuspended in the appropriate buffer for use in phenotyping and/or functional assays.

2.5 Phenotyping and Functional Assays

2.5.1 Cell Staining and Flow Cytometry

For all flow cytometric measurements, 50,000-500,000 cells were stained in 96-well plates, started by washing in PBS by centrifugation at 400 x g for three min. Cells were stained with Live/Dead Fixable Aqua Dead Cell Stain Kit (Life Technologies) to distinguish between live and dead cells in the analysis. Live/Dead stain was used at 1:100 dilution, for 12 minutes at room temperature. All subsequent steps were carried out in FACS buffer. Cells were washed and incubated with endogenous Fc receptor blockers. For human cells, normal human immunoglobulin (KIOVIG; Baxter, Stained-upon-Thames, UK) was used at a 1:1000 dilution and for mouse cells, heat inactivated rat serum

(ThermoFisher) was used at a 1:10 dilution for 15 minutes at 4^{o}C. Fluorochrome-conjugated mAbs directed against cell-surface antigens were added for 30 minutes at 4^{o}C. A complete list of antibodies, their clone and appropriate dilutions can be found in **Table 2.1**. Appropriate fluorescence minus one (FMO) control were used in all cases to enable accurate gating of cell populations. Cells were washed and resuspended in FACS buffer for acquisition.

Samples were acquired using a FACS Canto II or Fortessa instrument (BD, Oxford, UK). Analysis of raw data was performed using FlowJo software (Version 10.4, TreeStar Inc.). Intact cells were gated based on Forward Scatter – Area (FSC-A)/Side Scatter – Area (SSC-A), followed by single cell gating based on FSC Area vs. Height profile (FSC-A/FSC-H), live cells (Live/Dead^{-}) and expression of markers of interest.

Table 2.1. Fluorochrome-conjugated monoclonal antibodies used for flow cytometry

Antigen	Conjugate	Clone	Supplier	Reactivity
Cell-surface antigen				
CD11b	PE	S-HCL-3	BD	Human
CD11c	PE	61D3	eBioscience	Human
CD14	FITC	3G8	BD	Human
CD16	PE-Cy5	H1B19	BioLegend	Human
CD19	APC	SJ25C1	eBioscience	Human
CD19	BV421	UCHT1	BioLegend	Human
CD3	PerCP/Cy5.5	HI30	BioLegend	Human
CD45	PE	B159	BD	Human
CD56	PE	ICRF44	eBioscience	Human
CD4	BV421	RPA-T4	BD	Human
B220	Pacific Blue	RA3-6B2	BioLegend	Mouse
B220	APC	RA3-6B2	BioLegend	Mouse
CD117 (c-kit)	FITC	2B8	BioLegend	Mouse
CD117 (c-kit)	BV421	2B8	BioLegend	Mouse
CD11b	PE-Cy7	M1/70	BioLegend	Mouse
CD11b	FITC	M1/70	BioLegend	Mouse
CD11c	AF-700	N418	BioLegend	Mouse
CD3	PE	17A2	BioLegend	Mouse
CD3	AF-700	17A2	BioLegend	Mouse
CD3	BV785	17A2	BioLegend	Mouse
CD31	FITC	390	BioLegend	Mouse
CD34	PE	MEC14.7	BioLegend	Mouse
CD4	PE	PM4-4	BioLegend	Mouse
CD45	PerCP	30-F11	BioLegend	Mouse
CD64 (FcγRI)	Biotin	X54-5/7.1	BioLegend	Mouse
EpCAM (CD326)	PE	G8.8	BioLegend	Mouse
F4/80	BV421	BM8	BioLegend	Mouse
F4/80	APC-Cy7	BM8	BioLegend	Mouse
FcεRIa	PE-Cy7	MAR-1	BioLegend	Mouse
FcεRIa	PE	MAR-1	BioLegend	Mouse
Ly6C	BV421	HK1.4	BioLegend	Mouse
Ly6C	PE	HK1.4	BioLegend	Mouse
Ly6C	BV605	HK1.4	BioLegend	Mouse

Ly6G	AF700	1A8	BioLegend	Mouse
NK1.1	AF700	PK136	BioLegend	Mouse
NK1.1	FITC	PK136	BioLegend	Mouse
Sca-1 (Ly-6A/E)	FITC	E13-161.7	BioLegend	Mouse
Chemokine receptor				
CCR2	PerCP/Cy5.5	TG5	BioLegend	Human
CCR2	APC	K036C2	BioLegend	Human
CCR5	PE	2D7	BD	Human
CCR7	PE-Cy7	3D12	BD	Human
CCR7	PE-Cy7	G043H7	BioLegend	Human
CXCR3	FITC	49801.111	R&D	Human
CXCR4	PE	12G5	eBioscience	Human
CXCR4	BV421	12G5	BioLegend	Human
CXCR5	PE	51505.111	R&D	Human
CCR2	PE	475301	Biolegend	Mouse
CCR3 (CD193)	FITC	J073E5	Biolegend	Mouse
CCR3 (CD193)	PE	J073E5	Biolegend	Mouse
CCR3 (CD193)	PE	REA122	Miltenyi	Mouse
CX3CR1	PE-Cy7	SA011F11	Biolegend	Mouse
CXCR4 (CD184)	BV421	L276F12	Biolegend	Mouse

2.5.2 Labelling with Alexa Fluor® 647-CXCL14

AF-CXCL14 was used to label the putative CXCL14 receptor on the surface of cells. Binding of AF-CXCL14 (20 nM) was performed in combination with other antibodies against cell surface markers. Binding of AF-muCCL1 to cells was used as a negative control for non-specific binding, in order to take into account possible non-specific staining of cells by unrelated chemokines carrying the AF-fluorophore.

2.5.3 Transwell Chemotaxis Assay

Corning® HTS transwell 96 well plates with permeable supports with 5 µm pores or 8 µm pores (Sigma-Aldrich) were used in chemotaxis assays. Chemokines were resuspended in chemotaxis buffer to the desired concentration and placed in the lower chamber of the plate. In experiments, the synergistic effect of two chemokines was investigated, both chemokines were added to the lower wells of the plate. Cells were

resuspended at the desired concentration in chemotaxis buffer and placed in the upper chamber. Approximately 100,000 cells were used per well. A well containing chemotaxis buffer with no chemokine (buffer only control) was used as a negative control, by assessing random cell migration. The transwell plate was incubated at 37°C for between 1.5 and 5 hours, depending on the cell type tested. Upon termination of the assay, cells which had migrated to the lower chamber were collected and stained with antibodies against phenotypic markers, if required. Migration was assessed by flow cytometry. AccuCheck counting beads (ThermoFisher) were used to allow absolute cell counts and technical replicates were conducted in duplicate, when possible. Cell migration data were expressed either by a percentage of total input cells or a chemotactic index, which is defined as the number of cells migrated towards a chemokine divided by the number of cells migrated in response to buffer (buffer only control).

2.5.4 Intracellular Ca^{2+} Rise

Ca^{2+} mobilisation was measured by using a spectrophotometer (Fluorescence Spectrophotometer F-7000, Hitachi, Japan). Fura-2-acetoxymethyl ester (Fura-2AM; 1 mM; HelloBio, UK) was used for labelling of cells, which is a dual-wavelength ratiometric dye. Hence, the dual-excitation was set to 340 nm and 380 nm and emission at 510 nm to allow detection of Fura-2AM. Fura-2 emits light at 510 nm regardless of the amount calcium bound, therefore it can be used as a ratiometric calcium indicator. The excitation slit was set to 10.0 nm, emission slit to 5.0 nm and a recording was made every 1.5 s. The recordings were made using the FL Solutions 2.1 software. Samples were maintained at 37°C throughout the measurement by circulating warm water through the cuvette holder. Calcium concentration was calculated by the software using the Grynkiewicz formula (Grynkiewicz, Poenie and Tsien, 1985) which is defined as:

$$c = Kd + \frac{R - Rmin}{Rmax - R} + \frac{F2min}{F2max},$$

where,

$Kd = 224$ is defined as the dissociation constant,
R is the ratio of fluorescence intensity at 340 nm to the fluorescence intensity at 380 nM,
$Rmax$ is the limiting value of R when all indicator is saturated with Ca^{2+},

$Rmin$ is the limiting value of R when all indicator is in the Ca^{2+}- free form, $\frac{F2min}{F2max}$ is the ratio of the fluorescence intensity measured at 380 nm when all indicator is Ca^{2+}- free to the fluorescence intensity measured at 380 nm when all indicator is Ca^{2+}- bound.

Cells were prepared in cRPMI at a concentration of 10 x 10^6 cells/ml. Cells were incubated with a calcium-labelling cocktail containing 1mM Fura-2AM–at 2.5 µl/ml, Pluronic F-127 (20%, ThermoFisher) at 0.5 µl/ml and Probenecid (25 mM, Sigma-Aldrich) for 30 minutes at room temperature. Cells were then washed with cRPMI, followed by Calcium Buffer supplemented with Probenecid. Cells were finally counted and resuspended in Calcium Buffer/Probenecid at a concentration of 2 x 10^6 cells/ml. Fura-2-loaded cells were kept on ice until an aliquot (~1.8 x 10^6 cells) was incubated at 37°C for 10 minutes prior to measurement. Warmed-up cells were transferred to a cuvette and measurement was initiated. Upon stabilisation of the fluorescence emission baseline, chemokine diluted in cold calcium buffer was added. The contents of the cuvette were continuously mixed using a magnetic stirrer. Maximum calcium mobilisation was induced by addition of 5mM digitonin (Sigma-Aldrich) and minimum signal by addition of 20 mM Tris and 10 mM EGTA (Sigma-Aldrich). Recording was stopped after 300 seconds. Calcium concentration values were exported to Microsoft Excel and GraphPad Prism was used for generation of graphs.

2.5.5 Chemokine Receptor Internalisation

Cells (~100,000) were resuspended in chemotaxis buffer and incubated for one hour at 37°C with the indicated chemokine and concentration, in a 96-well plate. Cells were incubated in an acidic buffer (PBS, 100 mM NaCl and 50 mM Glycine, pH 3) for one minute in order to remove any ligand bound to receptors on the cell surface, followed by washing with FACS buffer twice. Level of expression of cognate receptors on the cell surface was determined by flow cytometry.

2.5.6 Immunohistochemistry

For cytospin analyses, 80,000-100,000 cells were cytocentrifuged onto a glass slides by spinning at 72.26 g (Cytospin 3, Shandon; UK) for 10 minutes at low acceleration and allowed to air-dry. Slides were fixed with cold methanol for five minutes and incubated

in 50% May-Grunwald stain (Merck, New Jersey, USA) for five minutes, followed by 14% Giemsa stain (Merck) for 15 minutes. Slides were washed with distilled water and allowed to air-dry. Finally, slides were mounted with mounting media (Vectashield), covered with coverslips and sealed using nail varnish. Images were acquired using a Zeiss Apotome microscope (Zeiss Apotome Axio Observer, Zeiss), with a 40x or 63x oil-immersion objective.

2.5.7 *In Vivo* Synergy of CXCL14 and CXCL12

Cell recruitment was tested in the peritoneal cavity or the knee cavity, in response to intraperitoneal or intra-articular injections of chemokines, respectively. Endotoxin free-CXCL14, CXCL12 or vehicle (PBS) were injected in female C57BL/6 mice, as previously described (Davies, Rosas, *et al.*, 2013). After 16 or 24 hours mice were culled. The peritoneal cavity was washed with PBS-2 mM EDTA and recovered cells were analysed by flow cytometry on a BD FACS Fortessa machine (BD). Absolute cell counts of recovered cells were determined using AccuCheck counting beads (ThermoFisher).

2.6 RNA Sequencing (RNA seq)

2.6.1 Sample Preparation

Cells recovered from murine skin and colon tissue as described above were purified on a flow cytometry cell sorter (BD Aria II, BD) by Dr Paul Collins. Dead cells were excluded during the sort by Live/Dead stain. CXCL14 target cells were sorted based on $CD45^{-}CXCL14^{+}F4/80^{+}$, skin tissue cells based on $CD45^{-}CXCL14^{-}F4/80^{-}$ and tissue resident macrophages based on $CD45^{+}CD11b^{+}CCR2^{-}CD64^{+}$. $2.5 \times 10^{3} – 1 \times 10^{5}$ cells were sorted into cRPMI + 20% FCS and kept cold at all times. Cells recovered from the sort were spun down, resuspended in Buffer RLT Plus (Qiagen, Netherlands) and stored at -80°C prior to shipping. Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) and its concentration and purity was assessed using a NanoDrop ND1000 (ThermoScientific).

All samples were shipped to the Institute for Computational Biology, Case Western Reserve University School of Medicine, Cleveland, Ohio.

2.6.2 Sequencing

Next-generation sequencing of mRNA was performed using the Illumina HiSeq 2500 System with TruSeq Technology (Illumina). Transcriptomes were characterised via paired-end, 50base pair-RNA sequencing runs (10 samples per lane), ensuring at least 30 x 10^6 mapped reads per sample. Sequencing files were received as FASTQ files, one forward and one reverse read file per sample. Reads were mapped to the mouse reference genome (GRCm38). Resulting BAM files (mapped reads) were processed to produce normalised expression counts per gene (reads mapped per kilobase length of transcript per million mapped reads; RPKM). Differentially expressed genes were identified using DESeq2 package in Bioconductor. This analysis was carried out by Dr You Zhou and Dr Robert Andrews.

2.7 Synthesis of Chemokine Variants

The study of CXCL14/CXCL12 structural variants was done in collaboration with Prof Paul Proost in KU Leuven, Belgium. Full-length CXCL14 and N-terminus CXCL14-CXCL12 variants were synthesised as previously described (Loos, Mortier and Proost, 2009). Briefly, peptides were synthesised based on solid-phase synthesis conducting cycles of single amino acid additions that involved chemical deprotection, activation coupling and washing (**Figure 2.3**). The α-carboxyl group of the COOH-terminal amino acid is attached to a stable and solid support [HMP-resin (4-hydroxymethyl-phenoxy-methyl-polystyrene, cross- linked by 1% divinylbenzene)], to which they remain coupled during chain assembly. Consecutive amino acids are coupled to the growing chain based on the amino acid sequence of the desired protein. The α-amino group of these amino acids is protected from inappropriate binding by a fluorenylmethoxy carbonyl- (Fmoc) protecting group. Because the side chains of some amino acids also contain chemically reactive groups, they are also blocked by protecting groups. Prior to each coupling step, the growing peptide chain was treated with 20% (v/v) piperidine in N-methyl-2 pyrrolidone (NMP) (Biosolve, Valkenswaard, The Netherlands), in order to remove the Fmoc protection groups from the N-terminus of the last coupled amino acid ("deprotection") (**Figure 2.2**). Following each piperidine treatment, the absorbance of the Fmoc-containing washing solvent was quantified by UV monitoring. Repeated piperidine treatments increased the yield of the synthesis, until the UV absorption

valued were 25 or lower, with a maximum of six piperidine treatments performed. Washing with NMP was followed by sequential activation, coupling, capping and further washing steps. When the last amino acid was coupled to the peptide chain, it was treated with piperidine to remove the Fmoc protection group of the last amino acid, following by washing and drying under nitrogen flow.

Following synthesis of the full-length protein, removal of side chain protection groups was performed by incubating with a mixture containing 88.9% (v/v) trifluoroacetic acid (TFA) (Biosolve), 3.3% (v/v) Thioanisole (Acros Organics), 2.2% (v/v) 1,2-ethanedithiol (Merck, Darmstadt, Germany) and 66.7 mg/ml crystalline phenol (Merck) for 105 minutes. The mixture was then filtered using a Bio-spin column (Bio-Rad laboratories, Hercules, California, USA) and washed with diethyl ether. The final precipitate was dissolved in ultrapure water, which was evaporated overnight using a SpeeVac Concentrator (SvC100 Savant, ThermoFisher Scientific).

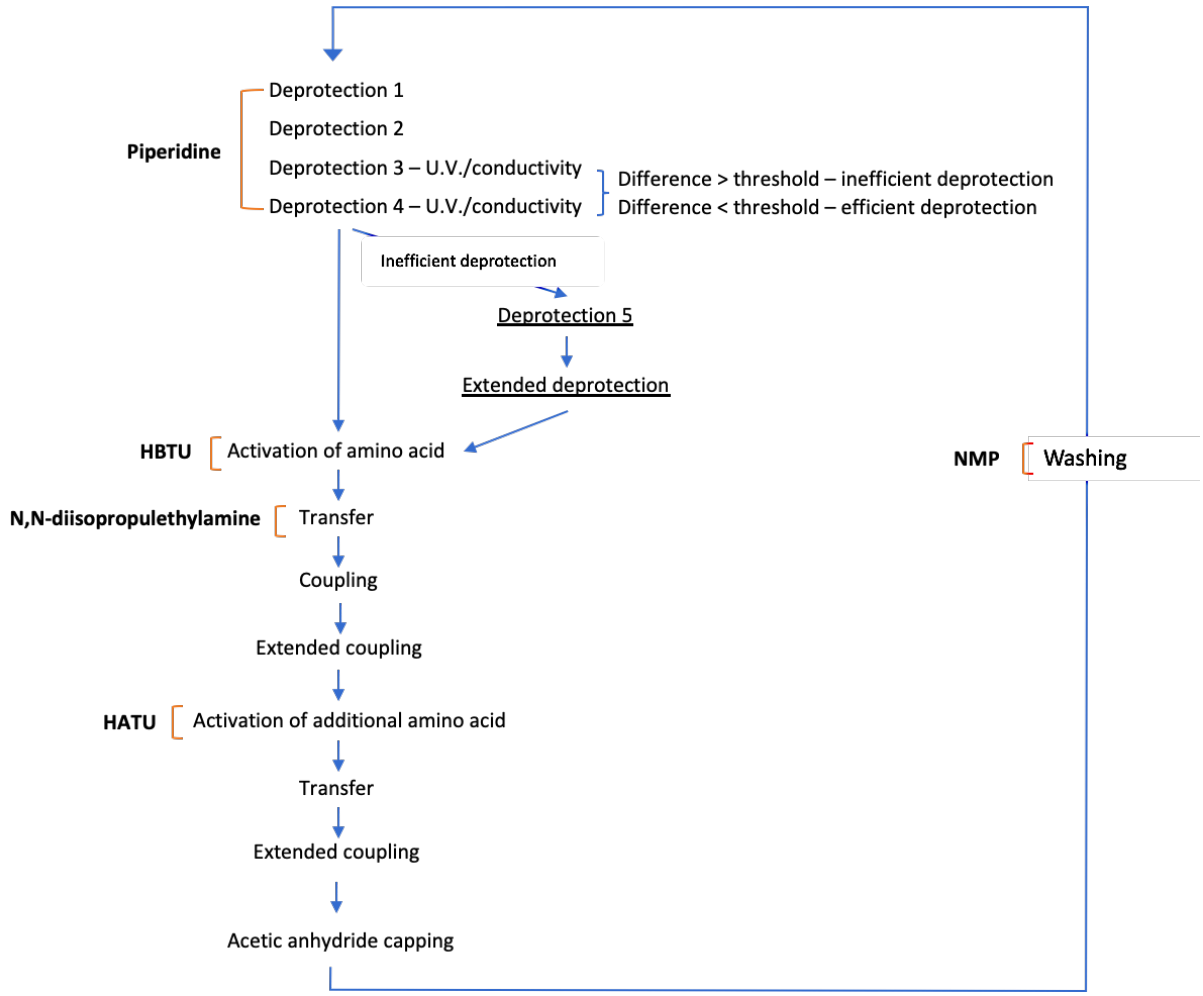


Figure 2.2. Modified protocol of single-amino acid cycle during solid-phase synthesis of proteins.

During solid-phase peptide synthesis, amino acids are coupled one by one to the growing peptide chain from the COOH- to the NH_2-terminus. The Fmoc protection group is removed from the α-amino group by treatment with piperidine, allowing for coupling of the next amino acid. When the UV or conductivity measurement after the fourth deprotection differs from the threshold, then a conditional deprotection is switched on (underlined). HBTU solution is added to a cartridge containing the next Fmoc-protected amino acid and activates it. N, N diisopropylethylamine (DIEA) is added and the activated amino acid is transferred to the reaction vessel to form a peptide bond with the amino acid-resin complex. In the case of CXCL14 synthesis, an additional coupling step was added, with HATU used as an activation agent. This step aims to increase coupling efficiencies and higher synthesis yield. Due to coupling never being 100% complete, the remaining free α-amino groups of the first amino acid are capped with acetic anhydride to prevent them to be coupled to amino acids added during the following cycles. Finally, the resin particles are washed 4 times with NMP. These cycles are repeated until the entire peptide chain is completed. The final Fmoc group of the last NH_2-terminal amino acid is removed with a last treatment with piperidine. Figure adapted from (Loos, Mortier and Proost, 2009).

Synthetic proteins were purified using reverse-phase high performance liquid chromatography (PR-HPLC) (Waters 600 Controller and Waters 600 Pump; Merck). For purification of desired proteins, a 4.6 x 150 mm Proto 300 C4 column or 10 x 150 mm Proto 300 C4 column (Higgins Analytical, Mountain View, CA, USA) was used. A gradient of acetonitrile (0-80%) in 0.1% TFA was applied at a flow rate of 1ml/min or 4ml/min, depending on column diameter, which resulted in sequential elution of individual protein variants. Elution fractions were collected (Frac-100^{TM}, Amersham Biosciences, UK) every one minute or 30 seconds, depending on the choice of flow rate. Detection of eluted proteins was performed by UV absorbance measurement at 214 nm and/or electrospray ion trap mass spectrometry (Amazon SL, Bruker Daltonics, Bremen, Germany), after splitting the eluate 1/50 online. In electrospray ion trap mass spectrometry, the combination of a strong electric field and the protons present in the acidic solvent lead to the ionisation of the peptides in the sample. This leads to the creation of fine highly charged droplets at the tip of the needle, which are then subjected to heated drying gas that causes the solvent molecules to be evaporated and the peptide ions to be in the gas phase. The positively charged gaseous ions are trapped in the negatively charged “ion trap” and are then detected by the detector, based on their mass-to-charge (*m/z*) ratio. Analysis of the mass spectra (Compass HyStar Software, Bruker) recorded during purification allowed identification of specific molecular masses eluted at each time point. Thus, identification and selection of fractions containing protein of interest was possible. Following Reverse-Phase High Performance Liquid Chromatography (RP-HPLC), selected fractions containing proteins of interest were manually injected into the mass spectrometer to confirm their purity.

The last stage of chemokine synthesis involved folding of purified protein chains to the correct three-dimensional conformation through incubation with folding buffer (see below), which enables formation of disulphide bridges between the cysteine residues of the chemokine. Elution fractions from HPLC containing the correct protein were identified using mass spectrometry and evaporated for removal of acetonitrile and incubated with folding buffer (1 M guanidine hydrochloride, 125 mM Tris-acetate, pH 8.5) for 18 hours, under continuous oxygenation. Following incubation with folding buffer, samples were acidified with TFA until pH 2-3 was achieved. Samples were then

purified with RP-HPLC and analysed via mass spectrometry. Fractions containing correct proteins were pooled and evaporated to produce lyophilised protein using a SpeedVac Concentrator.

2.8 Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software, Inc.). The D'Agostino-Pearson omnibus normality test was carried out as a first step to assess the distribution of data and to determine whether parametric or non-parametric tests were appropriate. Test selection was also based on the design of the experiment. For the comparison of two variables, either Student's *t* test (parametric data), the Mann-Whitney U test (unpaired, non-parametric data) or the Wilcoxon matched-pairs signed rank test (paired, non-parametric data) were used. For multiple variable comparison, either one-way ANOVA (parametric data), the Kruskal-Wallis test (unpaired, non-parametric data) or the Friedman test (paired, non-parametric data) was used. Following analysis, multiple comparisons were carried out using Dunn's multiple comparison test. Either all experimental conditions were compared within the experiment, or each experimental condition was compared to the negative control. Descriptive statistics are displayed as mean ± standard deviation of the mean (SD), unless otherwise stated. Significance of differences in experimental data was defined as p values of <0.05 and grouped according to *= $p<0.05$, **=$p<0.01$, ***=$p<0.001$), ****=$p<0.0001$, ns= non-significant.

2.9 Ethics

The study involving human samples was conducted according to the principles expressed in the Declaration of Helsinki and directed by local ethical guidelines. The study was approved by the South East Wales Local Ethics Committee (Reference Number 08/WSE04/17 and School of Medicine SMREC 04/18). All healthy blood donors provided written informed consent for the collection of samples and subsequent analysis and storage of material. All acquisition, analysis and storage or relevant material was performed under the Human Tissue Act guidelines.

Chapter 3: CXCL14 Synergism with Other Chemokines

3.1 Introduction

The main role of chemokines is the regulation of leukocyte migration and function, both in physiological and pathological immune responses (Griffith, Sokol and Luster, 2014). To accomplish proper tissue distribution of all distinct leukocyte subsets under normal and pathological conditions, chemokines and their receptors show promiscuity, but also synergy and cooperation. Both *in vitro* and *in vivo* studies have shown several mechanisms regarding the modes of chemokine synergism, summarised in **Figure 1.8**. These mechanisms include heterodimerisation of chemokine receptors, which are activated by their respective ligands simultaneously or sequentially. Additionally, a chemokine heterocomplex can be formed, with the ability to activate a single receptor. Finally, two chemokine receptors can be activated by their respective ligands to synergise at the level of downstream signalling.

Models of GPCR function indicate that ligand-free receptor conformations are in equilibrium between active and inactive states, where ligand binding induces a shift towards to, and stabilises an active state (Percherancier *et al.*, 2005; Thelen *et al.*, 2010). Besides different conformations being induced by their cognate ligands, receptors can also be altered by allosteric ligands. One example of allosterism was reported for CXCL14 and CXCL12. CXCL14 is co-expressed with the homeostatic chemokine CXCL12 in many sites of the body. Both CXCL12 and CXCL14 are considered to be archetypic on the basis of their sequence conservation across species and the presence of orthologs in lower vertebrates. CXCL12 controls the migration of haematopoietic stem cells from adult bone marrow, and plays a crucial role in organ development during embryogenesis (Nagasawa, 2014). Likewise, CXCL14 is also implicated in organ development. In fact, both chemokines are expressed constitutively in the developing organs of mouse, chicken and zebrafish embryos (Nomiyama *et al.*, 2008; García-Andreś and Torres, 2010; Gordon *et al.*, 2011; Ojeda, Munjaal and Lwigale, 2017). The key part played by both chemokines during development is demonstrated by homozygous deletions in the genes for either CXCL14, CXCL12 or the CXCL12 receptor, CXCR4 (Nagasawa *et al.*, 1996; Meuter *et al.*, 2007; Nara *et al.*, 2007; Tanegashima *et al.*, 2010; Nagasawa, 2014). The

striking breeding defect observed in CXCL14-KO mice suggests that CXCL14 fulfils a key role in early development, similar to CXCL12, and could control aspects of immune surveillance in adult peripheral tissues, where it is very highly expressed.

The allosteric relationship between CXCL14 and CXCL12 was first described to be inhibitory, with CXCL14 acting as a natural allosteric inhibitor (Tanegashima, Suzuki, *et al.*, 2013). However, this finding remains controversial as conflicting evidence soon followed, showing that CXCL14 may actually not act as an inhibitor of the CXCL12/CXCR4 pathway (Otte *et al.*, 2014). In clear contrast to the allosteric model, our group has recently reported that CXCL14 and CXCL12 show synergistic interaction in the induction of chemokine responses in primary human lymphoid cells and cell lines expressing CXCR4 (Collins *et al.*, 2017). Our evidence indicates that CXCL14 is a positive allosteric modulator of CXCR4, where CXCL14 is proposed to shift the balance of CXCR4 conformational state to that recognised by the functional ligand CXCL12 (**Figure 3.1**). This finding may have potential for therapeutic applications as it provides information for the design of allosteric modulators specific for CXCR4. These modulators could be used to target tissue repair processes, cancer or HIV infection.

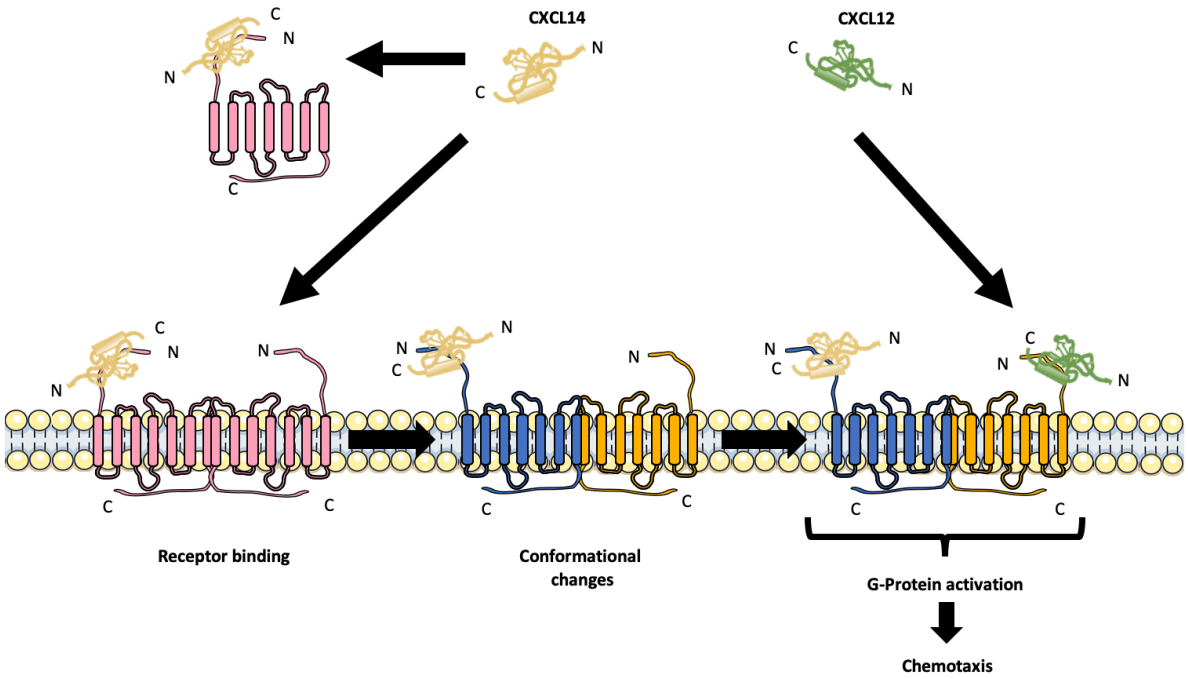


Figure 3.1. CXCL14 is a positive allosteric modulator for CXCR4.
This model explains how CXCL14 synergises with CXCL12 in the induction of CXCR4-mediated chemokine responses. Cell-surface CXCR4 exist in different conformational states, including empty receptors as monomers, dimers or oligomers. Conformational states can be altered by ligand binding (shown here by a shift from pink to blue conformation upon CXCL14 binding). CXCL14 binding alone does not induce any functional responses. However, CXCL14 binding causes allosteric changes in partner molecules that are present in CXCR4 oligomers, thereby lowering the threshold of receptor activation by CXCL12 (shown here by shift from pink to orange in the partner molecule). Figure adapted from (Collins *et al.*, 2017).

Although we currently have a lot of information regarding chemokine synergism and specifically the relationship between the two archetypic chemokines, there is a need to continue these studies, as many questions remain. The main functions of CXCL14 are still not clear due to the fact that its receptor remains unknown. Nevertheless, it is known that CXCL14 is ubiquitously and abundantly expressed in normal epithelial tissues, including the skin, breast, kidney, tongue, placenta and digestive and urinary tract. These tissues are also associated with other chemokines, both inflammatory and homeostatic. Homeostatic chemokines, like CXCL14, are constitutively expressed to maintain cell migration under physiological conditions, whereas inflammatory chemokine expression is induced upon infection or injury. The question remains whether CXCL14 can synergise with chemokines other than CXCL12, including both

homeostatic and inflammatory. Since our group has already shown that CXCL14 synergises with the homeostatic chemokine CXCL12, the next logical step is to examine whether it can have a greater role in the maintenance of immune surveillance by synergising with other homeostatic chemokines. As one of the major functions of homeostatic chemokines is the control of immune cell traffic within secondary lymphoid tissues, which is mainly controlled by the chemokines CCL19, CCL21 and CXCL13, and their cognate receptors CCR7 and CXCR5, respectively, it is important to assess potential synergism of these chemokines with CXCL14. In addition, inflammatory chemokine expression in response to inflammatory stimuli might coincide with CXCL14 expression. Therefore, we here sought to explore the ability of CXCL14 to synergise with such inflammatory chemokines. Some of the most prominent inflammatory chemokines include the CXCR3 ligands CXCL9, CXCL10, and CXCL11, as well as the CCR5 ligands CCL3, CCL4 and CCL5. The chemokine receptor CXCR3 and its ligands mainly control effector T cell trafficking to the periphery in response to inflammatory stimuli. Specifically, $CD4^+$ memory Th1 T cells express the chemokine receptors CXCR3 and CCR5, which enables them to reach sites of inflammation and deliver an adaptive immune response (Qin *et al.*, 1998; Groom and Luster, 2011). These chemokines are therefore prime candidates for assessing any potential synergism between CXCL14 and inflammatory chemokines.

3.2 Aims

- To examine the ability of CXCL14 to synergise with selected homeostatic chemokines in the induction of functional responses
- To examine the ability of CXCL14 to synergise with selected inflammatory chemokines in the induction of functional responses
- To examine synergism between CXCL14 and CXCL12 in mice

3.3 CXCL14 Synergism with Homeostatic Chemokines

3.3.1 CXCL14 Synergises with Homeostatic Chemokines CCL19 and CCL21 in the Induction of Chemotactic Responses

3.3.1.1 Primary Human Cells

Since our group previously established that CXCL14 synergises with CXCL12 in the induction of chemotactic responses (Collins *et al.*, 2017), I aimed to investigate whether this synergy extends to other homeostatic chemokines. The chemokine receptor CCR7 plays a vital role not only in DC trafficking but also in the recirculation of naïve and central memory T cells (T_{CM}) via secondary lymphoid organs, which make up the majority of peripheral blood T cells. The remainder cells in the blood are effector memory T cells (T_{EM}), which do not express CCR7 and therefore do not recirculate through the lymph nodes. To examine synergism of CXCL14 with CCR7-selective chemokines, PBMC were isolated from peripheral blood of healthy volunteers. Although the majority of T cells, including naïve and T_{CM} T cells, expressed CCR7 on their surface (**Figure 3.2a**), we have previously observed that freshly isolated T cells do not display strong chemotactic responses towards CCR7 (unpublished data). After resting the cells overnight at 37°C, robust responses towards both CCR7 ligands CCL19 and CCL21 were observed. Cells optimally migrated at concentrations ≥ 100 nM CCL19 and CCL21, which is typically seen with homeostatic chemokines, whereas concentrations below 100 nM were suboptimal (**Figure 3.2.b, c**). Of note, T cells showed no chemotactic responses towards CXCL14, in clear contrast to monocytes that responded well to CXCL14 (Collins et al. 2017). However, when combined with inactive or suboptimal concentrations of CCL19 and CCL21 (1 nM and 10 nM were tested), cells became highly responsive to CXCL14. Similar to the responses observed with CXCL12, the synergistic effect was maximal when 300 nM CXCL14 was used in combination with 1 nM or 10 nM CCL19 or CCL21.

Besides T cells, some peripheral blood B cells also express CCR7. In that way, they can enter secondary lymphoid organs, where they can interact with follicular B helper T (T_{FH}) cells in the B cell compartment in order to generate humoral immune responses (Breitfeld *et al.*, 2000; Schaerli *et al.*, 2000). Similar to T cells, CXCL14 on its own did not

induce B cell migration but synergised with suboptimal concentrations of CCL19 and CCL21 (**Figure 3.3**).

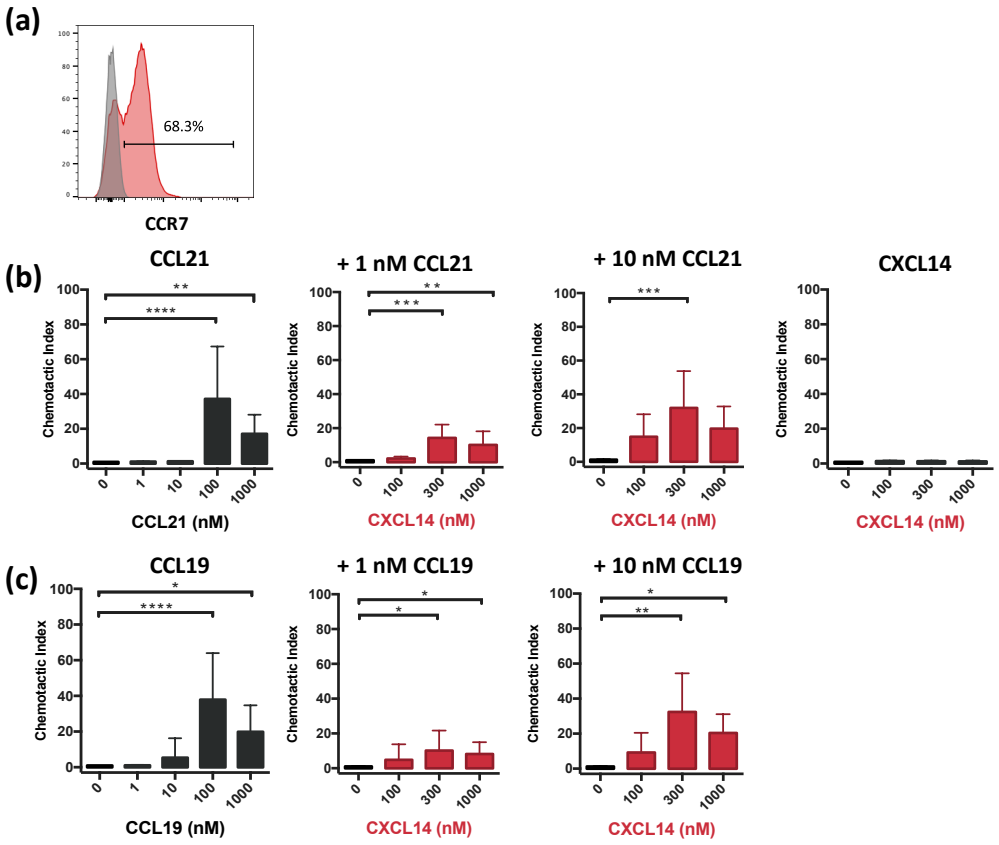


Figure 3.2. CXCL14 synergises with the CCR7 ligands CCL19 and CCL21 in the induction of chemotactic responses in primary human T cells.

PBMC were isolated from peripheral blood and rested overnight in medium. The next day, phenotyping was performed for expression of chemokine markers and migration towards chemokines was assessed by transwell chemotaxis assay. **(a)** Surface expression of CCR7 on T cells (red histogram), assessed by flow cytometry. Grey histogram indicates staining with fluorescence-minus one control. **(b)** Migration of T cells toward CCL21 (middle left), CXCL14 (middle right), 1 nM CCL21 & CXCL14 (middle centre left) or 10 nM CCL21 & CXCL14 (middle centre right). **(c)** Migration of T cells towards CCL19 (bottom left), 1 nM CCL19 & CXCL14 (bottom centre) or 10 nM CCL19 & CXCL14 (bottom right). Data are combined with results from Dr Paul Collins (Dr Collins contributed 2-3 donors). Data shown are means + SD of 6-7 independent experiments using PBMC from different donors. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$ compared to 0 nM using Friedman test followed by Dunn's multiple comparisons test.

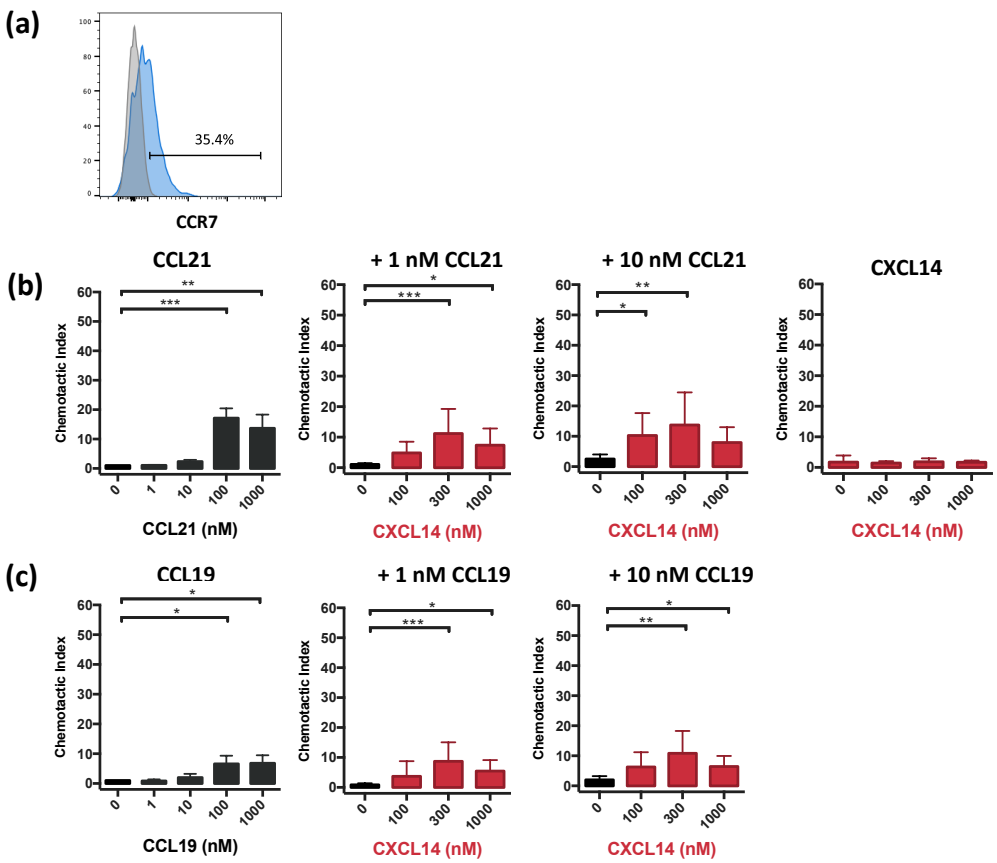


Figure 3.3. CXCL14 synergises with the CCR7 ligands CCL19 and CCL21 in the induction of chemotactic responses in primary human B cells.

PBMC were isolated from peripheral blood and rested overnight in medium. The next day, phenotyping was performed for expression of chemokine markers and migration towards chemokines was assessed by transwell chemotaxis assay. **(a)** B cells were gated based on expression of CD19. Surface expression of CCR7 on B cells (blue histogram), assessed by flow cytometry. Grey histogram indicates staining with fluorescence-minus one control. **(b)** Migration of B cells toward CCL21 (middle left), 1 nM CCL21 & CXCL14 (middle centre) or 10 nM CCL21 & CXCL14 (middle right). **(c)** Migration of B cells toward CCL19 (bottom left), 1 nM CCL19 & CXCL14 (bottom centre) or 10 nM CC19 & CXCL14 (bottom right). Data are combined with results from Dr Paul Collins (Dr Collins contributed 2-3 donors). Data are mean + SD of 6-7 independent experiments using PBMC from different donors. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 compared to 0 nM using Friedman test followed by Dunn's multiple comparisons test.

3.3.1.2 CCR7-Transfected Mouse 300-19 Pre-B Cell Lines

300-19 is a mouse pre-B cell line that is commonly used for stable transfection with either mouse or human chemokine receptors, allowing in-depth characterisation of their function. 300-19 cells stably transfected with CCR7 (300-19-CCR7 cells) were previously generated in our group (Willimann *et al.*, 1998), and CCR7 expression on the cell surface was confirmed by flow cytometry (**Figure 3.4a**). Similar to primary T and B cells, 300-19-CCR7 cells migrated substantially towards CCL21 (**Figure 3.4b**) and CCL19 (**Figure 3.4c**). Optimal responses were observed at 100 nM of either chemokine. No migration was observed at lower (1 nM or 10 nM) or higher concentrations (1000 nM) of CCL19 or CCL21. As seen before with primary human lymphocytes, CXCL14 on its own was inactive on 300-19-CCR7 cells. However, when combining 1 nM or 10 nM of CCL19 or CCL21 with CXCL14, strong migration of 300-19-CCR7 cells was observed, comparable to primary T and B cells. In contrast to primary lymphocytes, the combination of suboptimal concentrations of CCL21 with CXCL14 yielded strong responses at all concentrations of CXCL14 (**Figure 3.4a**). For CCL19, the highest synergistic effect was observed at 300 nM CXCL14, similar to primary lymphocytes (**Figure 3.4b**).

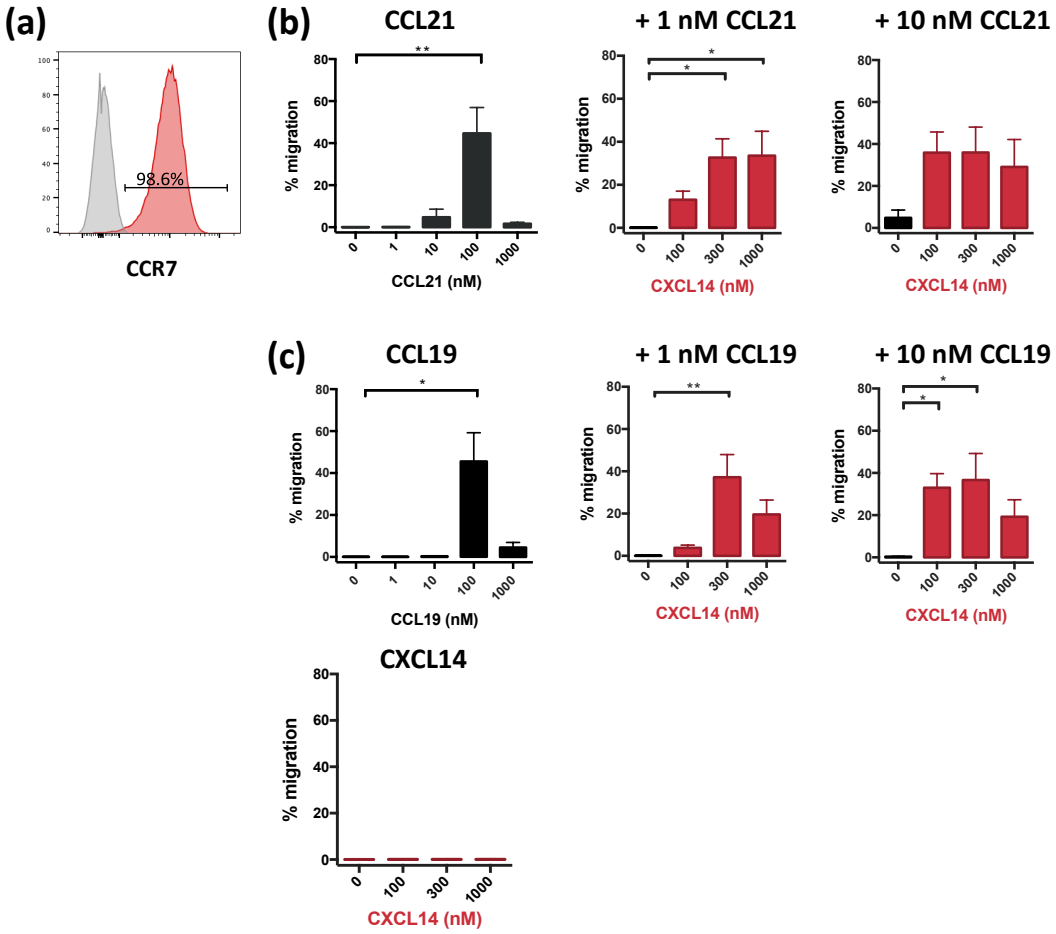


Figure 3.4. CXCL14 synergises with CCL21 and CCL19 in the induction of chemotactic responses in 300-19 cells stably transfected with CCR7.

(a) Surface receptor expression on 300-19 cells stably transfected with CCR7 was confirmed by flow cytometry (red histogram). Grey histogram shows staining with fluorescence-minus one control. **(b)** Migration of 300-19-CCR7 cells toward CCL21 (top left), 1 nM CCL21 & CXCL14 (top centre) or 10 nM CCL21 & CXCL14 (top right). **(c)** Migration of 300-19-CCR7 cells toward CCL19 (middle left), 1 nM CCL19 & CXCL14 (middle centre), 10 nM CCL19 & CXCL14 (middle right) or CXCL14 (bottom). Data are mean + SEM of 4 independent experiments, where *p< 0.05 and **p< 0.01 compared to 0 nM using Friedman test followed by Dunn's multiple comparisons test.

3.3.2 CXCL14 Synergises with Homeostatic Chemokines CCL19 and CCL21 in Triggering Ca^{2+} Mobilisation

Chemokine stimulation of GPCRs can induce the initiation of several downstream effectors that eventually lead to complex responses, such as actin polymerisation, morphological change and directional movement. Specifically, stimulation of the Gαi GPCR subunit can result in release of calcium [Ca^{2+}] ions from intracellular stores, followed by entry of Ca^{2+} via membrane channels (Mellado, Rodríguez-Frade, Mañes, *et al.*, 2001). Following addition of the cognate receptor agonist, cytoplasmic [Ca^{2+}] rises are almost instant, peaking within seconds before gradually restoring to basal levels. All chemokines show an optimal concentration for induction of chemotaxis, and higher or lower concentrations than this induce a partial response, resulting in a bell-shaped curve. With calcium responses, the response profile follows a sigmoidal response profile, in that adding higher concentration will not cause the signal to decrease. Using 300-19-CCR7 cells, CCL19 and CCL21 dilutions ranging from 0.1 nM to 1000 nM (0.1-10 nM shown) were tested to establish optimal, sub-optimal and inactive concentrations for triggering calcium release (**Figure 3.5**). It was observed that concentrations equal to or higher than 100 nM CCL19 and CCL21 elicited maximal rises in intracellular Ca^{2+}. Concentrations of 1 nM CCL19 elicited a response in some experiments, whereas 1 nM CCL21 failed to evoke a response. The differences observed between CCL19 and CCL21 in calcium responses do not agree with published findings (Ogilvie *et al.*, 2001; Bardi, Niggli and Loetscher, 2003; Kohout *et al.*, 2004; Otero, Groettrup and Legler, 2006). Surprisingly, in some experiments CXCL14 induced a response in 300-19-CCR7 cells, conflicting with chemotaxis results (data not shown). This could be experiment-to-experiment variation. Importantly, the combination of 0.1 nM CCL21 with 300 nM CXCL14 showed no induction of Ca^{2+} release, while 1 nM CCL21 showed a synergistic effect with 300 nM CXCL14. For CCL19, the synergistic effect was clearer and dose dependent, as both 0.1 nM and 1 nM combined with 300 nM CXCL14 induced transient [Ca^{2+}] spikes, with 1 nM having a greater effect. The synergy between CXCL14 and CCR7 ligands CCL19 and CCL21 is therefore not only limited to chemotaxis but extends to fast acting cellular responses like calcium flux.

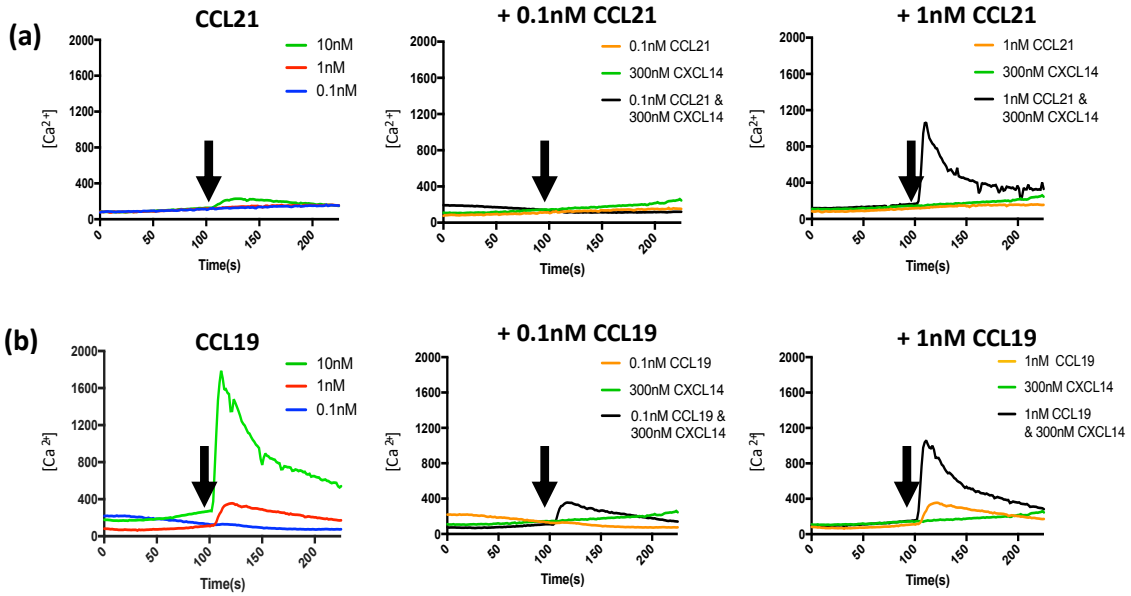


Figure 3.5. CXCL14 synergises with CCL19 and CCL21 in the induction of rapid cellular responses.

Changes in cytoplasmic free calcium [Ca^{2+}] concentration in 300-19-CCR7 cells upon addition of chemokines were monitored using a spectrophotometer. Cells were loaded with 1 µM Fura-2-AM and stimulated with chemokine. Chemokine was injected at 100 seconds (as indicated by arrows above), recording was stopped at 300 seconds and 5 mM digitonin was added at 230 seconds. **(a)** Changes in cytoplasmic free Ca^{2+} upon addition of various concentrations of CCL21 (top left), combination of 0.1 nM CCL21 and 300 nM CXCL14 (top centre) or 1 nM CCL21 and 300 nM CXCL14 (top right). **(b)** Changes in cytoplasmic free Ca^{2+} upon addition of various concentrations of CCL119 (bottom left), combination of 0.1 nM CCL19 and 300 nM CXCL14 (bottom centre) or 1 nM CCL19 and 300 nM CXCL14 (bottom right). One representative set of measurements from 4-5 independent experiments is shown.

3.3.3 CXCL14 does not Affect CCR7 Cell Surface Expression on CCR7-Transfectant 300-19 Cells

In addition to activation of signalling pathways, chemokine binding to its cognate receptor also induces regulatory processes, such as desensitisation of the receptor (**Figure 1.5**) (Baggiolini, 1995; Baggiolini, Dewald and Moser, 1997). This ensures decreased responsiveness of GPCRs to repeated or prolonged exposure to agonist. Since my results showed that CXCL14 synergises with CCL19 and CCL21 in the induction of chemotactic and calcium responses, I wanted to further examine the mechanism behind this synergy by looking at the induction of receptor internalisation. 300-19-CCR7 cells were incubated with chemokine for one hour at 37°C and surface receptor expression was subsequently assessed by flow cytometry. Mean fluorescence intensity (MFI) of CCR7 expression was normalised to cells incubated for one hour in medium alone (no chemokine). Incubation with 100 nM CCL21 induced minimal decrease in receptor expression (87.8 ± 6.6%), as opposed to incubation with 100 nM CCL19 (53.5 ± 7%) (**Figure 3.6**). This difference between CCL19 and CCL21 was consistent with published findings (Kohout *et al.*, 2004; Otero, Groettrup and Legler, 2006). As expected, suboptimal concentrations of CCL19 and CCL21 (1 nM), or 300 nM CXCL14 on their own showed reduction on receptor expression. Combining 300 nM CXCL14 with 1 nM CCL21 induced a slight reduction in chemokine receptor expression, compared to 1 nM CCL21 alone (92.4 ± 4% vs. 88.8 ± 10.5%, respectively) (**Figure 3.6a**). Similarly, combining 1 nM CCL19 with 300 nM CXCL14 produced a minor effect in receptor expression, compared to 1 nM CCL19 alone (92.9 ± 4.2% vs. 89.8 ± 2.9%, respectively) (**Figure 3.6b**). These results show that CXCL14 did not synergise with CCL19 and CCL21 in the decrease of cell surface receptor.

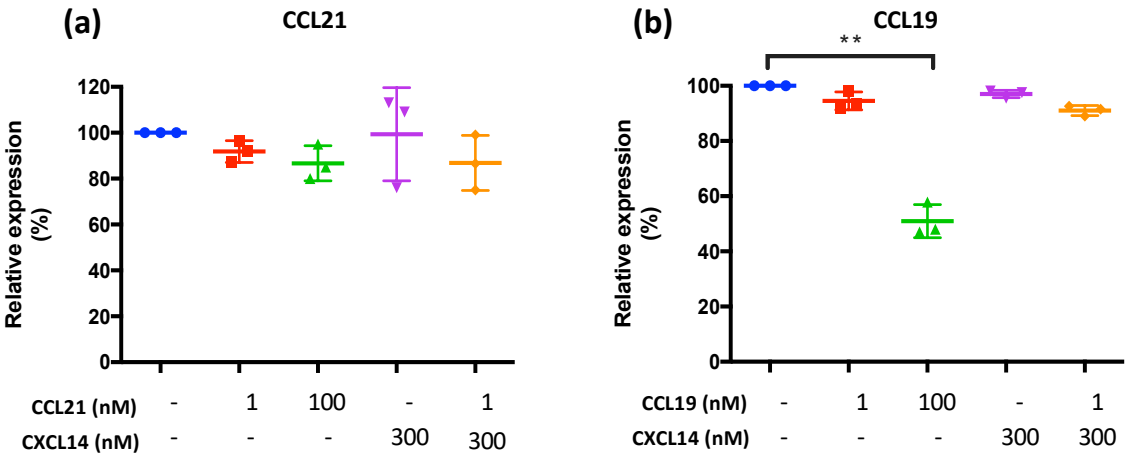


Figure 3.6. CXCL14 does not decrease cell surface expression of CCR7.
300-19-CCR7 cells were stimulated with medium only (blue), 1 nM CCL19/CCL21 (red), 100 nM CCL19/CCL21 (green), 300 nM CXCL14 (purple) or 1 nM CCL19/CCL21 and 300 nM CXCL14 (orange). Cells were stimulated for one hour at 37 °C, followed by surface CCR7 staining and detection by flow cytometry. MFI values (geometric mean) in each condition were collected and normalised to medium only (given as 100%). **(a)** Surface CCR7 expression in response to incubation with CCL21 and/or CXCL14. **(b)** Surface CCR7 expression in response to incubation with CCL19 and/or CXCL14. Mean + SD of three independent experiments are shown. **p<0.01 and compared to medium alone using the Friedman test followed by Dunn's multiple comparisons test.

3.3.4 CXCL14 Synergises with Homeostatic Chemokine CXCL13 in the Induction of Chemotactic Responses

Besides CCR7, the homeostatic receptor CXCR5 also plays a crucial role in lymphocyte traffic within secondary lymphoid tissues. It is highly expressed on B cells and involved in the formation of the B-cell compartment, whereas its expression on T cells is limited to follicular B helper T (T_{FH}). B cells isolated from healthy donors showed uniform expression of CXCR5 on the cell surface (**Figure 3.7a**). The potency of CXCL13 is known to be moderate, as chemotactic responses were only observed towards 1 µM CXCL13, which is comparable to earlier reports (Legler *et al.*, 1998). Lower concentrations of CXCL13 only became active when combined with varying concentrations of CXCL14 (**Figure 3.7b**). Combination of 100 nM CXCL13 with increasing concentrations of CXCL14 displayed robust B cell migration in a dose-dependent manner. These results were replicated in 300-19 cells stably transfected with CXCR5 (300-19-CXCR5 cells) (**Figure 3.8**) (Legler *et al.*, 1998). As with primary B cells, 300-19-CXCR5 cells demonstrated migration towards 1 µM CXCL13 only, with no migration detected at lower concentrations (**Figure 3.8b**). Combination of CXCL14 with either 10 nM or 100 nM CXCL13 resulted in significant migratory responses, compared to the respective concentration of CXCL13 alone, showing strong synergistic interaction between CXCL14 and CXCL13.

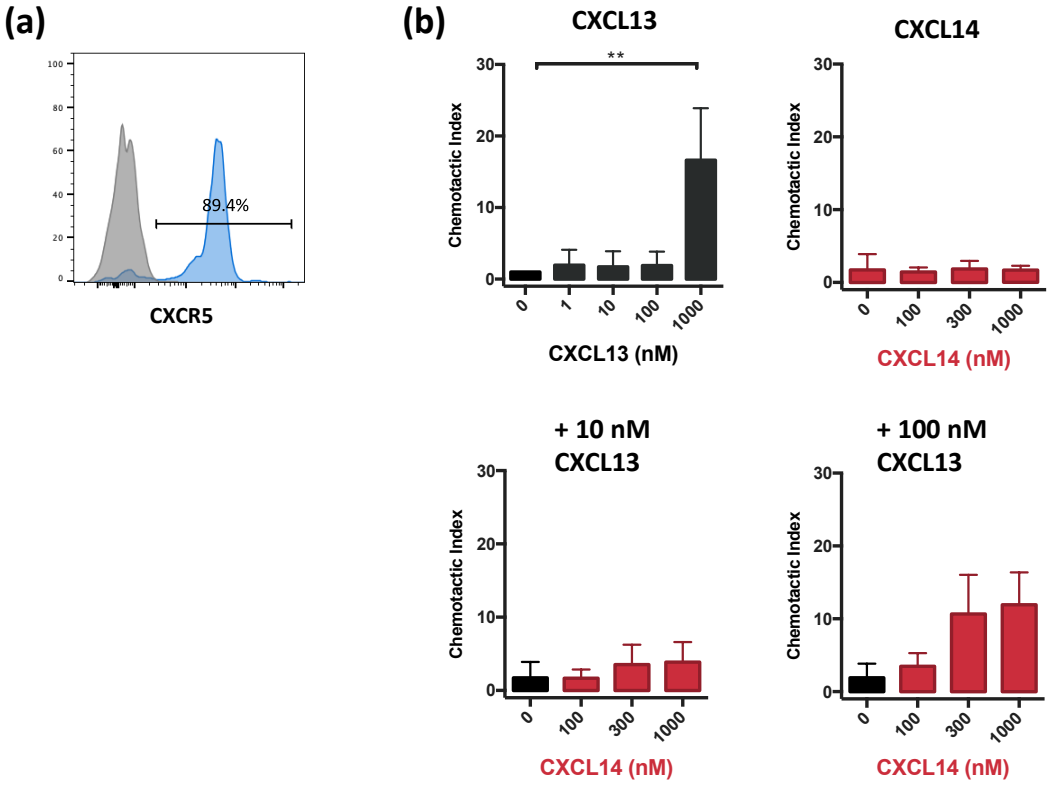


Figure 3.7. CXCL14 synergises with the CXCR5 ligand CXCL13 in the induction of chemotactic responses in primary human B cells.

PBMC were isolated from peripheral blood and rested overnight in medium. The next day, phenotyping was performed for expression of chemokine markers and migration towards chemokines was assessed by transwell chemotaxis assay. **(a)** Surface expression of CXCR5 on B cells (blue histogram), assessed by flow cytometry. Grey histogram indicates staining with fluorescence-minus one control. **(b)** Migration of B cells toward CXCL13 (top left), CXCL14 (top right), 10 nM CXCL13 & CXCL14 (bottom left) or 100 nM CXCL13 & CXCL14 (bottom right). Data are combined with results from Dr Paul Collins (Dr Collins contributed 2-3 donors). Data are mean + SD of 6-7 independent experiments using PBMC from different donors. *p<0.05 and **p<0.01 compared to 0 nM using Friedman test followed by Dunn's multiple comparisons test.

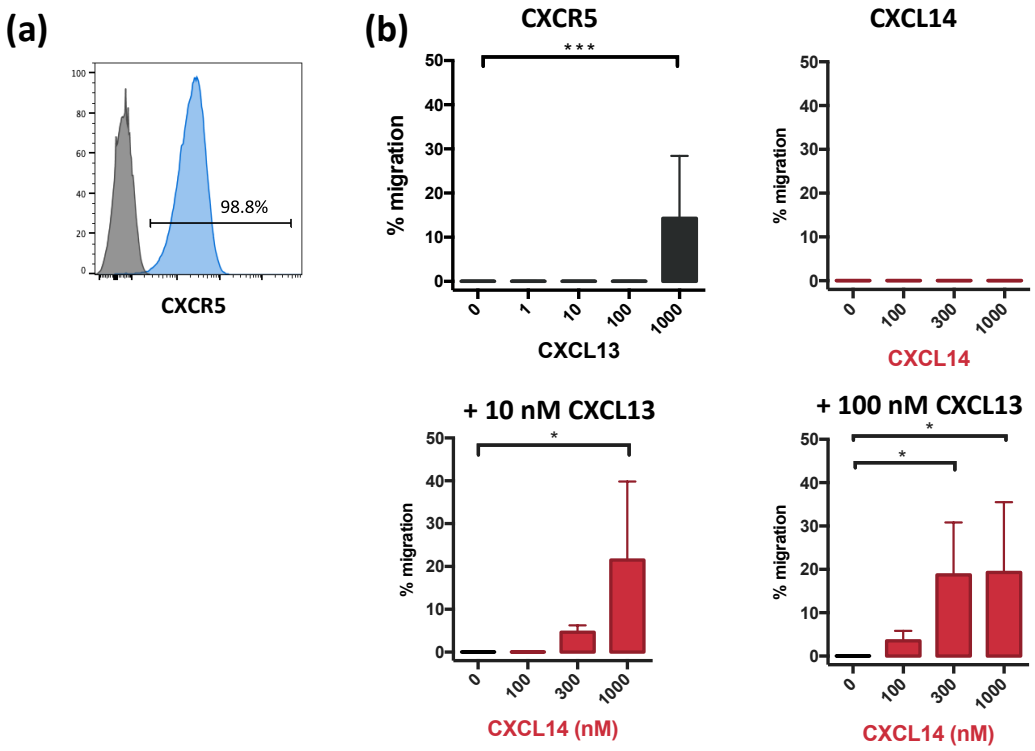


Figure 3.8. CXCL14 synergises with CXCL13 in the induction of chemotactic responses in 300-19 cells stably transfected with CXCR5.

(a) Surface receptor expression on 300-19 cells stably transfected with CXCR5 was confirmed by flow cytometry (blue histogram). Grey histogram shows staining with Fluorescence-minus one control. **(b)** Migration of 300-19-CXCR5 cells toward CXCL13 (top left), CXCL14 (top right), 10 nM CXCL13 & CXCL14 (bottom left) or 100 nM CXCL13 & CXCL14 (bottom right). Data are mean + SD of 4-8 independent experiments, where *p<0.05 and ***p<0.001 compared to 0 nM using either one-way ANOVA, followed by Holm-Sidak's multiple comparisons test (CXCL13) or Friedman test, followed by Dunn's multiple comparisons test.

3.3.5 CXCL14 Synergises with CXCL13 in the Induction of Ca^{2+} Release, but not in Receptor Cell Surface Expression

Considering that CXCL14 showed synergism with CCR7 and CXCR4 in the induction of Ca^{2+} mobilisation from intracellular stores, I next wanted to look into its induction of calcium responses with CXCR5. Using 300-19-CXCR5 cells, dilutions of CXCL13 ranging from 1 µM to 0.01 nM (100-0.1 nM shown) were tested to establish optimal, sub-optimal and inactive concentrations for triggering calcium mobilisation (**Figure 3.9**). Concentrations of as low as 10 nM CXCL13 induced responses, which was completely inactive in triggering chemotactic responses. This finding is consistent with published findings (Legler *et al.*, 1998). I observed that maximal rise in intracellular [Ca^{2+}] was achieved by 100 nM CXCL13, reaching a plateau at higher concentrations. Combination of 0.1 or 1 nM CXCL13 with 300 nM CXCL14 showed a synergistic interaction between the two chemokines in inducing calcium responses.

Receptor cell surface expression experiments showed a robust effect with 1 µM CXCL13 (11.81 ± 2.9%), compared to the suboptimal concentration 100 nM CXCL13 (72.9 ± 14.5%) (**Figure 3.10**). The sensitivity of cell surface receptor expression experiments seems to be comparable to migration experiments, in terms of chemokine concentration needed to induce a clear response, as opposed to calcium mobilisation. Combination of 100 nM CXCL13 with 300 nM CXCL14 showed a decreased but not significant receptor expression on the cell surface (66.5 ± 16.4%). Collectively, these results show that CXCL14 can not only synergise with CXCL12, but also with other homeostatic chemokines such as CCL19, CCL21 and CXCL13.

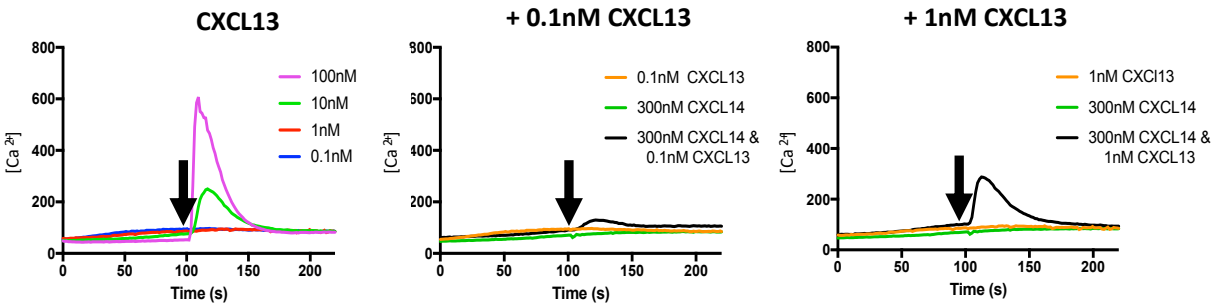


Figure 3.9. CXCL14 synergises with CXCL13 in the induction of rapid cellular responses. Changes in cytoplasmic free calcium [Ca^{2+}] concentration in 300-19-CXCR5 cells upon addition of chemokines were monitored using a spectrophotometer. Cells were loaded with 1 µM Fura-2-AM and stimulated with chemokine. Chemokine was injected at 100 seconds (as indicated by the arrows) and recording was stopped at 300 seconds. Changes in cytoplasmic free Ca^{2+} upon addition of various concentrations of CXCL13 (left), combination of 0.1 nM CXCL13 and 300 nM CXCL14 (centre) or 1 nM CXCL13 and 300 nM CXCL14 (right). One representative set of measurements from 4-5 independent experiments is shown.

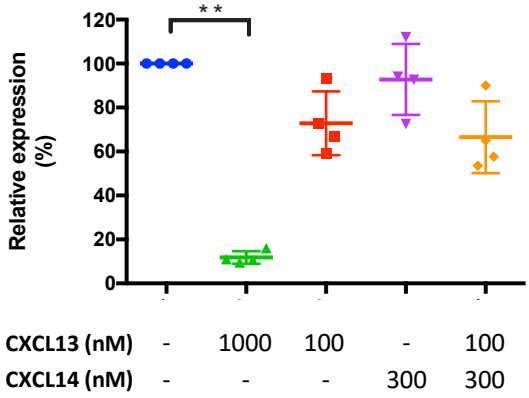


Figure 3.10. CXCL14 does not decrease cell surface expression of CXCR5. 300-19-CXCR5 cells were stimulated with medium only (blue), 1 µM CXCL13 (red), 100 nM CXCL13 (green), 300 nM CXCL14 (purple) or 100 nM CXCL13 and 300 nM CXCL14 (orange). Cells were stimulated for one hour at 37°C, followed by surface CXCR5 staining and detection by flow cytometry. MFI values (geometric mean) in each condition were collected and normalised to medium only (shown as 100%). Mean + SD of four independent experiments are shown. **p<0.01 compared to medium alone using Friedman test followed by Dunn's multiple comparisons test.

3.4 Study of CXCL14 Synergism with Inflammatory Chemokines

CXCL14 so far showed synergism with all homeostatic chemokines tested, including the CXCR4 ligand CXCL12 (Collins *et al.*, 2017), the CCR7 ligands CCL19 and CCL21 and the CXCR5 ligand CXCL13 (as shown above). Unlike homeostatic chemokines, inflammatory chemokines are not constitutively expressed at high levels in steady state conditions. Their expression is upregulated during inflammatory conditions and allows for a relatively transient attraction of inflammatory leukocytes to infected or inflamed tissues. There is already evidence that homeostatic and inflammatory chemokines can synergise, including CXCL12 with CCR5 ligand CCL5 and CXCR3 ligands CXCL9, CXCL10 and CXCL11 (Krug *et al.*, 2002; Vanbervliet *et al.*, 2003; Meller *et al.*, 2007; Venetz *et al.*, 2010; Gouwy *et al.*, 2011). Although CXCL14 is a homeostatic chemokine and its expression is lost in some inflammatory settings, it may be upregulated in others (Chen *et al.*, 2010). Given the fact that inflammatory chemokines are readily expressed in peripheral tissues in response to infection or tissue injury, I wanted to investigate the possibility of CXCL14 synergising with inflammatory chemokines. Our group previously saw that CXCL14 does not synergise with the monocyte chemoattractant CCR2 ligand CCL2 (Collins *et al.*, 2017). Therefore, I wanted to expand our investigation to other key inflammatory chemokines, including the CCR5 ligand CCL5, as well as the CXCR3 ligands CXCL10 and CXCL11. As already mentioned, these chemokines were selected as they are some of the most important players in the initiation of immune responses (Griffith, Sokol and Luster, 2014).

3.4.1 CXCL14 does not Synergise with the Inflammatory Chemokine CCL5

Synergy between CXCL14 and CCL5 was first tested on 300-19 cells stably transfected with CCR5 (300-19-CCR5 cells) (P. Loetscher *et al.*, 1998). Expression of CCR5 on the cell surface was confirmed by flow cytometry (**Figure 3.11a**). CCL5 was able to induce robust and reproducible chemotactic responses (**Figure 3.11b**). In contrast to homeostatic chemokines, CCL5 concentration as low as 0.01 nM was sufficient to induce migration, while peak responses were observed at concentrations as low as 1 nM CCL5 (0.01-1000 nM CCL5 tested). 0.1 and 1 nM CCL5 were tested as suboptimal concentrations for investigating synergism with CXCL14. There was no synergism observed between

CXCL14 and CCL5. In fact, addition of CXCL14 appeared to decrease the migration potential of the transfectants towards 0.1 and 1 nM CCL5.

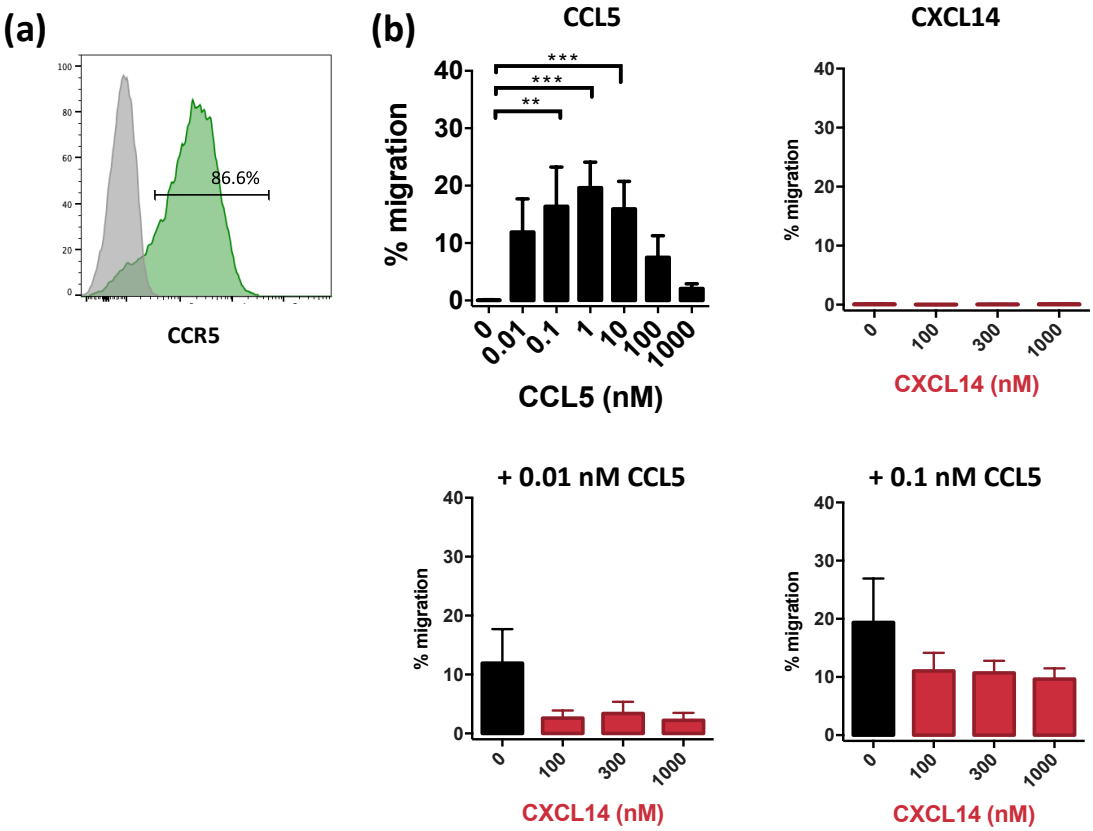


Figure 3.11. CXCL14 does not synergise with CCL5 in the induction of chemotactic responses in 300-19 cells stably transfected with CCR5.

(a) Surface receptor expression on 300-19 cells stably transfected with CCR5 was confirmed by flow cytometry (green histogram). Grey histogram shows staining with appropriate isotype control. **(b)** Migration of 300-19-CCR5 cells toward CCL5 (top left), CXCL14 (top right), 0.01 nM CCL5 & CXCL14 (bottom left) or 0.1 nM CCL5 & CXCL14 (bottom right). Data are combined with results from Dr Collins (Dr Collins contributed 1-3 repeats). Data are mean + SEM of 5-6 independent experiments, where **p<0.01 and ***p<0.001 compared to 0 nM using Friedman test, followed by Dunn's multiple comparisons test.

In order to confirm the absence of any synergistic interaction between CXCL14 and CCL5, human T cell lines expressing CCR5 were generated. Freshly isolated T cells from peripheral blood had low expression of inflammatory chemokine receptors on their surface (**Figure 3.12a**), which was increased upon activation and tissue culture according to established protocols (Loetscher *et al.*, 1996; Qin *et al.*, 1998). T cells were expanded for three weeks in the presence of IL-2 and IL-15 to establish high level expression of inflammatory chemokine receptors on their surface, as described in Chapter 2. Surface receptor expression on $CD4^+$ and $CD8^+$ T cells, and migratory responses towards CCL5 were confirmed on day 21 of expansion. Although $CD8^+$ T cells showed higher level of receptor expression on the cell surface, the migratory responses were almost identical to $CD4^+$ T cells (**Figure 3.12b**). The inhibitory effect of CXCL14 when combined with suboptimal concentrations of CCL5 was not as pronounced on primary T cells, compared to 300-19-CCR5 cells. This could be explained by the fact that the day 21 cell lines produced contained 60% and 30% CCR5-negative populations within the $CD4^+$ and $CD8^+$ populations, respectively. The presence of these populations could be neutralising the inhibitory effect observed in the transfected cells. Also, the 300-19-CCR5 cells showed a greater and more uniform receptor expression.

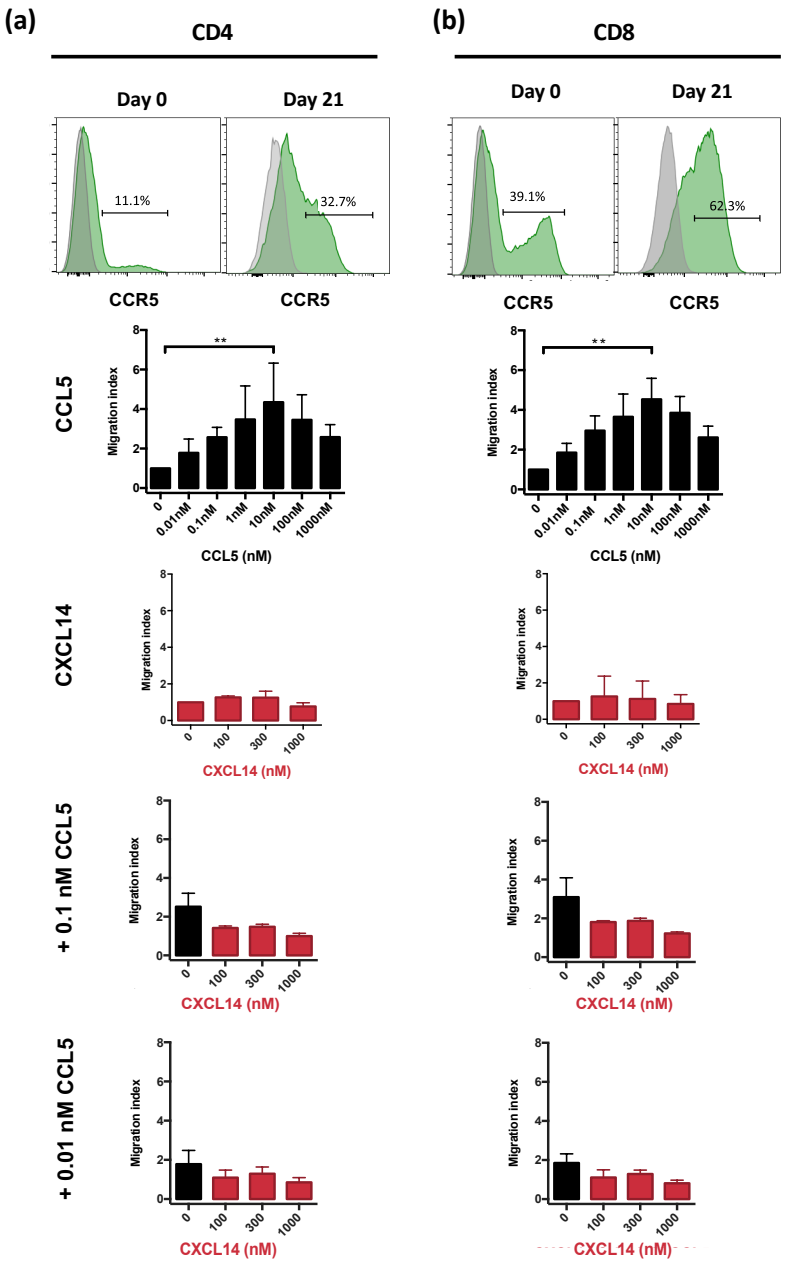


Figure 3.12. CXCL14 does not synergise with inflammatory chemokine CCL5 on primary human T cells.

Total CD3$^+$ T cells were isolated from PBMC using negative selection and stimulated with anti-CD3/CD28 beads in the presence of IL-2 and IL-15. **(a)** Representative histograms show expression of CCR5 by CD4$^+$ or CD8$^+$ T cells on day 0 and day 21 of expansion. Grey histograms represent isotype control staining. **(b)** Day 21-expanded T cells were used in transwell chemotaxis assays to test migration responses to CCL5. Migrated cells were collected after four hours. Migration of CD4$^+$ T cells toward CCL5 (top left), 0.1 nM CCL5 and CXCL14 (top centre) or 0.01 nM CCL5 and CXCL14 (top right). Migration of CD8$^+$ T cells toward CCL5 (bottom left), 0.1 nM CCL5 and CXCL14 (bottom centre) or 0.01 nM CCL5 and CXCL14 (bottom right). Data are mean + SD of 2-3 independent experiments using PBMC from different donors. *p<0.05 compared to 0 nM using Friedman test followed by Dunn's multiple comparisons test.

Next, the effect of CXCL14 on CCL5-mediated signal transduction was investigated in 300-19-CXCR5^{+} cells, including intracellular Ca^{2+} release and induction of receptor internalisation. CCL5 was titrated to establish maximal and non-optimal calcium responses (**Figure 3.13**). Conversely to chemotaxis, peak calcium responses were observed with 100 nM CCL5, whereas 0.01 nM induced a minor response. To answer the question of synergism, 0.01, 0.1 and 1 nM CCL5 were tested in combination with 300 nM CXCL14. Unexpectedly, CXCL14 showed reproducible synergistic interaction with CCL5, in contrast to its inhibitory effect on CCL5-mediated chemotactic migration. Additionally, CXCL14 alone induced a small rise in intracellular [Ca^{2+}]. Regarding receptor cell surface expression, incubation with 1 nM CCL5 somewhat reduced receptor expression, with around a 50% reduction of CCR5 MFI (**Figure 3.14**). Interestingly, 300 nM CXCL14 alone induced, approximately, a 12% decrease in MFI. Combination of 0.1 nM CCL5 and 300 nM CXCL14 caused a slight decrease in CCR5 MFI (50.14 ± 6.8% vs 63.3 ± 4.9%, respectively). Combination of CXCL14 with 0.01 nM CCL5 showed a less marked decrease in the level of cell surface CCR5 as compared to CCL5 alone.

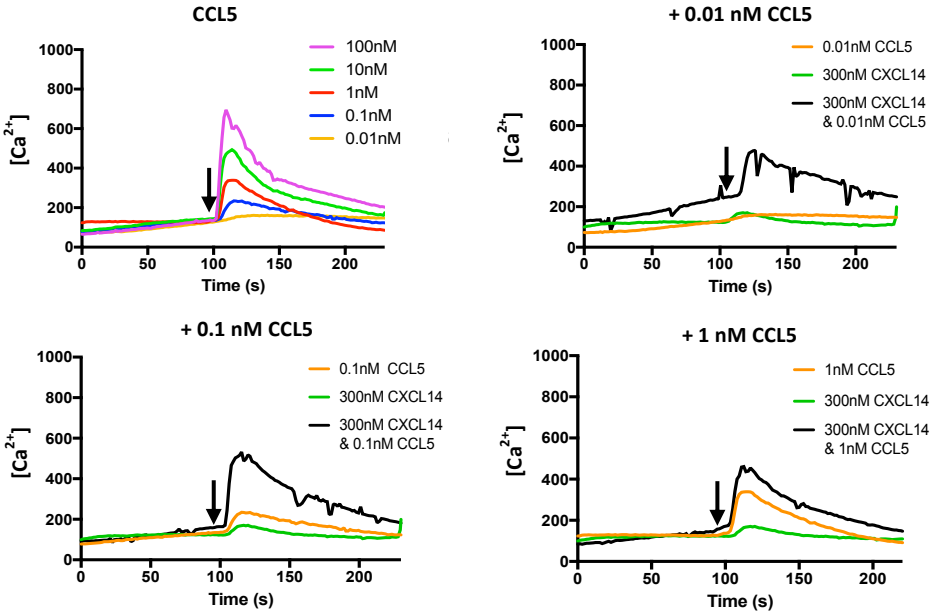


Figure 3.13. CXCL14 synergises with CCL5 in the induction of rapid cellular responses. Changes in cytoplasmic free calcium [Ca^{2+}] concentration in 300-19-CCR5 cells upon addition of chemokines were monitored using a spectrophotometer. Cells were loaded with 1 µM Fura-2-AM and stimulated with chemokine. Chemokine was injected at time indicated by arrow and recording was stopped at 300 seconds. Changes in cytoplasmic free Ca^{2+} upon addition of various concentrations of CCL5 (top left), combination of 0.01 nM CCL5 and 300 nM CXCL14 (top right), 0.1 nM CCL5 and 300 nM CXCL14 (bottom left) or 1 nM CCL5 and 300 nM CXCL14 (bottom right). One representative set of measurements from 3 independent experiments is shown.

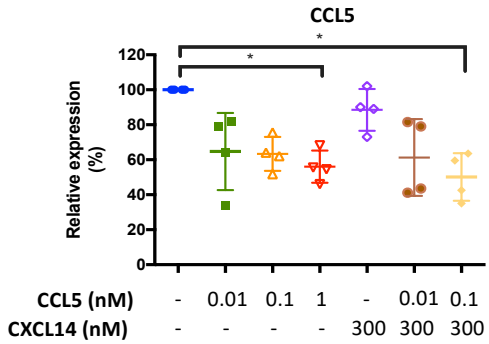


Figure 3.14. CXCL14 does not decrease cell surface expression of CCR5. 300-19-CCR5 cells were stimulated with medium only (blue), 0.01 nM CCL5 (green), 0.1 nM CCL5 (orange), 1 nM CCL5 (red), 300 nM CXCL14 (purple), 0.01 nM CCL5 and 300 nM CXCL14 (brown) or 0.1 nM CCL5 and 300 nM CXCL14 (yellow). Cells were stimulated for one hour at 37°C, followed by surface CCR5 staining and detection by flow cytometry. MFI values (geometric mean) in each condition were collected and normalised to medium only (shown as 100). Mean + SD of four independent experiments is shown. *$p<0.05$ and compared to medium alone using Friedman test followed by Dunn's multiple comparisons test.

3.4.2 CXCL14 does not synergise with the Inflammatory Chemokines CXCL10 and CXCL11

The other inflammatory chemokines tested were CXCL10 and CXCL11, ligands for the chemokine receptor CXCR3. CXCR3 is strongly expressed on type-1 helper $CD4^+$ (Th1) T cells, effector $CD8^+$ T cells and certain innate lymphocytes, such as natural killer (NK) cells and NKT cells (Groom and Luster, 2011). Its main role is navigating effector T cells to infected tissues under IFN-γ-driven inflammatory conditions (Loetscher *et al.*, 1996). To examine synergism between CXCL14 and CXCR3 ligands, I first tested 300-19 cells transfected with CXCR3 (300-19-CXCR3 cells) (Loetscher *et al.*, 1996). Following confirmation of receptor expression (**Figure 3.15a**), cell migration towards CXCL10 and CXCL11 was assessed. Both ligands induced robust migration, with a greater percentage of cells migrating towards CXCL11 (**Figure 3.15b, c**). This is consistent with published findings demonstrating that CXCL11 induces greater chemotaxis in both primary and transfected cells (Cole *et al.*, 1998). Peak migration for both ligands was observed at 100 nM, therefore 0.1 nM and 1 nM were selected as inactive concentrations to test synergism with CXCL14. One in six replicates gave a migratory response towards the lowest concentration of CXCL14, which deforms the figure (**Figure 3.15b)**. This was considered to be an experimental error, since the rest of the replicates were ~100 times lower. In contrast to CCL5, suboptimal concentrations of both CXCL10 and CXCL11 became active in the presence of CXCL14. This effect was more evident with 1 nM of CXCL10/CXCL11.

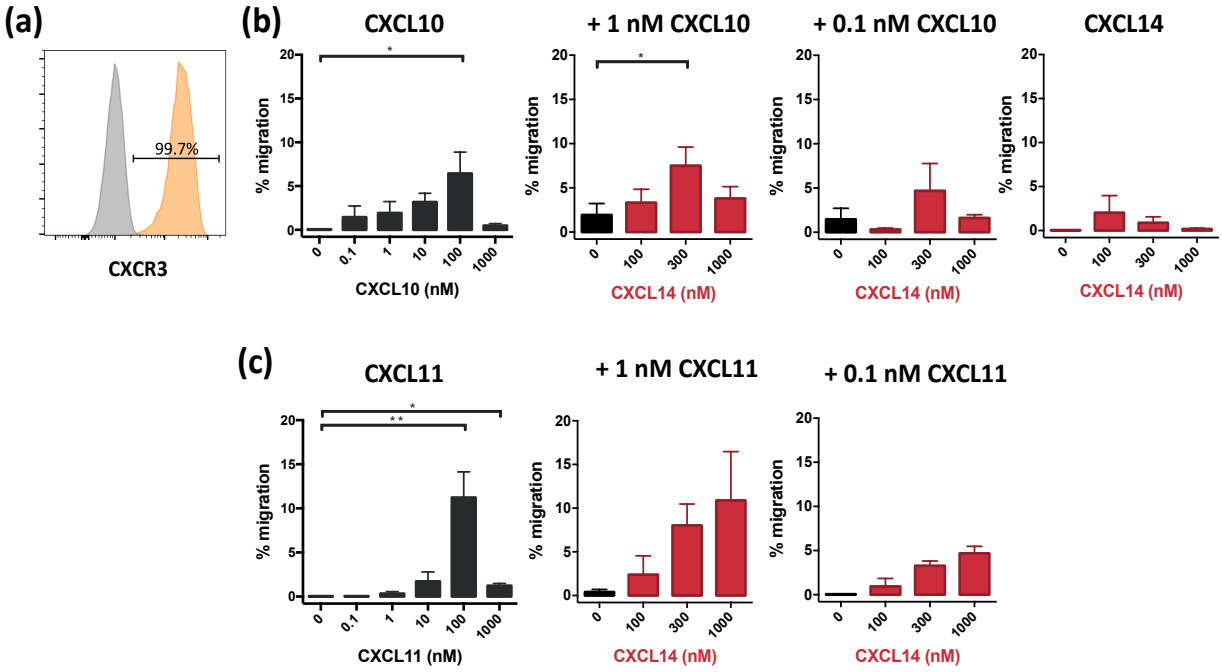


Figure 3.15. CXCL14 synergises with the inflammatory chemokines CXCL10 and CXCL11 in the induction of chemotactic responses in 300-19 cells stably transfected with CXCR3.

(a) Surface receptor expression on 300-19 cells stably transfected with CXCR3 was confirmed by flow cytometry (orange histogram). Grey histogram shows staining with fluorescence-minus one control. **(b)** Migration of 300-19-CXCR3 cells toward CXCL10 (top left), CXCL14 (top right), 0.1 nM CXCL10 & CXCL14 (top centre left) or 1 nM CXCL10 & CXCL14 (top centre right). **(c)** Migration of 300-19-CXCR3 cells toward CXCL11 (bottom left), 0.1 nM CXCL11 & CXCL14 (bottom centre) or 1 nM CXCL11 & CXCL14 (bottom right). Data are mean + SEM of 3-6 independent experiments, where *p< 0.05 and **p<0.01 compared to 0 nM using Friedman test, followed by Dunn's multiple comparisons test.

Taking into account that CXCR3 is mainly expressed on activated T cells, freshly isolated T cells were stimulated with anti-CD3-CD28 beads and then expanded for 21 days in the presence of IL-2 and IL-15. Receptor expression on the cell surface was confirmed by flow cytometry (**Figure 3.16a**) and cell migration towards CXCL10 was assessed (**Figure 3.16b**). Both $CD4^+$ and $CD8^+$ T cells migrated towards CXCL10, with maximal responses at 100 nM. CXCL14 alone was not active and combined with CXCL10 or CXCL11 did not induce synergistic T cell migration responses.

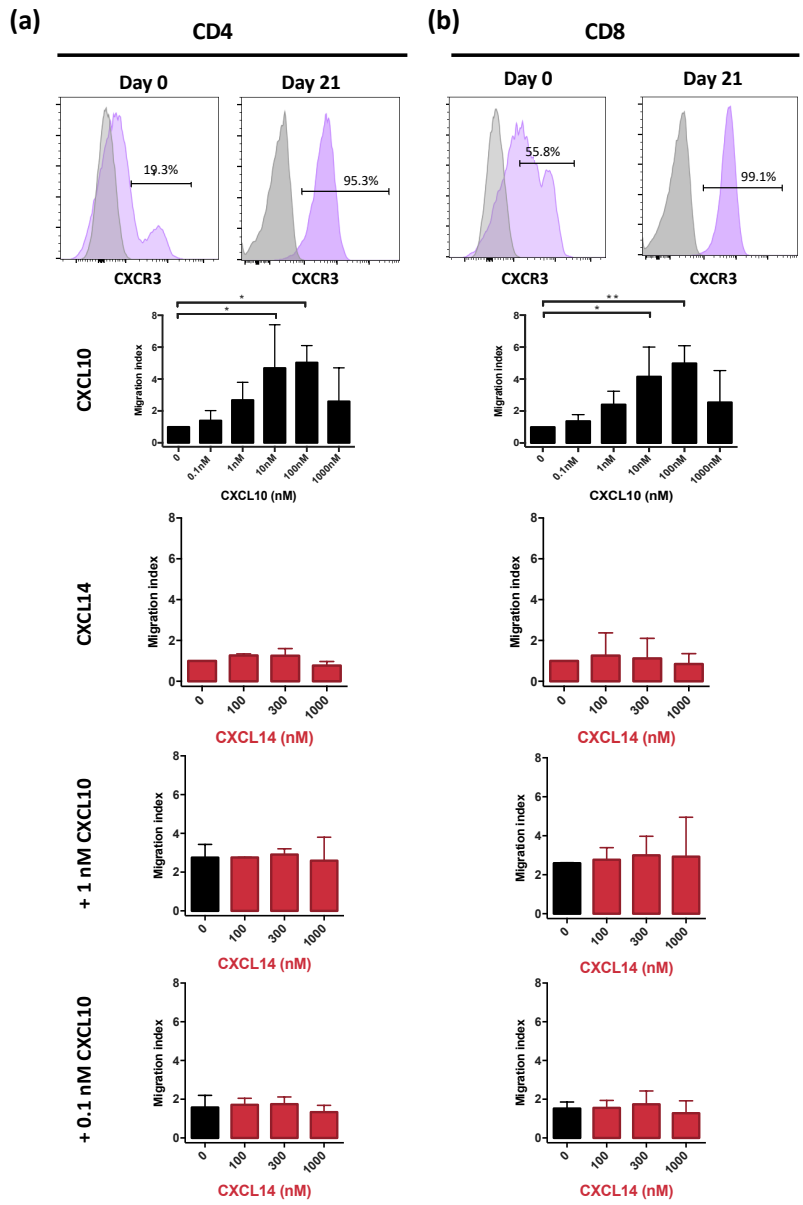


Figure 3.16. CXCL14 does not synergise with inflammatory chemokine CXCL10 on primary human T cells.

Total CD3$^+$ T cells were isolated from PBMC using negative selection and stimulated with anti-CD3/CD28 beads in the presence of IL-2 and IL-15. **(a)** Representative histograms show expression of CXCR3 by CD4$^+$ or CD8$^+$ T cells on day 0 and day 21 of expansion. Grey histograms represent isotype control staining. Populations shown are gated on single, live, CD3$^+$ cells. **(b)** Day 21- expanded T cells were used in transwell chemotaxis assays to test migration responses to CXCL10. Migrated cells were collected after four hours. Migration of CD4$^+$ T cells toward CXCL10 (top left), 1 nM CXCL10 & CXCL14 (top centre) or 0.1 nM CXCL10 & CXCL14 (top right). Migration of CD8$^+$ T cells toward CXCL10 (bottom left), 1 nM CXCL10 & CXCL14 (bottom centre) or 0.1 nM CXCL10 & CXCL14 (bottom right). Data are mean + SD of 2-4 independent experiments using PBMC from different donors. *p< 0.05 and **p<0.01 compared to 0 nM using Friedman test followed by Dunn's multiple comparisons test.

To further examine the synergistic interaction between CXCR3 ligands and CXCL14, I looked into calcium responses and receptor internalisation on 300-19-CXCR3 cells (**Figure 3.17**). CXCL11 is known to bind CXCR3 with highest affinity and induce greater calcium mobilisation than CXCL9 and CXCL10 (Cole *et al.*, 1998; Meyer *et al.*, 2001). Indeed, CXCL11 induced higher intracellular [Ca^{2+}] at 10 nM than CXCL10 but the signal observed at 100 nM was comparable to that of CXCL10. Of note, both concentrations of CXCL11 became active in combination with 300 nM CXCL14, as opposed to only 1 nM CXCL10 becoming active in the presence of CXCL14.

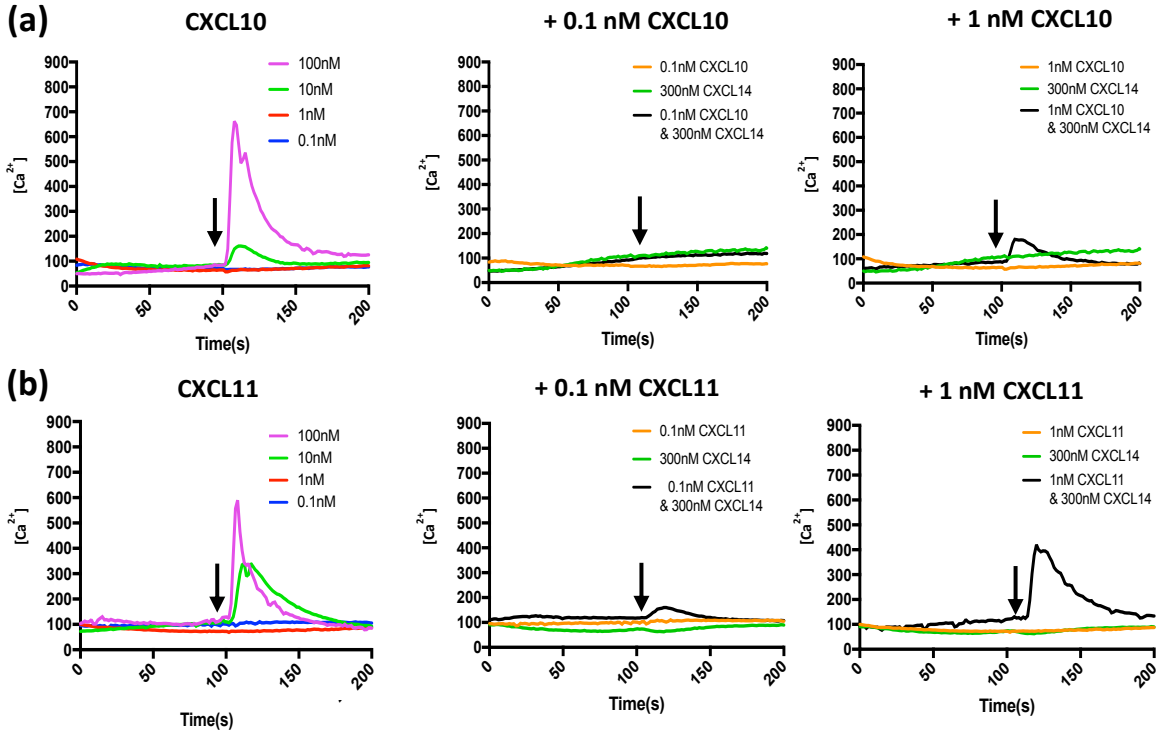


Figure 3.17. CXCL14 synergises with CXCL10 and CXCL11 in the induction of rapid cellular responses.

Changes in cytoplasmic free calcium [Ca^{2+}] concentration in 300-19-CXCR3 cells upon addition of chemokines were monitored using a spectrophotometer. Cells were loaded with 1 µM Fura-2-AM and stimulated with chemokine. Chemokine was injected at 100 seconds (as indicated by the arrows) and recording was stopped at 300 seconds. **(a)** Changes in cytoplasmic free Ca^{2+} upon addition of various concentrations of CXCL10 (top left), combination of 0.1 nM CXCL10 and 300 nM CXCL14 (top centre) or 1 nM CXCL10 and 300 nM CXCL14 (top right). **(b)** Changes in cytoplasmic free Ca^{2+} upon addition of various concentrations of CXCL11 (bottom left), combination of 0.1 nM CXCL11 and 300 nM CXCL14 (bottom centre) or 1 nM CXCL11 and 300 nM CXCL14 (bottom right). One representative set of measurements from 3-4 independent experiments is shown.

CXCL11 is also the predominant CXCR3 ligand responsible for induction of CXCR3 internalisation, both in primary and transfected cells (Sauty *et al.*, 2001; Rajagopal *et al.*, 2013). As expected, CXCL11 induced significant reduction of CXCR3 cell surface expression as opposed to CXCL10, with a notable 75% decrease in MFI, compared to medium alone (**Figure 3.18**). While 1 nM CXCL10 alone induced minimal decrease in receptor expression (93.5 ± 4.1%), its combination with 300 nM CXCL14 induced a greater but not significant effect (70.5 ± 14.6%). The difference between 1 nM CXCL11 alone (57.31 ± 3.2%) and combined with 300 nM CXCL14 (49.9 ± 11.3%) was not as striking.

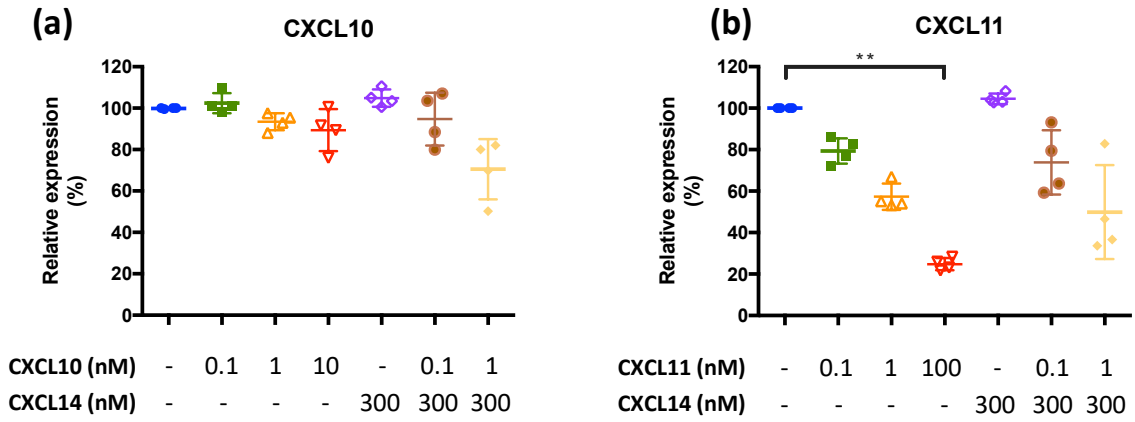


Figure 3.18. CXCL14 effect on CXCR3 cell surface expression.
300-19-CXCR3 cells were stimulated with medium only (blue), 0.1 nM CXCL10/CXCL11 (green), 1 nM CXCL10/CXCL11 (orange), 100 nM CXCL10/CXCL11 (red), 300 nM CXCL14 (purple), 0.1 nM CXCL10/CXCL11 and 300 nM CXCL14 (brown) or 1 nM CXCL10/CXCL11 and 300 nM CXCL14 (yellow). Cells were stimulated for one hour at 37 °C, followed by surface CXCR3 staining and detection by flow cytometry. MFI values (geometric mean) in each condition were collected and normalised to medium only (shown as 100%). **(a)** Surface CXCR3 expression in response to incubation with CXCL10 and/or combination with CXCL14. **(b)** Surface CXCR3 expression in response to incubation with CXCL11 and/or combination with CXCL14. Mean + SD of four independent experiments is shown. **p<0.01 compared to 0 nM using Friedman test followed by Dunn's multiple comparisons test.

Taken together, these results show that CXCL14 interacts with CCL5, as demonstrated by intracellular Ca^{2+} release and receptor internalisation. However, this interaction does not translate to migratory responses. In terms of synergism, CXCL14 appears to induce a decrease of migratory responses in combination with CCL5 but causes an increase in calcium mobilisation. Receptor internalisation was slightly, but not significantly, increased following incubation with CCL5 and CXCL14. For CXCR3, CXCL14 appears to synergise with both CXCL11 and CXCL10 in inducing migratory responses in 300-19 transfectants. However, this effect was not replicated in primary cells. Both CXCL10 and CXCL11 synergised with CXCL14 in the induction of intracellular Ca^{2+} release and there was a downward trend in CXCR3 expression, but there were no significant changes.

3.5 CXCL14 Synergism with CXCL12 in the Murine System

3.5.1 Analysis of *In Vitro* Synergism Between CXCL14 and CXCL12

The very high sequence conservation between human and mouse CXCL14 (95% amino acid identity) (see **Figure 1.7**), suggests that cross-species functional conservation is likely (Wolf and Moser 2012). Similarly, CXCL12 sequence is very highly conserved between species, with a 92% amino acid identity between mouse and human sequences. I therefore hypothesised that the synergism between human CXCL12 and CXCL14 would also be evident in the murine system. This two-pronged work aimed to examine whether human and murine chemokines can be used interchangeably, based on the sequence conservation, as well as to test the physiological context of the CXCL12-CXCL14 synergism *in vivo.*

I first embarked on answering the question of whether human and mouse CXCL14 can be used interchangeably to assess synergism with CXCL12. I isolated immune cells from murine spleens and assessed their migratory potential towards human CXCL14 (huCXCL14), murine CXCL14 (muCXCL14) and human CXCL12 (**Figure 3.19**). All three types of cells tested showed similar responses towards human CXCL12, although B cells showed marginally greater migration towards 10 nM CXCL12 compared to 100 nM CXCL12 for T cells and monocytes. The combination of CXCL12 (1 nM and 10 nM) with either human or murine CXCL14 resulted in strong migratory responses by all three subsets. Monocytes can be further divided into classical and non-classical monocytes, based on the level of expression of the marker Ly6C. Classical monocytes are characterised by Ly6C high expression, whereas non-classical monocytes have lower Ly6C expression (Geissmann, Jung and Littman, 2003). Our group previously saw that specifically classical monocytes migrate towards CXCL14 in the human system (Collins, 2016). Therefore, I would expect that this finding would translate to the mouse system. Surprisingly, both monocyte subsets showed migration towards 3000 nM human CXCL14, but Ly6C^{hi} monocytes migrated more towards murine CXCL14. For lymphocytes, the synergistic effect was more prominent with murine CXCL14, whereas human CXCL14 induced greater migration of both monocyte subsets, in combination with CXCL12. The magnitude of migratory responses towards the combination of CXCL14 with 10 nM CXCL12 was equal to or greater than that obtained with 10 nM or 100 nM CXCL12 alone.

Interestingly, 300 nM CXCL14 was by far the most effective CXCL14 concentration at enhancing the activity of CXCL12, which is consistent with our published findings using human cells (Collins *et al.*, 2017). Therefore, we can conclude that the synergistic interaction of CXCL14 and CXCL12 is also detectable *in vitro* using primary murine immune cells.

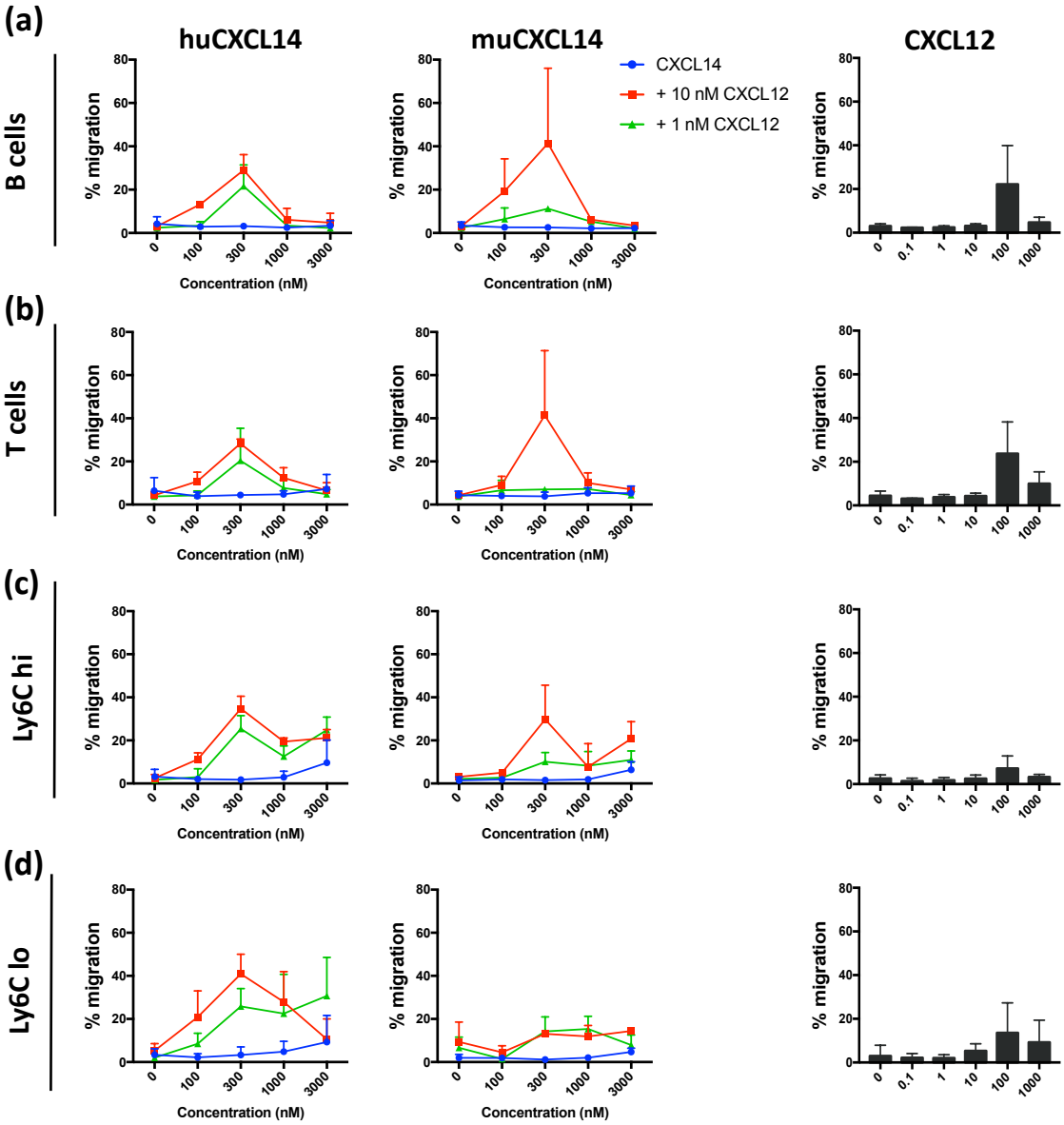


Figure 3.19. CXCL14 synergises with CXCL12 in the induction of chemotactic responses by murine cells *in vitro.*

Splenocytes were freshly isolated from wild-type Bl/6 mice and assessed for chemotactic response towards murine CXCL14 (muCXCL14), human CXCL14 (huCXCL14), CXCL12 or a combination of CXCL14 and CXCL12, by transwell chemotaxis assay. Migrated and input cells were stained and counted by flow cytometry, with gating on **(a)** $B220^+$ cells to distinguish B cells, **(b)** $CD3^+$ cells to distinguish T cells, **(c)** $Ly6C^{hi}$, $CD11b^+$ cells to distinguish classical monocytes and **(d)** $Ly6C^{lo}$, $CD11b^+$ cells to distinguish non-classical monocytes. Migration is expressed as the percentage of input cells of each cell type recovered from the lower chamber. Data are mean + SD of 2-5 independent experiments using cells from mice.

3.5.2 *In Vivo* Synergism Between CXCL14 and CXCL12

Considering the vast complexity of the chemokine system, in terms of the unique spatial and temporal expression patterns, it is difficult to define the physiological relevance of our *in vitro* observations. Nonetheless, chemokine synergism has successfully been demonstrated in various *in vivo* models, including monocyte recruitment to atherosclerotic lesions (Koenen *et al.*, 2009) and neutrophil recruitment to the peritoneum (Struyf *et al.*, 2005) in the mouse, and rat models of leukocyte recruitment to the CNS (Zwijnenburg *et al.*, 2003) and inflamed skin (Stanford and Issekutz, 2003). In order to investigate the synergistic effect of CXCL14-CXCL12 *in vivo*, I decided to use the well-established murine peritoneal cavity model (Proudfoot *et al.*, 2003; Struyf *et al.*, 2005). To this end, I first needed to optimise the experimental conditions for detection of immune cell migration into the peritoneum. Mice were injected with either 3000 nM CXCL14 or 100 nM CXCL12, concentrations which induced strong responses in our *in vitro* assays, or PBS as negative control. Two different incubation times were tested, 16 hours and 24 hours. After culling the mice, cells were harvested from the peritoneal cavity and analysed by flow cytometry. The gating strategy used for detection of specific cell types is shown in **Figure 3.20.** Previous studies from our group and others have shown that CXCL14 acts as a chemoattractant for various human blood immune cell subsets. CXCL14 is highly selective for blood monocytes, but not for any other type of immune cells within PBMC, including T cells, B cells, NK cells and DCs (Kurth *et al.*, 2001; Schaerli *et al.*, 2005; Meuter and Moser, 2008). However, robust migration of freshly isolated blood neutrophils was found at high CXCL14 concentrations (Collins, 2016). These data contrast findings by others who have shown responses to CXCL14 by NK cells, B cells or iDCs, in addition to monocytes and neutrophils (Hromas *et al.*, 1999; Cao *et al.*, 2000; Frederick *et al.*, 2000). These differences could be attributed to the use of different sources of CXCL14, including synthesised protein, protein present in conditioned media from transfected mammalian cells and commercially available recombinant proteins from various sources.

My studies in the mouse peritoneal recruitment model revealed that neutrophils show slightly increased migration towards CXCL14, whereas more macrophages were recruited in response to CXCL12 at 16 hours (**Figure 3.21**). T cells and monocytes showed

marginally higher migration towards CXCL14, while no response was observed by eosinophils and DCs. Unfortunately, the results from these experiments were not conclusive as to which incubation time was best, as there was too much variability across individual animals. These findings highlight the differences observed between *in vitro* and *in vivo* experiments, as the same cell populations may respond differently depending on the experimental setup.

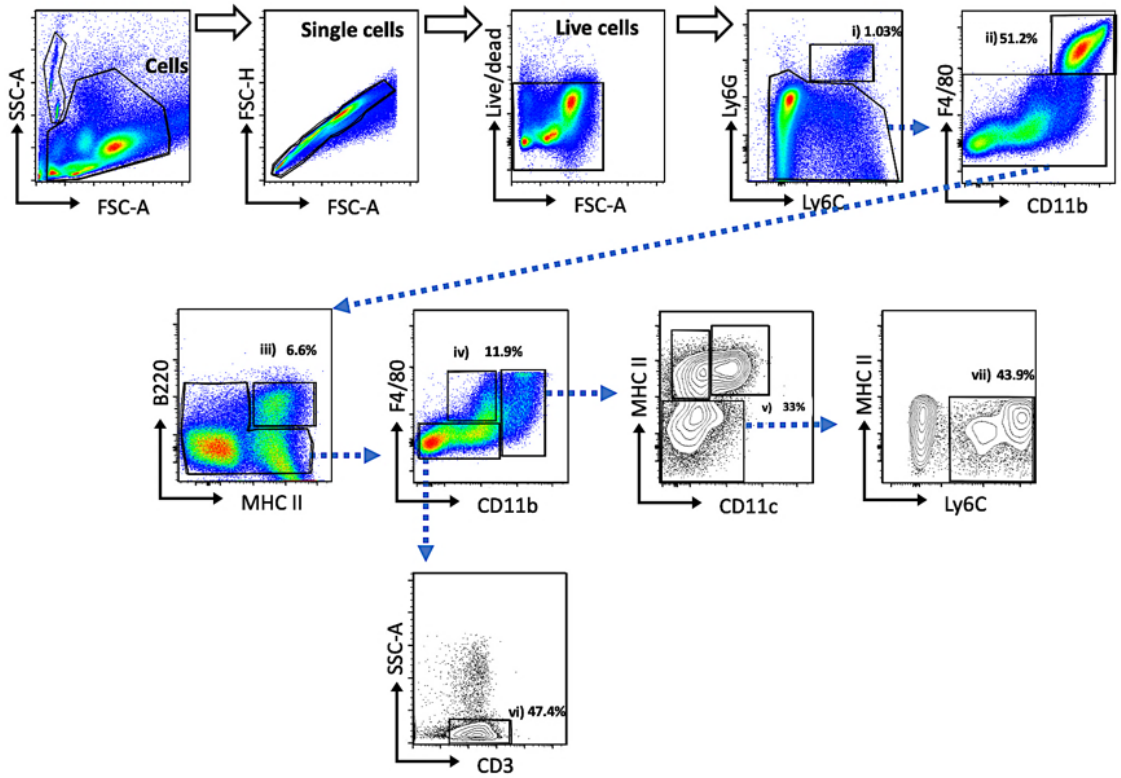


Figure 3.20. Gating strategy for identification of major cell subsets in the peritoneal cavity of mice.

Peritoneal cells were harvested from wild-type C57BL/6 mice and stained for flow-cytometric analysis. Live single cells were gated by excluding debris (top left panel), cell aggregates (top second panel) and dead cells (top third panel). Cells were stained with fluorochrome-conjugated mAbs directed against lineage markers, allowing the identification of **(i)** neutrophils ($Ly6G^{hi}Ly6C^{int}$), **(ii)** resident macrophages ($F4/80^{hi}CD11b^{hi}$), **(iii)** B cells ($B220^{+}$MHC II^{+}), **(iv)** eosinophils ($F4/80^{lo}CD11b^{lo}$), **(v)** dendritic cells (MHC $II^{+}CD11c^{+}$), **(vi)** T cells ($CD3^{+}SSC\text{-}A^{lo}$) and **(vii)** monocytes ($Ly6C^{hi}$MHC-II^{-}). Numbers indicate percentages of the parent populations of each cell type and are representative of two experiments, each with 3-4 mice per experimental group.

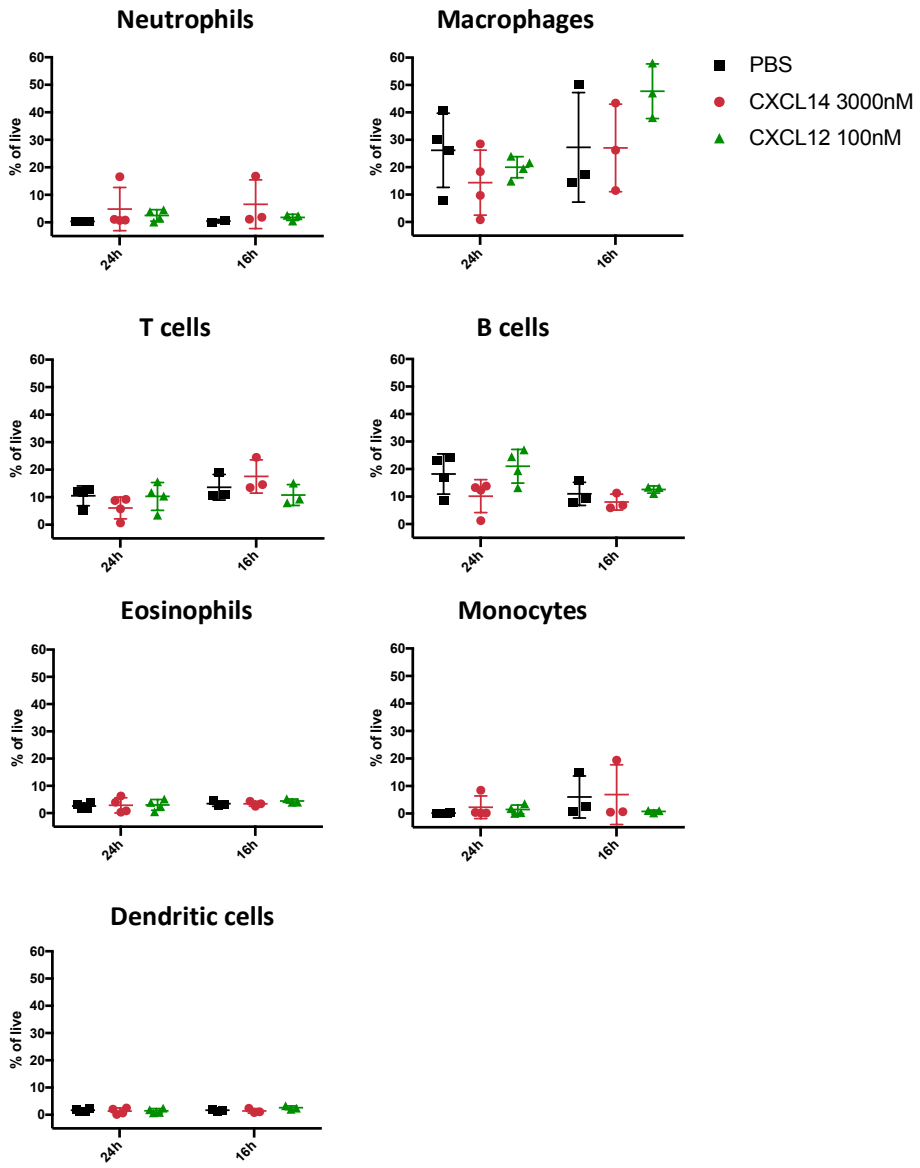


Figure 3.21. Cell recruitment in response to CXCL14 or CXCL12 *in vivo.*
Wild type C57BL/6 mice were intraperitoneally injected with PBS, 3000 nM CXCL14 or 100 nM CXCL12. Cells from the peritoneal cavity were harvested after 16 or 24 hours and stained by flow cytometry. Cell subsets were identified as outlined in **Figure 3.20** and cell numbers were expressed as percentage of total live cells. Data shown are mean + SD of two independent experiments, each with 3-4 mice per experimental condition.

3.6 Discussion

In summary, the work outlined in this chapter aimed to assess the ability of CXCL14 to synergise with chemokines other than CXCL12 in the induction of functional responses. The chemokine receptors selected included the most prominent homeostatic and inflammatory receptors. The homeostatic chemokines and their receptors tested play a pivotal role in the initiation of adaptive immune responses. Namely, CCR7 ligands CCL19/21 mediate the co-localisation of naive and T_{CM} cells as well as mature DCs in secondary lymphoid tissues, which is critical for the initiation of adaptive immune responses (Sallusto *et al.*, 2014). A similar role is played by CXCR5 and its ligand CXCL13, since they are responsible for the co-localisation of B cells and T_{FH} cells in B cell follicles during the initiation of antibody responses (Breitfeld *et al.*, 2000; Schaerli *et al.*, 2000). The inflammatory chemokine receptors included in this work, namely CXCR3 and CCR5, are key players in controlling T cell traffic in the effector phase of adaptive immune responses. They both have a role in the regulation of Th1-dominated immune responses and have been implicated in many diseases, including rheumatoid arthritis and multiple sclerosis (Loetscher *et al.*, 1996; Qin *et al.*, 1998). CCR5, along with CXCR4, is also implicated in HIV-1 infection. It acts as a co-receptor used by HIV-1 for cell entry, and R5-tropic HIV-1 strains require CCR5 as HIV-1 co-receptor and are mostly involved in the early stages of infection, whereas X4-tropic HIV-1 strains require CXCR4 as HIV-1 co-receptor and become dominant in the late stages of the disease (Lusso, 2006).

The data presented in this chapter provide clear evidence that CXCL14 can synergise with several chemokines. The synergistic effect was more prominent with the homeostatic chemokines tested. In fact, CXCL14 synergised with CCL19, CCL21 and CXCL13 in the induction of migratory responses by both primary and transfected cells. The synergistic effect was also apparent in the induction of calcium responses in transfected cells. Nevertheless, as opposed to the interaction with CXCL12 which leads to internalisation of CXCR4 (Collins *et al.*, 2017), CXCL14 did not induce a decrease in cell surface expression of CCR7 or CXCR5. The interaction of CXCL14 with CCR7 and CXCR5 did not appear to involve the activation of traditional signalling pathways, as neither migratory responses nor a rise in calcium mobilisation and decrease in receptor expression were induced in CCR7 and CXCR5-expressing cells by CXCL14 alone. This

indicates a mechanism comparable to that of CXCL14 being a positive allosteric modulator, since the presence of CXCL14 lowered the threshold of CCR7 and CXCR5 activation by their cognate ligands. Further examination of the interaction between CXCL14 and these receptors would allow confirmation of CXCL14 positive allosteric modulator activity, for instance by binding studies of CXCL14 to CXCR5 and CCR7 and Förster resonance energy transfer (FRET) analysis of cell surface chemokine receptor interactions.

The data regarding CXCL14 synergism with inflammatory chemokines are less striking. CXCL14 appears to synergise with inflammatory chemokines in some aspects of functional responses following chemokine stimulation but not in others. Specifically, CXCL14 synergised with all inflammatory chemokines tested (CCL5, CXCL10, CXCL11) in the induction of calcium responses, as well as the induction of migratory responses in combination with CXCL10 and CXCL11 in 300-19 transfectants. Research into CXCR3 ligands demonstrated that CXCL11 has higher affinity for CXCR3 and induces chemotaxis and calcium mobilisation most potently, compared to the other CXCR3 ligands (Cole *et al.*, 1998). My results showed that the synergistic effect of CXCL14 was stronger with CXCL11 than CXCL10, which is consistent with observed allosteric interaction characteristics describing that the higher the efficacy of the orthosteric agonist is, the greater the positive allosteric modulator effect is (Christopoulos, 2014).

Another characteristic of allosteric modulators is biased agonism and/or modulation of GPCRs. This refers to the ability of different ligands to preferentially stabilise certain GPCR conformations so that different signalling outputs are accentuated, as opposed to others (Stallaert, Christopoulos and Bouvier, 2011; Kenakin and Christopoulos, 2013; Rajagopal *et al.*, 2013). This mechanism is evident in the synergy of CXCL14 with CCL5 and its receptor CCR5, where chemotactic responses to CCL5 were inhibited by CXCL14, whereas synergistic effects were observed in calcium mobilisation responses. Our group has previously shown that the CXCL14 positive allosteric modulator activity on CXCR4 also extends to HIV-1 infection, where CXCL14 surprisingly enhanced entry of CXCR4-tropic (X4) HIV-1 strains into CXCR4-expressing $CD4^+$ target cells (Collins *et al.*, 2017). In this case, CXCL14 did not synergise with the HIV-1 inhibitory activity of CXCL12, i.e. the inhibitory activity of suboptimal CXCL12 concentrations were not boosted by the

addition of 300 nM CXCL14. Quite the contrary, CXCL14 neutralised the HIV-1 inhibitory activity of CXCL12 and even enhanced uptake of X4 HIV-1 particles by $CXCR4^{+}CD4^{+}$ target cells (Collins *et al.*, 2017). Of note, our group has also observed a similar enhancement effect on infection by CCR5-tropic (R5) HIV-1 strains, which require CCR5 as a co-receptor for entry into $CD4^{+}$ target cells. My findings with CCL5 and CCR5 support our previous HIV-1 infection studies by showing that CXCL14 indeed induced CCR5 signalling, i.e. Ca^{2+} mobilisation despite the fact that CXCL14 had a negative effect on CCL5-mediated chemotaxis of CCR5-expressing cells.

Our group has also previously tested synergism of CXCL14 with the inflammatory monocyte chemoattractant CCL2. No synergism between the two chemokines was observed in the induction of migratory responses or modification of basal FRET efficiency (Collins *et al.*, 2017). However, combination of CXCL14 with a sub-optimal concentration of CCL2 triggered a slight increase in receptor internalisation compared to CCL2 alone, in a single experiment (not published). Synergism between the two chemokines was also observed in calcium mobilisation in two out of three experiments (not published). In the present study I have only included a fraction of the homeostatic and inflammatory chemokines that are normally expressed in physiological and inflammatory conditions. Therefore, our results so far demonstrate strong synergism with homeostatic chemokines, whereas synergistic responses with inflammatory chemokines were varied, in general weak and, importantly, mostly absent in cell migration responses.

Several researchers have undertaken the difficult task of defining the physiological relevance of chemokine synergism *in vivo*. There are various reports of the impact of synergism between chemoattractants on leukocyte recruitment in different rodent models. My results show that the CXCL14-CXCL12 synergism is detectable in the murine system *in vitro*. To test this interaction *in vivo*, I used the peritoneal cavity, since it has proven to be a reliable model by other researchers (Proudfoot *et al.*, 2003; Struyf *et al.*, 2005). Unfortunately, my initial experiments aiming to determine the correct incubation time failed to show the expected results in control conditions, such as the recruitment of cells in response to CXCL12. This could be explained by truncation of CXCL12 by CD26/dipeptidyl peptidase 4, which has been tested *in vivo* using intra-articular

lymphocyte migration (Janssens *et al.*, 2017). Janssens et al. used orally administered CD26 inhibitor sitagliptin to reverse the inhibitory effect. Therefore, in any future efforts to address *in vivo* synergism of CXCL12-CXCL14, protease inhibitors should be included to prevent truncation of CXCL12. Furthermore, a different model such as intra-articular injection or the air-pouch model (Sin *et al.*, 1986; Schiraldi *et al.*, 2012), could provide a more controlled setting for such delicate experiments given the absence of resident cells in these cavities.

My findings provide novel evidence that CXCL14 potently synergises with homeostatic chemokines other than CXCL12. The mechanism behind this interaction is not entirely clear, yet it could be postulated that CXCL14 acts as an allosteric modulator of all homeostatic chemokine receptors tested. As a result, CXCL14 appears to have a much bigger role than anticipated in the localisation of immune cells and possibly the initiation and maintenance of immune responses. In fact, it may be that the main function of CXCL14 is to potentiate other chemokines (probably mostly of the homeostatic class) in epithelial tissues where CXCL14 is strongly and constitutively expressed. Under these steady state conditions, local CXCL14 in synergy with trace amounts of homeostatic chemokines could control the tissue localisation of $CXCR4^+$ as well as $CXCR5^+$ and $CCR7^+$ cells, which outnumber those cells expressing alternative chemokine receptors. Additionally, our group previously showed that CXCL14 is expressed in Peyer's patches in mice, where CXCL13 also plays a major role (Debard, Sierro and Kraehenbuhl, 1999; Meuter and Moser, 2008). CXCL14 expression is down regulated in most inflammatory settings (Kurth *et al.*, 2001; Maerki *et al.*, 2009; Frick *et al.*, 2011). However, there are examples where its expression is upregulated, such as chronic inflammatory lesions in the joint of mice with induced rheumatoid arthritis (Chen *et al.*, 2010). Such chronic inflammatory disorders are often associated with the presence of ectopic or tertiary lymphoid organs in the tissue, which enable the local activation of adaptive immune responses. As a result, pro-inflammatory cytokines are released, leading to an influx of inflammatory cells and autoantibody production. Homeostatic chemokines including CCL19/21 and CXCL13 have been detected in these ectopic lymphoid structures (Hjelmström *et al.*, 2000; Pitzalis *et al.*, 2014). It may be postulated that CXCL14 synergises with these chemokines to exacerbate the inflammatory cell recruitment. In

fact, CXCL14 expression has been shown to be up-regulated in the joint in a murine model of collagen-induced arthritis, and transgenic mice over-expressing CXCL14 developed a more severe arthritis than wild type controls (Chen *et al.*, 2010).

Collectively, the synergism of CXCL14 with homeostatic chemokines may well go beyond the control of immune cell traffic in epithelial tissues under steady-state conditions and may extend to chronic inflammatory conditions and even HIV-1 infection. This hypothesis of chemokine synergy as a main function of CXCL14 fully agrees with the fact that a selective receptor for CXCL14 has not been identified despite multiple and intensive efforts. Future CXCL14 research should focus on the potential importance of CXCL14 in synergising with the homeostatic chemokines CXCL12, CXCL13 and CCL19/CCL21 during diseases with secondary lymphoid tissue involvement, including chronic inflammation and cancer. The HIV-1 boosting activity of CXCL14 will be more difficult to study since a convenient *in vivo* model for HIV-1 does not exist.

Chapter 4: Structure-Function Analysis of CXCL14

4.1 Introduction

In view of the major role of chemokines and chemokine receptors in disease pathology, numerous attempts have been undertaken to target them with small molecule drug candidates, albeit with limited success. Despite the importance of chemokines in disease, the structural basis of receptor-chemokine interactions remains unclear. Chemokine receptors are GPCRs and, thus, are fully inserted in cell membranes. Due to the difficulties of studying membrane-bound proteins because of their hydrophobic nature, crystal structure analyses only became possible a few years ago. Site-directed mutagenesis in combination with binding and functional assays have prevailed in the efforts to determine molecular details of chemokine-receptor binding and activation. This work was pioneered by Clark-Lewis, whose work on CXCL8 revealed the critical role of the chemokine N-terminus in receptor activation and the fact that receptor binding and activation could be uncoupled (Clark-Lewis, Schumacher, *et al.*, 1991). Those studies showed that variants containing N-terminal modifications of CXCL8, including deletions or mutations, had antagonistic activities (Clark-Lewis *et al.*, 1995). Subsequent studies demonstrated that proteolytic modification of chemokine N-termini acted as a natural mechanism for regulating chemokine function (Pease *et al.*, 1998). More recently, work into the post-translational modifications of chemokines further highlighted the importance of the N-terminus for chemokine function (Moelants *et al.*, 2013).

As far as chemokine receptors are concerned, mutagenesis studies showed that N-termini of chemokine receptors are vital for binding of the structured chemokine "core domain" (Monteclaro and Charo, 1996, 1997). Together, these findings gave rise to the paradigm of chemokine-chemokine receptor interactions referred to as the two-site model (Monteclaro and Charo, 1996, 1997; Crump, 1997) **(Figure 4.1)**. According to this model, the chemokine receptor N-terminus interacts with the chemokine core domain (chemokine recognition site 1, CSR1), which includes the N-loop that immediately follows the second Cys residue and regions defined by the three antiparallel β-strands. This step is vital for providing affinity and specificity. The chemokine N-terminus interacts with the receptor ligand-binding pocket (chemokine recognition site 2, CSR2),

which elicits allosteric changes in the receptor and subsequent G protein activation. This model was developed based on biochemical, biophysical and functional studies of different chemokine-receptor pairs.

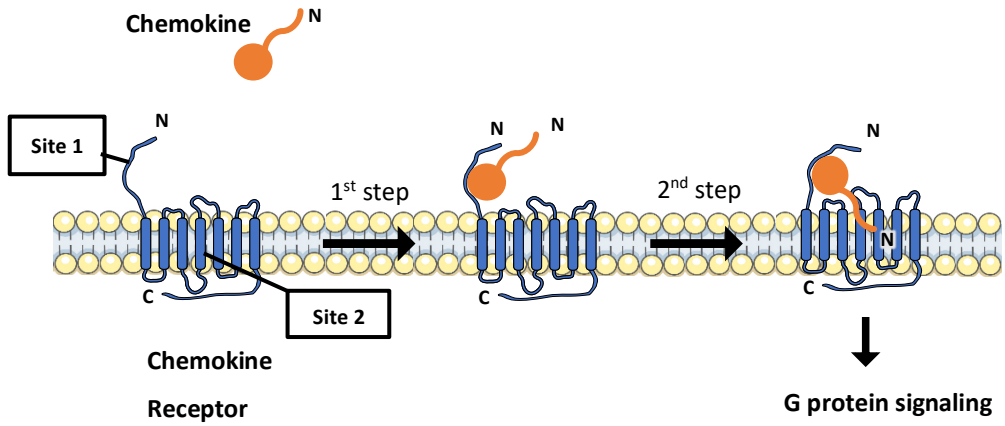


Figure 4.1. Schematic diagram of the two-step mechanism for chemokine receptor-chemokine interaction.
The 1st step interaction occurs between the chemokine N-loop and the extracellular N-terminal domain of the receptor (Site 1). In the 2nd step, the chemokine N-terminal residues bind to the receptor transmembrane residues (Site 2). This step triggers conformational changes to the receptor transmembrane region to induce G protein signalling.

The two-site model has guided the field for many years, as higher resolution understanding of the interaction was not available until a few years ago. In 2015, the first structure of a chemokine receptor in complex with a chemokine was solved, namely that of human CXCR4 with the viral chemokine antagonist vMIP-II (Qin *et al.*, 2015). That study was shortly followed by the report of another structure, the viral chemokine receptor US28 in complex with the human chemokine CX3CL1 (Burg *et al.*, 2015). Solving the first three-dimensional structure of a chemokine-chemokine receptor complex provided crucial information about the second step, the interaction of the N-terminal region of chemokines with site 2 of the chemokine receptors that is buried by the transmembrane domains. Those studies did not, however, reveal structural details of step 1, which involves the flexible N-terminus of chemokine receptors, because density was missing for much of the receptor N-termini. Combination of these site 1 and site 2

structures, as well as modelling studies has allowed construction of the most detailed chemokine-chemokine receptor interaction model to date. The current refined model proposes that additional contacts take place in this interaction, that fall outside the defined site 1 and site 2. In particular, a new intermediate interaction point was identified, which is located between CRS1 and CRS2. This site acts as a flexible pivot allowing chemokines to interact with the extracellular loops of the receptor, without interfering with the CRS1/2 interactions (Kufareva *et al.*, 2017). This model could explain the structural adaptability that allows for the promiscuity seen between chemokines and receptors. For instance, a single chemokine receptor can bind multiple ligands that share little sequence homology. Our group and others have shown that both CXCL14 and CXCL12 can interact with CXCR4, the specific receptor for CXCL12 (Tanegashima, Suzuki, *et al.*, 2013; Tanegashima, Tsuji, *et al.*, 2013; Collins *et al.*, 2017). Both CXCL14 and CXCL12 are highly conserved through evolution and they share 23.4% amino acid sequence homology (**Figure 4.2**). The interaction of CXCL14 with CXCR4 is non-functional (Collins *et al.*, 2017), i.e. does not lead to CXCR4-mediated cell responses. It is therefore tempting to speculate that step 2, i.e. the N-terminus of CXCL14, is not involved in the synergistic activation of CXCR4.

(a)

```
CXCL12  KPVSLSYRCPCRFFESHVARANVKHLKIL-NTPNCALQIVAR-----LKNNNRQVCIDPK 54
CXCL14  ------SKCKCSRKGPKIRYSDVKKLEMKPKYPHCEEKMVIITTKSVSRYRGQEHCLHPK 54
               :* *     ::  ::**:*::  : *:*  ::*        : ..:: *:.**

CXCL12  LK-------WIQEYLEKALNKRFKM    72
CXCL14  LQSTKRFIKWYNAWNEKRRVYE--E    77
        *:       * : : **    .
```

(b)

	CXCL12	CXCL14
CXCL12	100.00	23.44
CXCL14	23.44	100.00

Figure 4.2. Structural similarities of CXCL14 and CXCL12.
(a) Amino acid sequence alignment of human CXCL12 and CXCL14. Mature (secreted) chemokines were aligned using Clustal Omega multiple sequence alignment tool (Goujon *et al.*, 2010; Sievers *et al.*, 2011; McWilliam *et al.*, 2013) .Mature human CXCL14 is a 77 amino acid peptide, whereas mature human CXCL12 is a 72 amino acid peptide. CXCL14 and CXCL12 show a degree of amino acid sequence conservation, with 4 cysteines, 2 valines. 3 lysines, 2 leucines, 1 proline, 1 tryptophan and 1 glutamic acid in common * (asterisk) indicated positions which have a single, fully conserved residue. : (colon) indicates conservation between groups of strongly similar properties. (period) indicates conservation between groups of weakly similar properties. **(b)** Percent Identity Matrix between CXCL12 and CXCL14 were performed using Clustal Omega multiple alignment tool.

Our group and others have previously shown that CXCL14 can bind to CXCR4 with high affinity, as demonstrated by SPR experiments (Tanegashima, Suzuki, *et al.*, 2013; Collins *et al.*, 2017). Yet, CXCL14 was not able to trigger CXCR4-mediated signalling events, possibly due to its short N-terminus. Chemokine receptors can coexist in a variety of conformational states on cell surfaces, each of which is associated with a distinct functional outcome (Christopoulos, 2014). Our model of CXCL14-CXCL12 synergism proposes that CXCL14 is able to synergise with CXCL12 by acting as a positive allosteric modulator of CXCR4 (**Figure 4.1**). In particular, we propose that CXCL14 induces conformational changes in CXCR4 receptors existing at different conformational states (conformers) within receptor homodimers (oligomers), thereby lowering the threshold for receptor activation by CXCL12.

CXCL14 exhibits unique structural and functional features among the chemokine family.

Its very short N-terminus of just two amino acids prior to the first cysteine residue suggests that its mechanism of receptor activation may be different to that of other chemokines. In fact, there is no known chemokine variant with an equally short N-terminus that has retained chemokine function. Nonetheless, this hypothesis can only be addressed once the specific CXCL14 receptor is known. Using a chimera model, Crump and colleagues showed that insertion of the CXCL12 N-loop into unrelated CXC-family chemokines (CXCL1 and CXCL10) rendered them capable of binding and activating CXCR4 (Crump, 1997), thereby highlighting the importance of the N-loop motif for chemokine receptor recognition. Moreover, the 40s loop that connects the β2- and β3-strands in the β-pleated sheet of CXCL14 contains an additional 5-amino acid (VSRYR) insertion that can potentially affect the selectivity for its cognate (yet unknown) receptor. We have already established that CXCL14 interacts with CXCR4 and hypothesise that the N-terminus of CXCL14 is not involved in this interaction, as mature CXCL14 does not trigger signalling in CXCR4-expressing cells. However, it is tempting to speculate that substitution of the two-amino acid N-terminus in CXCL14 with the N-terminus of CXCL12 could render this hybrid chemokine a functional ligand for CXCR4. This Chapter, therefore, intends to investigate the functionality of CXCL12-CXCL14 hybrid proteins on cells expressing CXCR4 and cells that migrate in response to CXCL14 alone.

4.2 Aims

- To synthesise functional human CXCL14 and CXCL14-CXCL12 hybrids by automated solid-phase synthesis
- To test whether modification of the N-terminus of CXCL14 can render CXCL14 an agonist for CXCR4
- To examine whether modification of the CXCL14 N-terminus affects the observed CXCL14-CXCL12 synergism

4.3 Synthesis of Functional CXCL14 and CXCL14-12 Hybrids Using Solid-Phase Synthesis

To study the functional characteristics of CXCL14 and CXCL12 we designed and synthesised CXCL14-12 hybrids (**Figure 4.3**). The synthesis of sufficient quantities of pure CXCL14 structural variants was an obvious requirement. In order to achieve that, automated solid-phase synthesis was selected as the optimal technique to manufacture these chemokines as it allows the protein sequence to be manipulated. Chemical synthesis of chemokines is a well-established method, although it requires specialised laboratory equipment and chemical knowhow. For that reason, this work was done in collaboration with Prof. Paul Proost at the Rega Institute of KU Leuven (Belgium), whose lab generously welcomed me and helped me learn the process of chemokine synthesis.

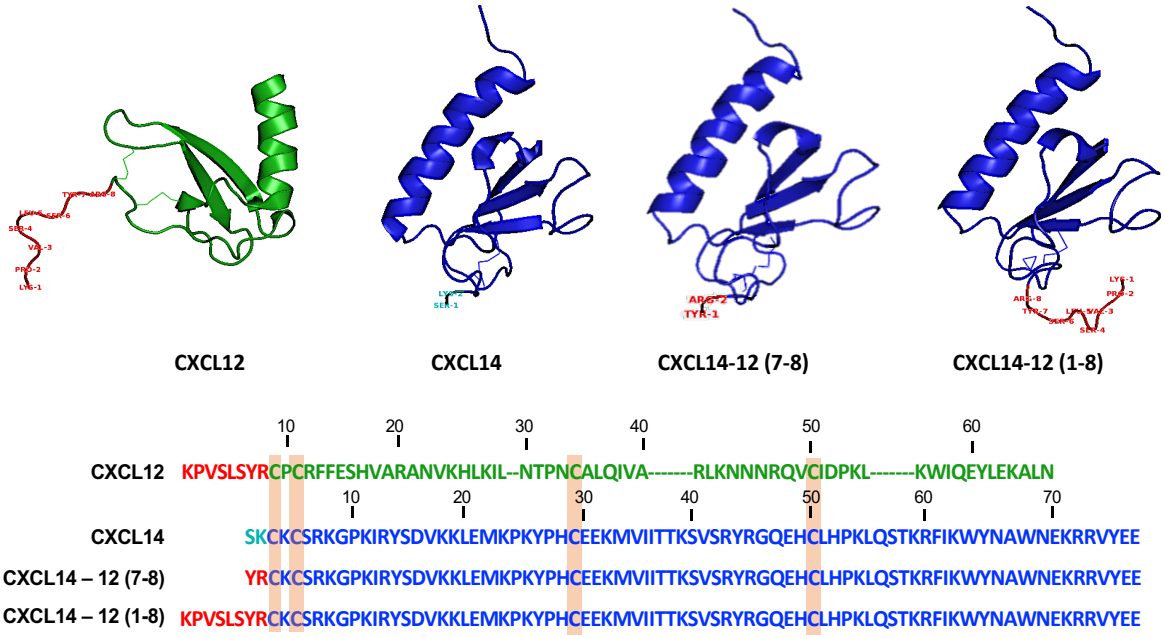


Figure 4.3. Structure and peptide sequence of CXCL12, CXCL14 and CXCL14-12 hybrids. Mature human CXCL12 is a 68 amino acid protein with an 8 amino acid N-terminus (highlighted in red). Mature human CXCL14 is a 77 amino acid protein with a 2 amino acid N-terminus (highlighted in cyan). CXCL14–12 hybrids were synthesised by solid-phase synthesis based on Fmoc chemistry. In the CXCL14-12 (7-8) hybrid, the CXCL14 N-terminus was replaced by the first two amino acids of the CXCL12 N-terminus (highlighted in red). In the CXCL14-12 (1-8) hybrid, the CXCL14 N-terminus was replaced by all 8 amino acids of the CXCL12 N-terminus (highlighted in red). Three-dimensional chemical structures were generated using Pymol (Version 0.99rc6, New York, NY).

The chemokines were synthesised using fluorenyl methoxycarbonyl (Fmoc) chemistry (Loos, Mortier and Proost, 2009). In brief, after coupling of the first C-terminal amino acid to the HMP-resin (4-hydroxymethyl-phenoxy-methyl-polystyrene), the Fmoc protection group is removed from the resin-coupled amino acid. The next Fmoc-protected amino acid is then activated and coupled to the resin-peptide chain. These deprotection, activation and coupling steps are automatically repeated (for further details, please refer to Chapter 2). For synthesis of the CXCL14 and hybrids, two flasks of each amino acid were used for each round to be able to perform a conditional double coupling for each amino acid. Each flask contained a 10-fold molar excess of amino acid compared to the amounts of active groups in the growing chain. This step has a disadvantage of added time and cost, but it increases the yield and purity of the final protein, which was important as our synthesised material had to be split into three aliquots. Optimisation into the optimal deprotection conditions included adjustment of the incubation time as well as water content of the deprotection mixture. The optimal conditions for deprotection of the synthesised proteins was determined as 1:45 hour incubation with the specified deprotection solution (please refer to Chapter 2 for more details). Deprotected proteins were purified using Reverse-Phase High Pressure Liquid Chromatography (RP-HPLC). The proteins were eluted with a linear acetonitrile gradient acidified with 0.1% (v/v) Trifluoroacetic acid (TFA). Initially, a Proto300 C4 column was used but following optimisation experiments, we established that a Proto300 C18 column provided better separation of our desired proteins from any contaminant proteins, as well as better yield. Fractions containing the desired proteins were identified by UV absorbance measurement at 214 nm in combination with online mass spectrometry (MS), which allows mass determination of proteins with a very high accuracy (± 0.01-0.1%). A mass spectrometer generates gas phase ions from a sample, separates the ions according to mass-to-charge (*m*/*z*) ratios and records a spectrum of their abundancies. Mass spectra show the detected *m*/*z* values and the intensity of the detected ions, both of which were used to determine the presence of synthesised proteins with specific relative molecular masses (M_r) (**Figure 4.4**). It was evident that our proteins of interest where not the most abundant within the deprotected mixtures. Nevertheless, contaminant proteins would include fragments from the synthesis that could be eliminated in the next purification steps.

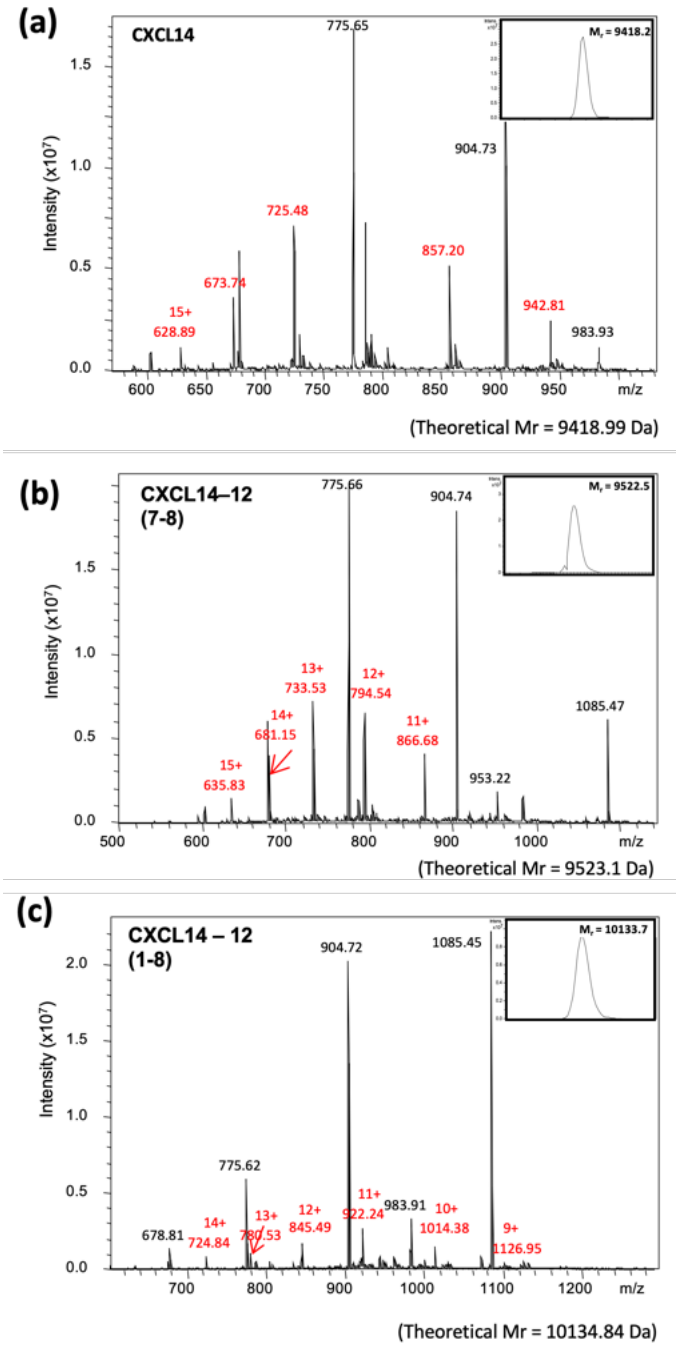


Figure 4.4. Mass spectrometry analysis of unfolded synthetic CXCL14 and CXCL14-12 hybrids.

CXCL14 **(a)**, CXCL14-12 (7-8) **(b)** and CXCL14-12 (1-8) **(c)** were chemically synthesised using solid-phase peptide synthesis, deprotected and purified using RP-HPLC. Deprotected proteins were subjected to electrospray ion trap mass spectrometry. Fractions with correct relative molecular mass (M_r) and sufficient purity were selected and pooled for each protein. The averaged spectra of one of these fractions are shown with the ion intensities, the number of charges and the corresponding mass over charge ratio (*m*/*z*) for multiple charged ions shown in red. The deconvoluted mass spectrum, as calculated by the Bruker deconvolution software, with the M_r of the uncharged proteins is shown as insert.

Finally, the deprotected and purified proteins were folded into their correct conformation through incubation with folding buffer. This solution enabled formation of disulphide bridges between the cysteine residues of natural chemokines. For the majority of synthesised chemokines, a folding buffer containing specific ratios of reduced and oxidised glutathione, among other components, is added to purified proteins for several hours depending on the chemokine. Nevertheless, optimisation of the appropriate folding conditions is required for each protein. The most commonly used folding mixture was tested on the synthesised hybrids but the proteins were not folded adequately, as determined using RP-HPLC and MS. Instead, an alternative folding solution was used, containing guanidine hydrochloride and Tris-acetate, and stirred vigorously overnight (Clark-Lewis, Moser, *et al.*, 1991). These conditions had also been used for folding of CXCL8 and CXCL7, and are thought to promote formation of the disulfide bridges by oxidation of the appropriate half-cystines (Clark-Lewis, Moser, *et al.*, 1991). Folding of proteins was assessed by purification using a PepMap C18 column and MS. The advantage of using two different columns is that different contaminant proteins can be eliminated more efficiently. The final purity and yield of the synthesised proteins was determined by MS (**Figure 4.5**). Samples were considered pure if the experimental M_r and *m*/*z* values agreed with the theoretical values of each CXCL14 hybrid (**Table 4.1.).** Along with the major folded chemokine containing the correct disulfide pairings, as assessed by M_r and *m*/*z* values, additional proteins could be detected, which are generally not functionally active. Normally, folded proteins reduce in size by 4 Daltons with all 4 cysteines being oxidised (Veldkamp *et al.*, 2016). Ion trap MS has an accuracy of 0.1% for each peak thus for the synthesised proteins where the prominent peak was either 11 or 12 charges (**Figure 4.5**), we expected an error of approx. 1 Da. Therefore, based on the experimental M_r obtained, the synthesis of the proteins was considered successful. **Table 4.1.** also shows the yield of the final proteins obtained. To prevent any further loss of material due to adherence to the plastic wall of the vials, a low concentration of detergent that was compatible with mass spectrometry and was unlikely to interfere with functional responses was added to the samples.

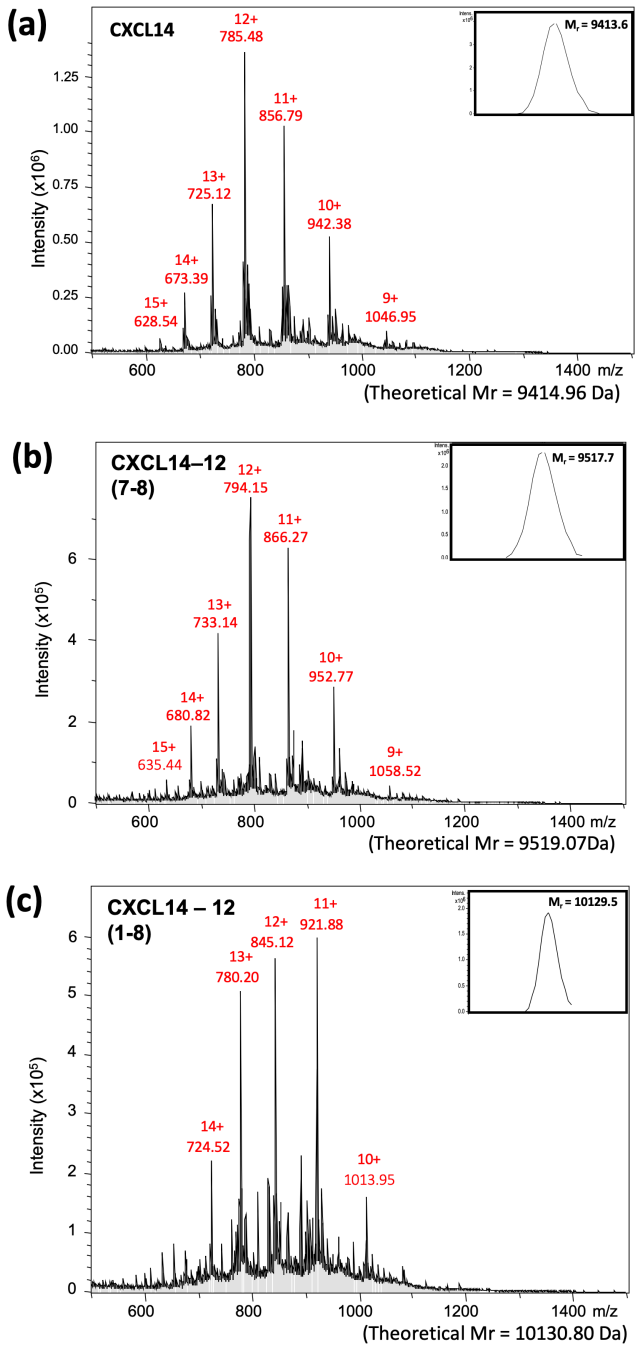


Figure 4.5. Mass spectrometry of folded synthetic CXCL14 and CXCL14-12 hybrids. Following deprotection and purification, fractions containing correct relative molecular mass and sufficient purity were selected and pooled for each protein. Folded and purified CXCL14 **(a)**, CXCL14-12 (7-8) **(b)** and CXCL14-12 (1-8) **(c)** proteins were subjected to electrospray ion trap mass spectrometry. The averaged spectra of these pools are shown with the ion intensities, the number of charges and the corresponding mass over charge ration (*m*/*z*) for multiple charged ions. The deconvoluted mass spectrum, as calculated by the Bruker deconvolution software, with the M_r of the uncharged proteins is shown as insert.

Table 4.1. Experimental M_r values of CXCL14 and CXCL14-12 hybrids determined by mass spectrometry, compared to theoretical values, and yield of synthetic proteins obtained.

Protein	Experimental M_r	Theoretical M_r	Yield
CXCL14	9413.6 Da	9414.96 Da	510 µg
CXCL14-12 (7-8)	9517.66 Da	9519.07 Da	320 µg
CXCL14-12 (1-8)	10129.45 Da	10130.80 Da	390 µg

4.4 Assessment of Functionality of Synthesised Proteins

Based on mass spectrometry data, the synthesised proteins obtained were expected to be fully functional. In order to assess the success of the synthesis, the bioactivity of newly synthesised CXCL14 ('new CXCL14') was compared to previously synthesised and tested stocks of CXCL14 existing in our laboratory ('old CXCL14') (Kurth *et al.*, 2001). These experiments focused on the migration of blood monocytes towards CXCL14 as it is a well-established and reproducible experimental readout. As shown in **Figure 4.6,** classical $CD14^{++}CD16^{-}$ monocytes migrated towards 3 µM old CXCL14, as expected. In contrast, new CXCL14 induced very low migration by classical monocytes. Intermediate monocytes also migrated towards 3 µM old CXCL14, in contrast to non-classical monocytes which showed no migration. No migration of intermediate or non-classical monocytes was observed towards new CXCL14. For the subsequent experiments presented in this Chapter, analysis was focused on classical monocytes as they are the primary targets of CXCL14 among monocyte subsets. At 3 µM, new CXCL14 was 10-fold less potent than old CXCL14 (old CXCL14: 11.7 ± 5.5 migration index, compared to 1.32 ± 0.9 for new CXCL14). Looking at the functionality of CXCL14-12 variants in classical monocytes, moderate responses were observed. CXCL14-12 (7-8) induced highest responses at 3 µM (3.3 ± 2.7), compared to CXCL14-12 (1-8) which induced lower responses at the same concentration (2.43 ±1.9). Although not statistically significant at this point, these data showed a moderate response to CXCL14-12 (7-8), which peaked at 3 µM. While the newly synthesised CXCL14 was less active as the previously used stock of CXCL14, CXCL14-12 hybrids showed moderate activity on primary monocytes.

However, it is not clear whether this activity was mediated through the cognate (and unknown) CXCL14 receptor or CXCR4.

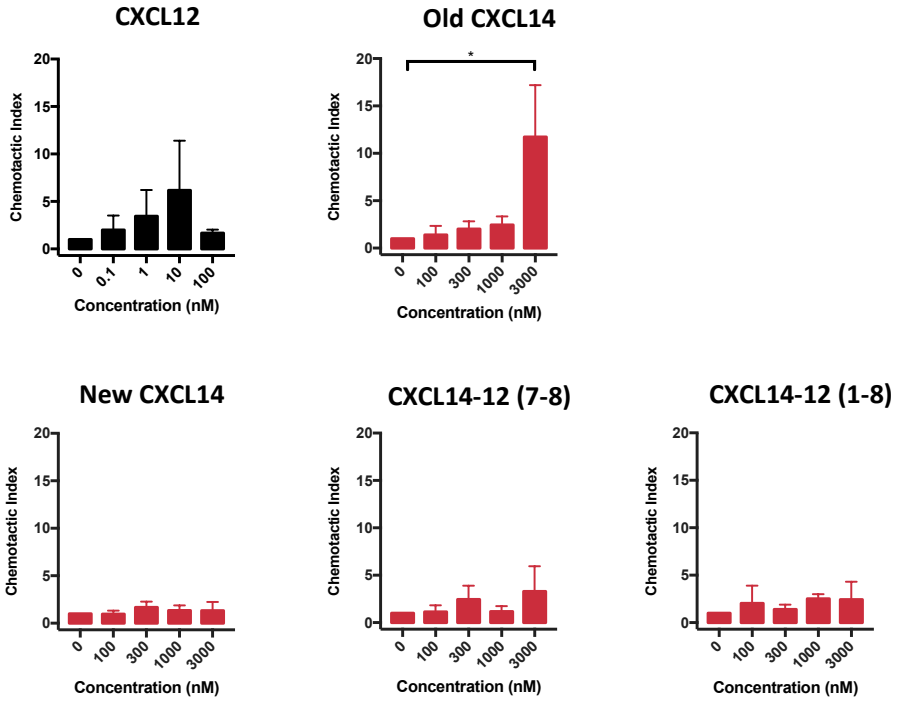


Figure 4.6. Synthetic CXCL14 and CXCL14-12 hybrids show moderate activity on freshly isolated monocytes.

Migration of freshly isolated blood monocytes towards chemokines was assessed by transwell chemotaxis assay. Chemokines tested included CXCL12, existing old stock of CXCL14, newly synthesised CXCL14, CXCL14-12 (7-8) hybrid and CXCL14-12 (1-8) hybrid. Data shown are mean + SD of 3-5 independent experiments using monocytes from different donors. *$p<0.05$ compared to no chemokine (0 nM) using Friedman test followed by Dunn's multiple comparisons test.

4.5 Interaction of Synthetic Hybrids with CXCR4-Expressing Cells

4.5.1 Induction of Migratory Response with Primary Cells

The earliest CXCL12 structure-function analysis established that the N-terminal amino acids Lys1 and Pro2 play an important role in receptor activation, showing complete loss of Ca^{2+} flux activity upon deletion or substitution of either residue (Crump et al., 1997). The same group showed that N-terminally modified CXCL12 proteins retained high affinity for the receptor and could potentially function as potent CXCR4 antagonists. They therefore suggested that site 2 contacts at the base of the orthosteric binding pocket play a role in signal transduction but contribute little to the overall binding energy. Additionally, N-terminal peptides of CXCL12 have been shown to trigger responses in CXCR4-expressing cells at ≥10 μM concentrations (Pius Loetscher *et al.*, 1998). In that study, the CXCL12 1-9 and 1-8 N-terminal peptides showed chemotactic activity, albeit much lower than CXCL12, with peptide 1-9 amino acids (aa) exhibiting 1000-fold less potency than CXCL12, and the peptide 1-8 aa a further 7-fold less potency than peptide 1-9. In the same study, researchers looked at the effect of adding a folded fragment corresponding to CXCL12 9-67, therefore allowing the entire CXCL12 structure to interact with CXCR4, but in two separate molecules. Addition of the second molecule had no effect on the functionality of the N-terminal peptides. Our own group previously showed that CXCL14 can synergise with CXCL12, but that it is not a ligand for CXCR4 (Collins *et al.*, 2017), as opposed to reports by others (Tanegashima, Suzuki, *et al.*, 2013; Tanegashima, Tsuji, *et al.*, 2013). The next step of the present study was therefore to examine the ability of the CXCL14 N-terminal hybrids to interact with CXCR4.

We hypothesised that addition of the CXCL12 N-terminus would render the hybrid a partial agonist of CXCR4. In order to test this hypothesis, freshly isolated PBMCs were allowed to migrate towards CXCL12, CXCL14 and the newly synthesised hybrid chemokines. Distinct cell types were differentiated by flow cytometry into $CD3^+$ T cells, $CD19^+$ B cells and $CD14^{++}CD16^-$ classical monocytes. CXCL12 was used as a positive control, as all cells tested expressed CXCR4 on their surface (data not shown). Additionally, CXCL12 activity can be blocked by the prototype non-peptide antagonist of CXCR4, AMD3100 (Tasker and Sklar, 1975). Monocytes migrated strongly towards the existing stock of CXCL14, as opposed to T or B cells, which showed no migration towards

CXCL14, as expected (**Figure 4.7**). Monocytes showed moderate migration towards both hybrids, with CXCL14-12 (7-8) inducing slightly higher migration. CXCL14-12 hybrids induced no migratory responses in T or B lymphocytes at the concentrations tested (100 nM-3 µM) (**Figure 4.7**). New CXCL14 showed no induction of migration in all cell subsets tested (data not shown).

In order to establish whether CXCR4 was involved in the moderate induction of migration of monocytes towards the synthesised hybrids and in particular CXCL14-12 (7-8), migration following blockade of CXCR4 with AMD3100 was tested. AMD3100 is a specific CXCR4 antagonist that inhibits the binding and function of CXCL12 with high affinity and potency (Rosenkilde *et al.*, 2004). As expected, migration of T, B cells and monocytes in response to CXCL12 was inhibited by AMD3100 (**Figure 4.7**). In agreement with the observation that T and B cells did not migrate in response to CXCL14 on its own, the CXCR4-specific inhibitor AMD3100 had no effect on CXCL14-induced migration by those cells. Unexpectedly, migration of monocytes towards CXCL14 was enhanced by the addition of AMD3100, although the results from a single experiment did not allow for statistical analyses. Notably, AMD3100 did not have a noticeable enhancing effect on CXCL14-12 hybrids. Taken together, these findings confirm that CXCL14 induces migration of monocytes via binding to a receptor distinct from CXCR4.

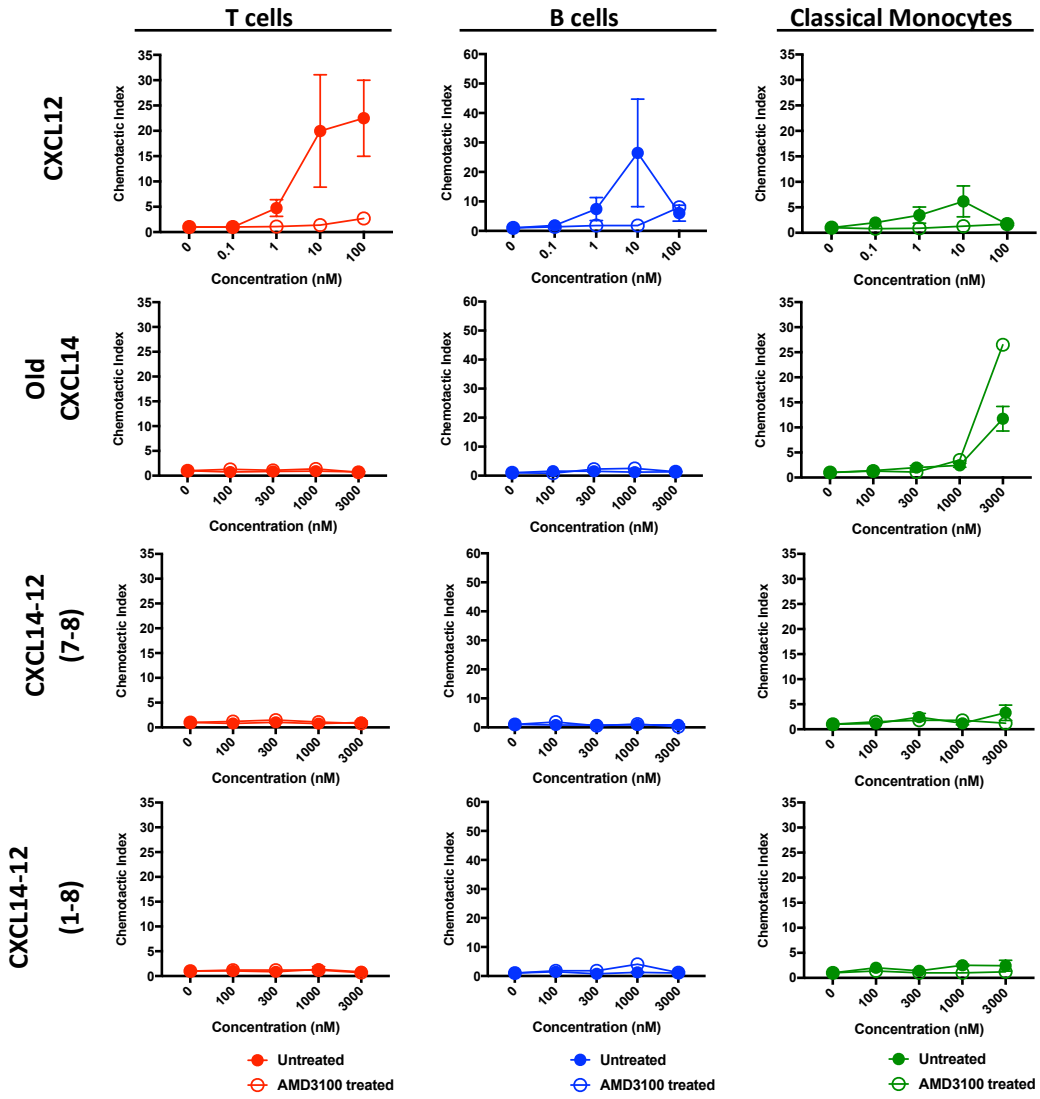


Figure 4.7. Synthetic CXCL14 and CXCL14-12 hybrids activity is not affected by AMD3100 treatment in primary peripheral blood cells.

PBMC were isolated from peripheral blood and migration was assessed by transwell chemotaxis assay. To test AMD3100 effect on migration, cells were incubated with 10 µM AMD3100 at 37°C for 1 hour prior to transwell migration. Chemokines tested included CXCL12, existing stock of synthesised CXCL14, CXCL14-12 (7-8) hybrid and CXCL14-12 (1-8) hybrid. Following migration, cells were stained with antibodies in order to differentiate between cell types, including CD3 (T cells), CD19 (B cells) and CD14 (monocytes), among others. Data are mean + SEM of 1-3 independent experiments using cells from different donors.

4.5.2 Induction of Migratory Responses by 300-19-CXCR4 Transfected Cells

In order to confirm the functionality of the synthesised hybrids on CXCR4 expressing cells, 300-19 cells stably transfected with CXCR4 (300-19-CXCR4) were used, which were previously generated in our group (Loetscher *et al.*, 1994; Oberlin *et al.*, 1996). CXCR4 expression on the cell surface was confirmed by flow cytometry (data not shown). Similar to primary T cells and in agreement with published findings (Collins *et al.*, 2017), peak migration towards CXCL12 was observed at 100 nM (**Figure 4.7**). In accordance to previously published findings from our group, no migration was observed towards CXCL14 (Collins *et al.*, 2017) (**Figure 4.8**). Old synthetic CXCL14 also failed to induce a migratory response. Interestingly, moderate migration was observed towards CXCL14-12 (1-8), peaking at 1 µM (4.6 ± 0.7%), correlating to a chemotactic index of ~ 65, given the low background migration of 300-19 cells. This response is thus presumably solely mediated by CXCR4.

Collectively, data on primary and transfected CXCR4-expressing cells indicate that CXCL14-12 hybrids can induce moderate migratory responses in CXCR4 expressing cells. Potentially, higher concentrations of hybrids could induce migration responses by primary lymphocytes, similar to those observed by primary monocytes (**Figure 4.7**).

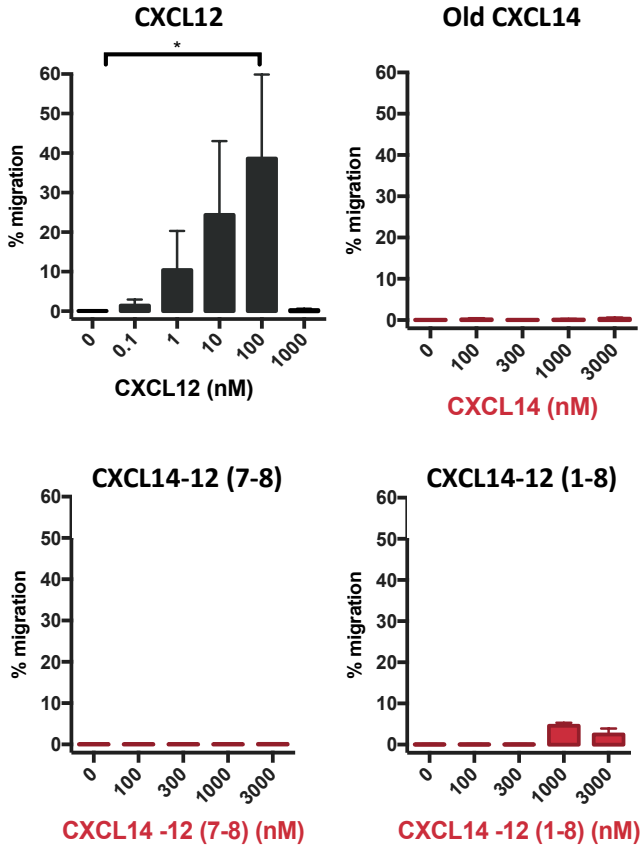


Figure 4.8. Synthetic CXCL14 and CXCL14-12 hybrids do not show clear interaction with 300-19 cells stably transfected with CXCR4.

Migration of 300-19-CXCR4 towards chemokines was assessed by transwell chemotaxis assay. Chemokines tested include CXCL12 (top left), existing stock of synthesised CXCL14 (top right), CXCL14-12 (7-8) hybrid (bottom left) and CXCL14-12 (1-8) hybrid (bottom right). Data are mean + SD of 3 independent experiments. *$p<0.05$ compared to 0 nM using Friedman test followed by Dunn's multiple comparisons test.

4.6 CXCL14 and CXCL14-12 Hybrids Synergise with CXCL12 in the Induction of Migratory Responses

4.6.1 300-19-CXCR4 Transfected Cells

One of the major structural differences between CXCL14 and CXCL12 is the length of the N-terminal sequence preceding the first cysteine (**Figure 1.7**). CXCL12 contains eight amino acids, which are critical for its function (Crump, 1997), whereas CXCL14 only contains two (Ser-Lys). The CXCL12 N-terminus is vital for GPCR activation by binding to site 2 within CXCR4. Given the short length of the CXCL14 N-terminus, we postulated that CXCL14 would primarily occupy site 1 on CXCR4, leaving site 2 free for binding by CXCL12. Previously, our group also established that although synthetic CXCL12 N-terminus (residues 1-9) induced chemotaxis in 300-19-CXCR4 at micromolar concentrations, no synergism was observed when combining CXCL14 and CXCL12 (1-9) (Collins *et al.*, 2017). Nevertheless, combination of both these structures within the same molecule had not been tested. Another aim of the present study was therefore to examine the effect of modification of the CXCL14 N-terminus on its synergistic activity with CXCL12. It was hypothesised that the synthesised hybrids would maintain their ability to synergise with CXCL12.

In order to test this hypothesis, migration of 300-19-CXCR4 cells towards CXCL12 alone, or suboptimal concentrations of CXCL12 in combination with CXCL14 or the synthesised hybrids was assessed (**Figure 4.9**). In accordance to published findings (Collins *et al.*, 2017), combination of CXCL14 with a fixed concentration of CXCL12 (1 nM) resulted in synergistic chemotactic migration peaking at 300 nM CXCL14. Interestingly, although new CXCL14 had no activity on primary monocytes, it was able to trigger chemotactic responses in combination with 1 nM CXCL12. Chemotactic responses peaked at 300 nM CXCL14, similar to our existing stock of CXCL14. The synthetic hybrids showed similar patterns of activity, although CXCL14-12 (1-8) induced greater chemotactic responses than CXCL14-12 (7-8) at 300 nM (32 ± 15.2% vs. 19.9 ± 17.8%, respectively), although this difference was not statistically significant. Synergism of these hybrids with 0.1 nM CXCL12 was also tested, which showed a lower but dose-dependent migration of cells towards increasing concentrations of CXCL14-12 (7-8) or CXCL14-12 (1-8) (data not shown). These data show that both CXCL14-12 hybrids showed comparable responses,

thus demonstrating that the length of the N-terminus does not play a role in the synergistic activity.

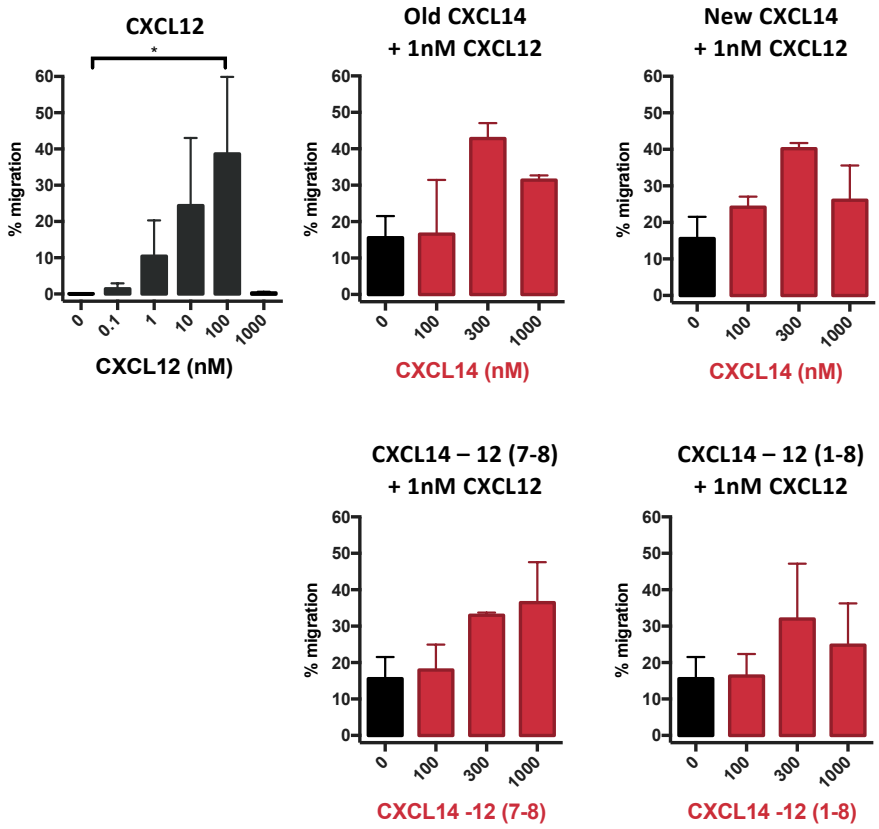


Figure 4.9. Synthetic CXCL14 and CXCL14-12 hybrids synergise with CXCL12 in the induction of chemotactic responses in 300-19 cells stably transfected with CXCR4.
Migration of 300-19-CXCR4 towards combination of chemokines was assessed by transwell chemotaxis assay. Chemokines tested included CXCL12, and combination of 1 nM CXCL12 with existing stock of synthesised CXCL14 (old CXCL14), newly synthesised CXCL14 (new CXCL14), CXCL14-12 (7-8) hybrid and CXCL14-12 (1-8) hybrid (bottom right). Data are mean + SD of 2-3 independent experiments. *p<0.05 compared to 0 nM CXCL14 or CXCL14-12 hybrid using Friedman test followed by Dunn's multiple comparisons test.

4.6.2 Primary Cells

Next, the synergistic potential of CXCL14-12 hybrids was tested on primary cells, using PBMC isolated from healthy volunteers. In these experiments, T and B cells displayed normal responses toward CXCL12 (**Figure 4.10**). Due to the high donor-to-donor variation, the data are presented in such a way that responses from each donor are displayed individually. Combination of suboptimal concentration (1 nM) of CXCL12 with CXCL14 resulted in migratory responses by both lymphocyte subsets. Similar to data with 300-19-CXCR4, both old and new batches of CXCL14 induced strong migratory responses in these subsets, peaking at 300 nM CXCL14, in the presence of suboptimal concentrations of CXCL12 but not in the absence of CXCL12. CXCL14-12 hybrids were also able to induce migratory responses when combined with 1 nM CXCL12. In the case of T cells, this response was dose-dependent for both hybrids. In contrast, B cells displayed peak migration towards CXCL14-12 (1-8) at 300 nM, whereas CXCL14-12 (7-8) was more variable between the donors. NK cells were also tested, which showed similar patterns of activity as B cells, although peak migration towards CXCL12 was observed at 10 nM (data now shown). Even though there was high donor-to-donor variation, these results clearly show that CXCL14-12 hybrids can synergise with suboptimal concentrations of CXCL12 in the induction of chemotactic responses by primary CXCR4-expressing cells.

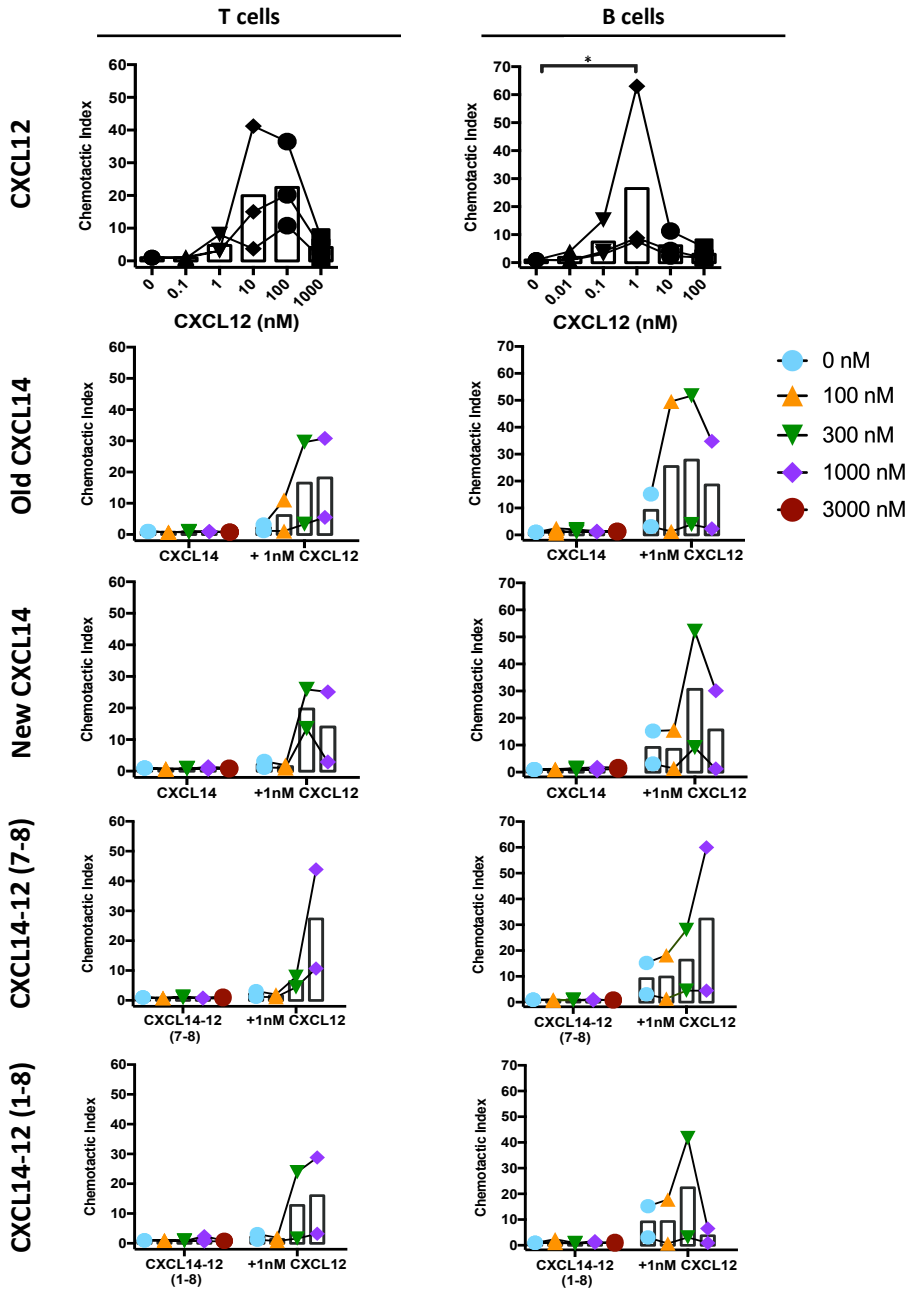


Figure 4.10. Synthetic CXCL14 and CXCL14-12 hybrids synergise with CXCL12 in the induction of chemotactic responses by primary T and B cells.

PBMC were isolated from peripheral blood and migration was assessed by transwell chemotaxis assay. Chemokines tested include CXCL12, and chemokine alone or in combination of 1 nM CXCL12 with the existing stock of synthesised CXCL14, newly synthesised CXCL14, CXCL14-12 (7-8) hybrid and CXCL14-12 (1-8) hybrid. Following migration, cells were stained with antibodies in order to differentiate between $CD3^+$ T cells and $CD19^+$ B cells. Each traced line and data point represent a different donor. Data are mean of 2-3 independent experiments using cells from different donors. *$p<0.05$ compared to 0 nM using Friedman test followed by Dunn's multiple comparisons test.

4.6.3 Blockade of CXCR4 Abolishes the Synergy Between CXCL14-12 Hybrids and CXCL12 on Primary Lymphocytes

Previous results from our group showed that treatment with the CXCR4 antagonist AMD3100 inhibited the synergism between CXCL14 and CXCL12, confirming that it was indeed CXCR4-mediated (Collins *et al.*, 2017). I therefore hypothesised that the synergism observed between CXCL14-12 hybrids and CXCL12 was similarly mediated through CXCR4 and could be inhibited by AMD3100. To test this hypothesis, PBMC were treated with AMD3100 for 30 minutes prior to use in chemotaxis assays (**Figure 4.11**). Migratory responses of T and B cells towards 1 nM CXCL12 + 300 nM old CXCL14 were completely abolished by prior treatment with AMD3100, in agreement with published findings (Collins *et al.*, 2017). Migration towards new CXCL14 combined with 1 nM CXCL12 was also completely inhibited by addition of AMD3100, confirming that old and new synthetic CXCL14 had similar bioactivities, thereby validating the new synthesis. Confirming my hypothesis, the synergism between CXCL14-12 hybrids and CXCL12 was similarly inhibited by AMD3100, indicating that this mechanism was indeed mediated by CXCR4. Synergism between CXCL14-12 hybrids and CXCL12 observed in NK cells was also abolished following treatment with AMD3100 (data not shown).

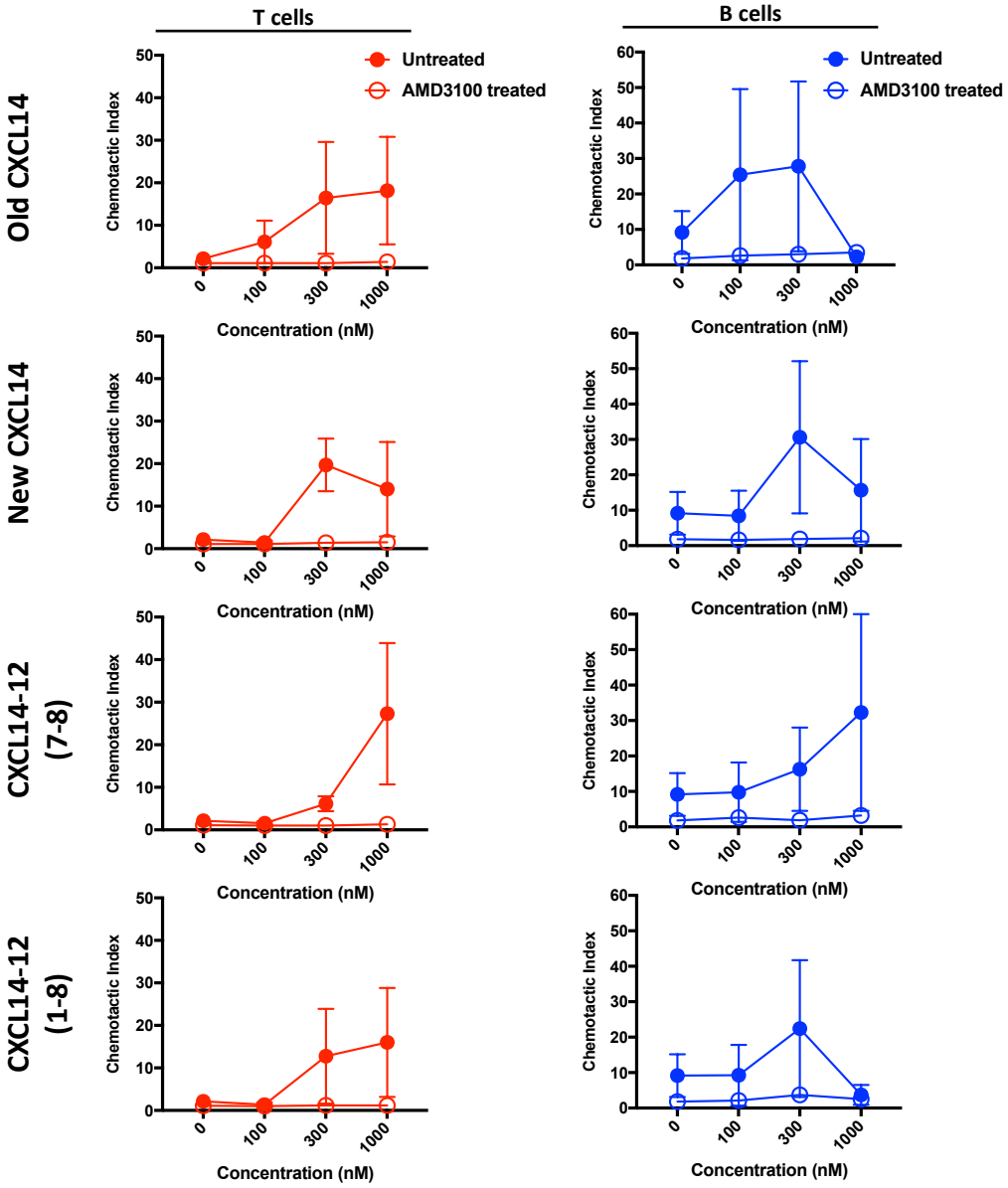


Figure 4.11. CXCR4-dependent synergism between CXCL14-12 hybrids and CXCL12 in the induction of chemotactic responses by primary T and B cells.

PBMC were isolated from peripheral blood and migration was assessed by transwell chemotaxis assays. To test an AMD3100 effect on migration, cells were incubated with 10 µM AMD3100 at 37°C for 1 hour prior to transwell migration. Chemokines tested included CXCL12, and combination of 1 nM CXCL12 with the existing stock of synthesised CXCL14 (old CXCL14), newly synthesised CXCL14 (new CXCL14), CXCL14-12 (7-8) hybrid and CXCL14-12 (1-8) hybrid. Following migration, cells were stained with antibodies in order to differentiate between $CD3^+$ T cells and $CD19^+$ B cells. Each traced line and data point represent a different donor. Data are mean + SEM of 1-2 independent experiments.

4.7 CXCL14-12 Hybrids Induce Calcium Mobilisation Responses in 300-19-CXCR4 Transfected Cells

Along with chemotaxis, additional cellular responses are activated in response to chemokines. For instance, following binding to the receptor, chemokines induce release of calcium ions [Ca^{2+}] from intracellular stores followed by entry of Ca^{2+} via membrane channels. This response is very rapid, peaking within seconds before gradually returning to basal levels. In order to examine the ability of synthetic CXCL14 and CXCL14-12 hybrids to induce calcium mobilisation, 300-19-CXCR4 cells were incubated with the ratiometric dye Fura-2-AM prior to calcium release measurement using a spectrophotometer. As a positive control, calcium release in response to different concentrations of CXCL12 was determined, in order to confirm the responsiveness of the cells and the sensitivity of the experimental approach (**Figure 4.12**). Maximal rise in intracellular Ca^{2+} was observed with 100 nM CXCL12, whereas 1 nM induced no detectable response. To determine the functionality of the CXCL14-12 hybrids a range of concentrations were tested, varying from 100-1000 nM. The CXCL14-12 (7-8) hybrid failed to induce a calcium response in 300-19-CXCR4 cells, which was consistent with the lack of a chemotactic migration response (**Figure 4.8**). In contrast to CXCL14-12 (7-8), the CXCL14-12 (1-8) hybrid induced a small response at 300 nM and 1000 nM. These data show that the moderate response of 300-19-CXCR4 cells to CXCL14-12 (1-8) seen in chemotactic migration extended to activation of calcium mobilisation, providing more evidence that CXCL14-12 (1-8) is a partial CXCR4 agonist. When comparing the magnitude of responses induced by 1000 nM CXCL14-12 (1-8) to those induced by CXCL12, the peak was most comparable to that observed with 1 nM CXCL12. This suggests that CXCL14-12 (1-8) is approx. 1000 times less potent than CXCL12.

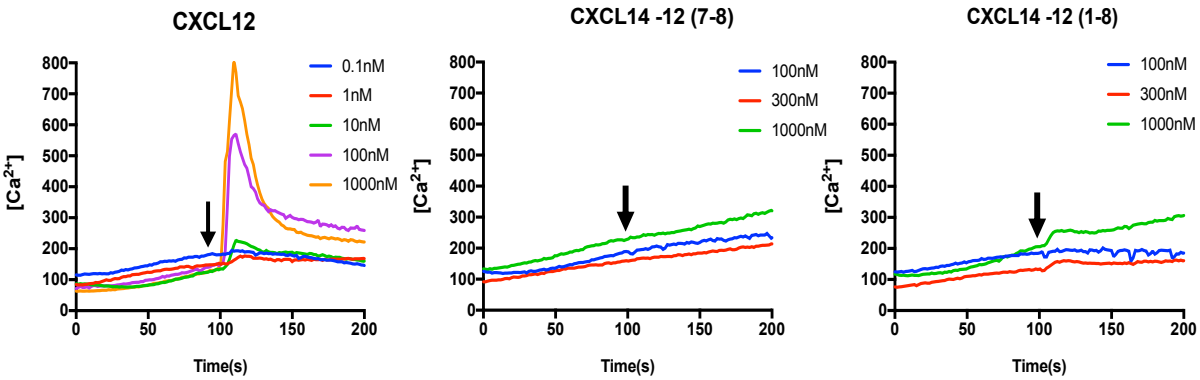


Figure 4.12. CXCL14-12 hybrids can induce calcium mobilisation responses in 300-19-CXCR4 transfected cells.

Changes in cytoplasmic free calcium [Ca^{2+}] concentration in 300-19-CXCR4 cells upon addition of chemokines were monitored using a spectrophotometer. Cells were loaded with 1 µM Fura-2-AM and stimulated with chemokine. Chemokine was injected after 100 seconds (indicated by the arrow). Changes in cytoplasmic free Ca^{2+} upon addition of various concentrations of CXCL12 (left), CXCL14-12 (7-8) (centre) or CXCL14-12 (1-8) (right) are shown. One representative set of measurements from up to four independent experiments is shown.

4.8 CXCL14-12 Hybrid Synergism with CXCL12 is not Evident in Calcium Mobilisation Responses

Considering that CXCL14 has the ability to synergise with the CXCL14-12 hybrids in the induction of chemotactic migration responses, I next aimed to examine whether this synergism can be detected in calcium mobilisation responses. Having established that 1 nM CXCL12 was a suboptimal concentration for triggering calcium release in 300-19-CXCR4 responder cells (**Figure 4.12**), I examined the effect of combining 1 nM CXCL12 with different concentrations of either CXCL14 or CXCL14-12 hybrids. Combination of 300 nM of our existing stock of CXCL14 (old CXCL14) with 1 nM CXCL12 resulted in clear transient [Ca^{2+}] spikes (**Figure 4.13**). A similar response, although slightly lower, was seen with new CXCL14, which was inconsistent with the absence of robust migratory responses to new CXCL14 observed earlier. Combining either of the CXCL14-12 hybrids with 1 nM CXCL14 resulted in a marginally higher response than in the presence of either chemokine alone. These findings were consistent between experimental repeats, indicating that the synergism between the CXCL14-12 hybrids did not induce prominent fast acting cellular responses.

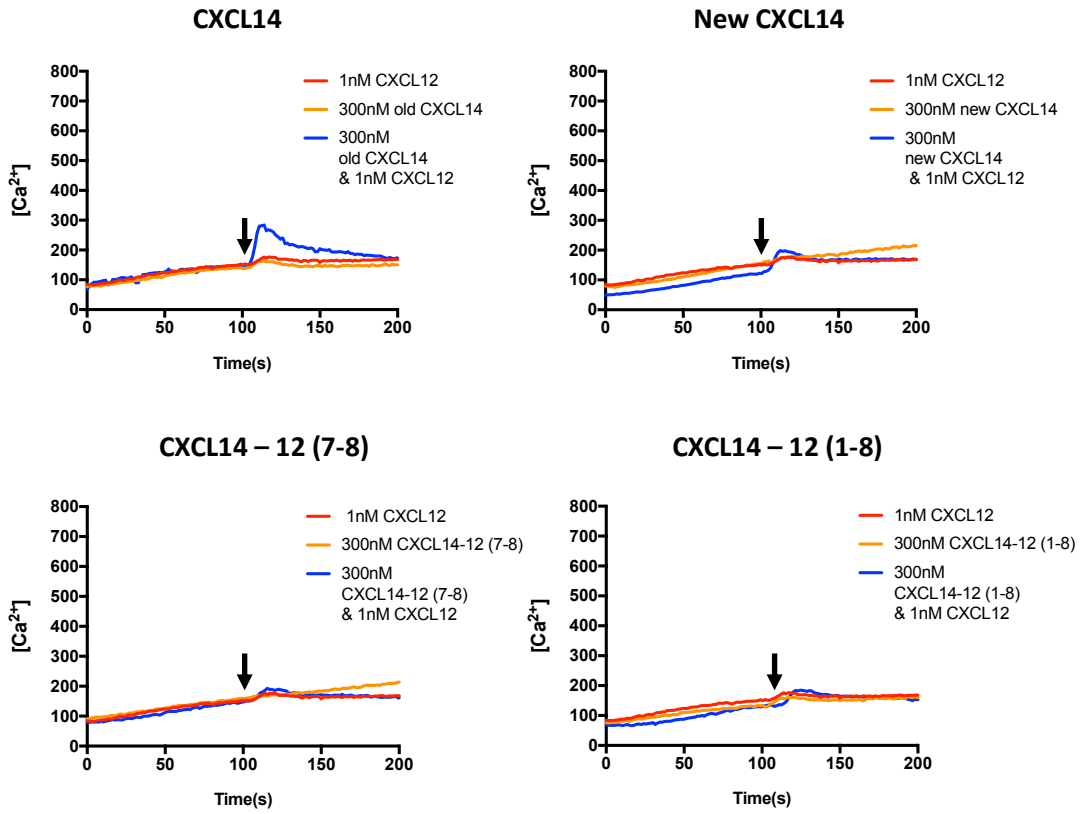


Figure 4.13. CXCL14-12 hybrids do not synergise with CXCL12 in the induction of calcium mobilisation responses in 300-19-CXCR4 cells.

Changes in cytoplasmic free calcium [Ca^{2+}] concentration in 300-19-CXCR4 cells upon addition of chemokines was monitored using a spectrophotometer. Cells were loaded with 1 µM Fura-2-AM and stimulated with chemokine. Chemokine was injected at the time indicated by the arrow. Changes in cytoplasmic free Ca^{2+} upon addition of combination of 1 nM CXCL12 and 300 nM CXCL14, 1 nM CXCL12 and 300 nM synthetic CXCL14, 1 nM CXCL12 and 300 nM CXCL14-12 (7-8), or 1 nM CXCL12 and 300 nM CXCL14-12 (1-8). One representative set of measurements from 2-3 independent experiments is shown.

4.9 Discussion

The work reported in this Chapter aimed to study the structure-function relationship of CXCL14. Structural hybrids of CXCL14 with an altered N-terminus were designed on the basis of the synergism observed between CXCL14 and CXCL12 (Collins *et al.*, 2017). These hybrids were synthesised using solid-phase chemical synthesis to facilitate modification of the N-terminus. They were then tested in a variety of functional tests in order to establish whether the synthesis and purification process produced bioactive proteins and to fully characterise their functional characteristics. The main aim of this part of the PhD project was to establish whether replacing the N-terminus of CXCL14 with either a partial or the full-length N-terminus of CXCL12 would render it an agonist for CXCR4. Additionally, this work aimed to establish whether modification of the CXCL14 N-terminus would affect its observed synergism with CXCL12.

Studies by others investigating the structure-function relationship of chemokines often employ solid phase peptide synthesis as their preferred method of synthesising N-terminally modified or native chemokines. Being an established method of protein synthesis, it has many advantages including superior purity, yield and ease to modify the sequence of proteins. Although many of these advantages held true during the present study, the need for optimisation of purification methods for specific proteins of interest meant that purity and yield of the synthesis were not optimal. In order to establish the success of the synthesis and purification process, CXCL14 was used as an internal control. The much lower activity of newly synthesised CXCL14 on monocytes was a surprise, since the mass spectra and experimentally obtained molecular mass values did not reveal structural faults. Specifically, very low levels of contamination were observed in the mass spectra of folded CXCL14 and the experimental M_r achieved was within the error values expected. However, these contaminations cannot explain the drop of activity of newly synthesised CXCL14. Although the mass spectrometry data of the folded proteins showed that the synthesis and purification process was of satisfactory quality, additional NMR spectroscopy might have been able to confirm the correct folding of the synthetic proteins. Interestingly, during the purification process we observed that CXCL14 contains a stretch of amino acids where the synthesised protein is likely to break. CXCL14 contains an extra 5-aa (VSRYR) insertion within the 40s loop

that connects the β2 and β3 strands in the β-pleated sheet. We found that following deprotection of the synthesised material, two fragments of the protein were present, along with the full-length protein. The larger fragment was CXCL14 (1-45 aa), whereas the smaller one was CXCL14 (46-77 aa), thus the fragmentation occurred just prior to the VSRYR insertion. While these contaminating side products were unlikely to interfere with the functionality of the synthesised full-length proteins, they were eliminated following purification of the folded protein. We did not hypothesise that they would interfere with the bioactivity of the synthesised proteins. However, their presence as well as the optimisation steps that had to be undertaken for their elimination led us to postulate that the yield of the synthesis was not optimal.

To determine the structural requirements for the function of CXCL12, Crump and colleagues used a chimera model. This chimera approach was successfully used for defining the structural elements of chemokines that are important for their function (Clark-Lewis *et al.*, 1994). That study showed that insertion of the CXCL12 N-terminus, in particular residues before or including the first Cys residue, into unrelated CXC-family chemokines (CXCL1 and CXCL10) rendered them capable of binding and activating CXCR4 (Crump, 1997) and highlighted the importance of the N-terminal motif of the chemokine for chemokine receptor activation. Replacement of the CXCL14 N-terminus with that of CXCL12 could, according to these findings, render the CXCL14-12 hybrids as partial agonists of CXCR4. In particular, the CXCL14-12 (1-8) was hypothesised to be more active on CXCR4-expressing cells than CXCL14-12 (7-8), based on the studies by Crump *et al*. and Loetscher *et al*. (Crump, 1997; Pius Loetscher *et al.*, 1998). Data collected so far confirmed our hypothesis, by showing that CXCL14-12 (1-8) is a partial agonist of CXCR4. CXCL14-12 (1-8) induced both migration and calcium mobilisation responses on CXCR4 transfectants. Experiments with CXCR4-transfected mouse pre-B cells provide a clean system to test interaction with this receptor, since primary cells express an array of additional receptors. In migration experiments, in order to achieve a response similar to that induced by the optimal concentration of CXCL12 (10 nM), it is estimated that one would require over 10 μM of the hybrid. When comparing the magnitude of calcium mobilisation responses induced by 1000 nM CXCL14-12 (1-8) to those induced by CXCL12, the peak was most comparable to that induced by 1 nM

CXCL12. This suggests that CXCL14-12 (1-8) is approx. 1000-fold less potent than CXCL12 and is consistent with previous studies by Crump and Loetscher. Although calcium mobilisation experiments are a good tool for determining the potency of these chemokines, the present research was restricted by the limited amount of synthesised material obtained. Further synthesis of these hybrid proteins at larger scale would thus be required to repeat these experiments to quantify the potency of these hybrids. In future experiments, use of CXCR4 transfectant cells expressing higher numbers of cell surface receptors per cell would be the best approach for determining the activity of these hybrids.

In the previously published study investigating the synergism between CXCL14 and CXCL12 from our group (Collins *et al.*, 2017), synthetic CXCL12 (N-terminal residues 1-9) peptide induced chemotaxis by freshly isolated primary T cells at micromolar concentrations. However, synergy between CXCL14 and these short CXCL12 peptides was not observed. Nevertheless, the lack of synergism between CXCL14 and N-terminal peptides of CXCL12 could be due to the lack of simultaneous interaction with the receptor. The second aim of this work therefore was to establish whether modification of the N-terminus of CXCL14 with CXCL12 would affect the observed synergism between the two chemokines. The synergistic activity of the hybrids was still detectable, which provided evidence that the N-terminus of CXCL14 does not play any role in the synergistic activity observed. It is worth noting that the synergism was inhibited by AMD3100, emphasising the fact that the responses were mediated by CXCR4 as opposed to the endogenous (as yet unidentified) CXCL14 receptor. The migratory responses which occurred in response to CXCL14-12 hybrids and CXCL12 were not stronger than those observed with CXCL14 alone or in combination with CXCL12. This evidence agrees with the hypothesis that the core part of CXCL14 interacts with CXCR4 and is responsible for the positive allosteric activity. Similar to the rest of the work in this Chapter, data on CXCR4-transfected cells provide more reliable evidence of this activity than primary cells, due to the donor-to-donor variation and possible interference by additional chemokine receptors on primary cells. Nevertheless, data of synergism on primary cells provided a confirmation of our findings on CXCR4 transfectants. Synergistic activity of these hybrids on calcium mobilisation responses was not as striking as migration

responses. Throughout the work studying synergism in this project, it was observed that calcium mobilisation responses can be variable. The majority of published studies studying synergism between chemokines have looked at migration responses instead of calcium mobilisation, indicating that this method of detection might not be the most accurate for evaluating chemokine synergism. More experiments are required to understand the full functionality of the CXCL14-12 hybrids. FRET analysis on CXCR4 expressing cells would be able to indicate whether they are indeed able to induce homodimerisation of CXCR4 molecules, similar to CXCL14. Additionally, it would be important to establish direct interaction of CXCL14-12 hybrids with CXCR4 using SPR, as it does not interfere with the native receptor conformation or binding activity (Rodríguez-Frade *et al.*, 2016). Interestingly, although the newly synthesised CXCL14 was not active on freshly isolated monocytes, it was still able to synergise with CXCL12 in the induction of both migratory and calcium responses. The reason for the observed lack of activity of the newly synthesised batch of CXCL14 with monocytes is not known.

Despite the fact that the responses observed were very small, certain conclusions regarding the functionality of the CXCL14 N-terminus and its receptor can be drawn from the data collected to date. Data with the CXCL14-12 (7-8) hybrid, which induced moderate responses on monocytes at 3 µM but was not active on CXCR4 transfected cells, would suggest that this hybrid signals through the yet unknown CXCL14 receptor. Although there was a slight decrease in the migration of monocytes following incubation with AMD3100, this experiment was only performed once. Therefore, more experimental repeats would be required to fully elucidate the functional characteristics of the hybrids and determine whether they interact with the CXCL14 receptor. Previously, Schaerli *et al.* showed that amino-terminal extensions of CXCL14 were inactive, demonstrating that slight amino acid sequence variations at the amino terminus had a profound effect on CXCL14 activity (Schaerli *et al.*, 2005). One of the main limitations of this work is the lack of a clean experimental system to test responses mediated through the CXCL14 receptor. Primary cells including monocytes have additional chemokine receptors such as CXCR4 on their surface, and donor-to-donor variation is a common problem within experimental repeats.

In summary, structure-function studies of CXCL14 show that by replacing its N-terminus with that of CXCL12 converts this chemokine into a partial CXCR4 ligand. This is an exciting finding that should be further investigated to determine the exact signalling cascades activated by this interaction. In particular, further experiments would include investigation of β-arrestin recruitment, cAMP activation, actin polymerisation and ERK activation, among others. Moreover, this study confirms previous findings of synergism between CXCL14 and CXCL12 and confirms the original hypothesis of this Chapter that the C-terminal part of CXCL14 is responsible for this activity. Potentially testing higher concentrations of the newly synthesised CXCL14 on monocytes could show whether this chemokine is indeed active. Larger quantities of synthetic CXCL14-12(1-8) would allow us to fully clarify its function with primary (monocytes) and CXCR4-transfected cells lines.

Chapter 5: CXCL14 Target Cells in Peripheral Tissues

5.1 Introduction

CXCL14 has been studied for >18 years, yet its function remains largely unresolved. CXCL14 is very highly expressed in a wide range of healthy epithelial tissues, including the skin, lung and kidney, as well as gastrointestinal and reproductive tracts (both in humans and mice). Its absence from secondary lymphoid tissues sets it apart from other homeostatic chemokines such as CXCL12, CCL19, CCL21 and CXCL13. Moreover, whereas other non-ELR CXC chemokines are able to attract T cells, CXCL14 does not (Cao et al., 2000; Sleeman et al., 2000). Although much research has been conducted investigating the target cells of CXCL14, progress has been hampered by not knowing its cognate receptor, which has led to conflicting experimental findings from different groups. Published findings reported different human immune cell subsets displaying chemotaxis towards CXCL14, including neutrophils, immature dendritic cells, monocytes (especially following PGE_2 activation) and NK cells from blood and uterus (Cao *et al.*, 2000, 2013; Sleeman *et al.*, 2000; Kurth *et al.*, 2001; Shellenberger *et al.*, 2004; Starnes *et al.*, 2006; Tanegashima, Suzuki, *et al.*, 2013). In the mouse, tissue expression of CXCL14 has been examined, yet its chemoattractant function is largely undefined with only a single report on migration of immature DCs and NK cells towards CXCL14 (Salogni *et al.*, 2009). Inconsistencies in reported target cell selectivity can be attributed to the use of CXCL14 from different sources. Sources included synthetic protein, CXCL14 isolated from conditioned media by transfected mammalian cells and commercially available recombinant CXCL14 from various suppliers. It is important to keep in mind that CXCL14 has a poor chemotactic potency, i.e. micromolar concentrations of chemokine are needed to induce migratory responses *in vitro*. Therefore, slight differences in concentration of recombinant, commercial or synthetic chemokines might lead to failure to detect its activity. Additionally, differences in isolation methods of primary cells as well as donor-to-donor variation may cause contradictory reports between different laboratories.

Work previously performed by Dr Paul Collins in our group focused on the identification of leukocyte subsets in human blood and peripheral tissues that represent the major

targets for CXCL14. During this work, target cells were assessed for their migratory potential towards CXCL14. For these studies, a custom-made synthetic CXCL14 reagent was used, which contained an Alexa Fluor 647 fluorochrome covalently attached at its C-terminus (AF-CXCL14), for staining of potential CXCL14 target cells. The approach of utilising fluorescently labelled chemokines was successfully used by our group as well as others in the past to study the distribution of chemokine receptors on immune cells (Strong *et al.*, 2006; McCully *et al.*, 2015). This work examining target cells in peripheral blood demonstrated that monocytes and in particular $CD14^+$ monocytes are major targets of CXCL14, while neutrophils did not bind AF-CXCL14 but showed weak and consistent migration responses. Other subsets of human peripheral blood, including T cells, B cells, NK cells and DCs did not stain with AF-CXCL14 and did not migrate towards CXCL14 in our hands (Collins, 2016). Migration of monocytes towards CXCL14 correlated well with binding of the custom-made AF-CXCL14. In contrast, binding of AF-CXCL14 to neutrophils was not observed, which could be due to the lower levels of CXCL14 receptor(s) below the limit of detection by our reagent.

The site of chemokine production is often the site where responsive cells migrate to. Therefore, since CXCL14 is highly and constitutively produced in epithelial tissues such as skin, part of this work by our group aimed to identify target cells within the skin. Previous work has shown that epidermal keratinocytes as well as macrophages and mast cells in the dermis produce CXCL14 during steady-state conditions (Schaerli *et al.*, 2004; Meuter *et al.*, 2007; Maerki *et al.*, 2009). Using an *in vitro* tissue model, human epidermal equivalents were shown to be capable of inducing the differentiation of $CD14^+$ monocytes into Langerhans-like cells. It was thus proposed that CXCL14 has the important role of guiding $CD14^+$ cells from blood to distinct epidermal niches, where they could differentiate into Langerhans cells (LCs) (Schaerli *et al.*, 2005). Further looking into skin for CXCL14 target cells, healthy human skin was proteolytically separated into the epidermal and dermal layers of the skin, followed by incubation of skin tissue fragments for approx. 48 hours in order to allow cells to migrate out of the tissue. Emigrated cells were examined, as well as tissue resident cells following further digestion of each tissue compartment. Interestingly, LCs did not show migration or binding of AF-CXCL14, demonstrating that CXCL14 does not play a role in the localisation

of LCs during steady state conditions (Collins, 2016). This finding agreed with previous work showing that CXCL14 deficient mice possess normal numbers of LCs (Meuter *et al.*, 2007). Unpublished findings from our group show that the subset of human skin $CD14^+$ DCs showed binding of AF-CXCL14, but did not migrate towards CXCL14, in clear contrast to the larger subset of $CD1a^+$ DCs that did not bind AF-CXCL14 (Collins, 2016). As opposed to dermal DC, macrophages were largely resident and did not spontaneously migrate out of skin tissue. Following tissue digestion, some of these macrophages, identified based on their autofluorescence, showed binding of AF-CXCL14. During this work, an unexpected discovery was made (Collins, 2016). A population of cells expressing the myeloid markers CD14 and CD1a but lacking the pan-leukocyte marker CD45 was identified. All immune cells are characterised by their expression of CD45. Therefore, this finding was particularly interesting because these cells did not express CD45, suggesting they were not part of the immune cell compartment of the tissue. However, they uniformly expressed the antigen presentation protein CD1a and showed more variable expression of the pathogen-sensing protein CD14. These cells demonstrated binding of AF-CXCL14, as well as strong migratory responses towards CXCL14, thereby identifying this novel population as CXCL14 target cells (Collins, 2016). **Figure 5.1** shows data from emigrant cells within the dermis, though similar findings were observed with cells from the epidermis. This cell subset was present among the emigrant cells recovered from both the dermis and epidermis. Enzymatic digestion of the dermis yielded greater numbers, indicating that they constitute a significant compartment of healthy human skin. Although some of these cells can migrate out of the tissue, the fact that higher numbers were retrieved following enzymatic digestion indicates that they are primarily resident cells. Moreover, they are not detectable in peripheral blood, which provides more evidence of their "resident" characteristics (Collins, 2016). Identification of non-immune ($CD45^-$) cells binding AF-CXCL14 is noteworthy. The majority of studies examining cell populations in the skin both in human and mouse mainly aim to study the immune cell compartment of the tissue, therefore focusing on $CD45^+$ cells, while $CD45^-$ cells are discarded (Haniffa, Collin, *et al.*, 2009; McGovern *et al.*, 2014; Malosse and Henri, 2016). It could thus be anticipated that these cells were overlooked in the past due to their lack of CD45 expression. A summary of AF-CXCL14

binding cells that have been identified in healthy human skin by our group is shown in **Table 5.1.**

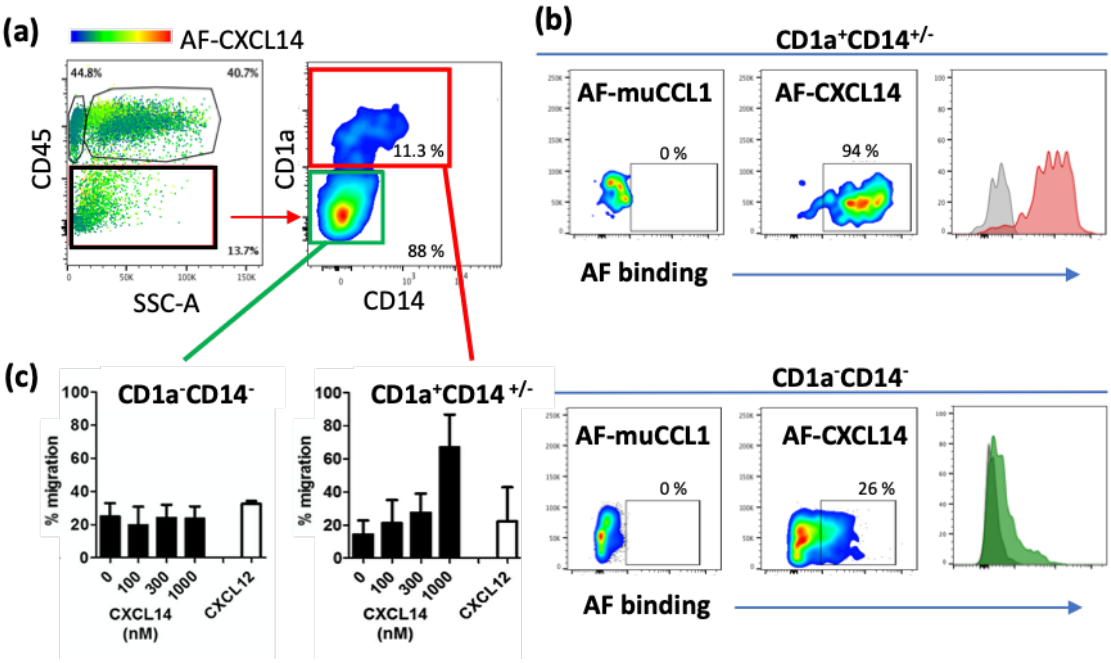


Figure 5.1. Identification of a novel subsets of CXCL14 target cells expressing myeloid cell markers in the CD45⁻ fraction of human dermis.
(a) Representative heatmap dot plot shows expression of AF-CXCL14 according to CD45 expression (left panel) and CD1a and CD14 expression by the CD45⁻ fraction of dermal emigrant cells. **(b)** Representative dot plots and histograms show binding of AF-muCCL1 and AF-CXCL14 to CD1a⁺CD14$^{+/-}$ cells (top), and CD1a⁻CD14⁻ cells (bottom). Numbers indicate the percentage of AF-CXCL14⁺ cells. Grey filled histogram indicates binding of AF-muCCL1 serving as negative control. **(c)** Migration of CD1a⁺CD14⁻ cells (left) and CD1a⁻CD14^{int} cells (right) towards 100-1000 nM CXCL14 or 100 nM CXCL12. Data are mean + SEM of three donors; (Collins, 2016).

Table 5.1. Summary of human skin cells examined during this work and their responsiveness to CXCL14

Cell type	Migration responses	Binding of AF-CXCL14
Langerhans (epidermis)	-	- [a]
$CD3^+$ T cells (dermis)	-	-
$CD14^+$ DC (dermis)	-	++
$CD1a^+$ DC (dermis)	-	-
Macrophages (dermis)	(not tested)	+
$CD45^-CD1a^+CD14^{int}$ (dermis and epidermis)	++	++
$CD45^-CD1a^-CD14^-$ (dermis and epidermis)	-	-

[a] Binding of AF-CXCL14 within each population. Gates were set based on AF-CXCL14 FMO. ++ = >50% AF-CXCL14 binding, + = <50% binding, - = <10% AF-CXCL14 binding (Collins, 2016).

5.2 Aims

The very high sequence conservation of CXCL14 between human and mouse suggests that CXCL14 has similar functions in both species (Wolf and Moser, 2012). Considering the difficulties associated with gaining regular access to fresh healthy human skin tissue, we have decided to continue our investigations into CXCL14 target cells in the mouse, which gave access to multiple tissue and organs. Mouse studies were carried out in order:

- To confirm unpublished findings from our group about CXCL14 target cells in human blood
- To identify CXCL14 binding cells in mouse tissues
- To characterise murine CXCL14 target cells

5.3 CXCL14 Target Cells in Human Peripheral Blood

Previous research from our group has indicated that monocytes, in particular classical monocytes, are the primary targets of CXCL14 within human peripheral blood. I have confirmed this finding in Chapter 4. Before focusing on CXCL14 target cells in the mouse, pilot experiments aimed to confirm the correlation between staining with AF-CXCL14 and migration experiments. In order to show that, single cell suspensions of PBMC were stained with fluorochrome-conjugated monoclonal antibodies (mAbs) directed against CD3, CD14, CD16, CD19 and CD56. Following exclusion of dead cells and cell aggregates, this staining strategy enabled identification of monocyte subsets following further exclusion of T, B and NK cells. A representative gating strategy is shown (**Figure 5.2a**). The three monocyte subsets were identified based on expression of CD14 and CD16. These included classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$) monocytes. Chemotactic responses of PBMC to CXCL14 were assessed using the transwell chemotaxis assay. Collective data from three independent experiments using different donors show the chemotactic response of each monocyte subset (**Figure 5.2b**). Classical and intermediate monocytes showed maximal migration towards 3 µM CXCL14. It should be noted that donor-to-donor variation was substantial in these responses (classical monocyte migration was 12.1 ± 14.3% towards 1 µM CXCL14 and 62.5 ± 69.4% towards 3 µM CXCL14). **Figure 8.1** in Appendix shows detailed responses for each donor and demonstrates the clear migratory response of monocytes towards CXCL14, with responses starting at 1 µM CXCL14. Intermediate monocytes also showed migratory responses towards 3 µM CXCL14, albeit more modest than classical monocytes (33.1 ± 30.5% migration towards 3 µM CXCL14). This subset demonstrated the highest background migration among all monocyte subsets (8.6 ± 14.4% migration to buffer alone). In contrast, non-classical monocytes did not display migration towards CXCL14. In agreement with the migration data, binding of AF-CXCL14 revealed that the majority of classical monocytes express CXCL14 receptor(s), as shown in representative plots in **Figure 5.2c**. Intermediate monocytes also demonstrated binding of AF-CXCL14, whereas non-classical monocytes showed no clear binding of AF-CXCL14.

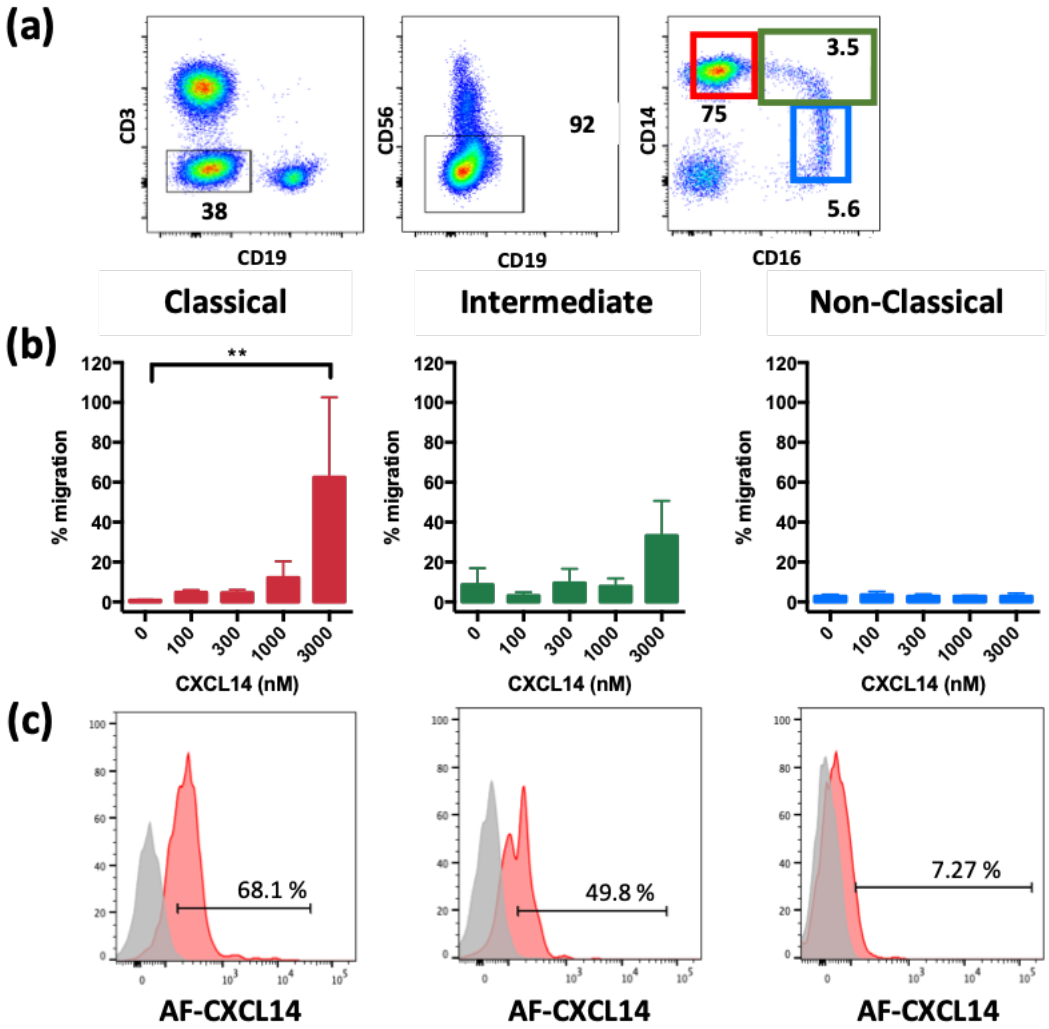


Figure 5.2. Classical and intermediate monocytes are the major targets of CXCL14 while non-classical monocytes are not CXCL14 targets.

Migratory responses of PBMC toward CXCL14 were tested by transwell chemotaxis assay. **(a)** Gating strategy for detection of monocytes following depletion of T, B and NK cells. Classical ($CD14^{++}$ $CD16^{+}$; red gate), intermediate ($CD14^{++}$ $CD16^{+}$; green gate) and non-classical ($CD14^{+}$ $CD16^{++}$; blue gate) monocytes are shown. **(b)** Migration of classical, intermediate and non-classical monocytes in response to CXCL14. Data are mean + SEM of 3 donors from 3 independent experiments. $^{**}p<0.01$, Friedman test followed by Dunn's multiple comparisons test. **(c)** Representative FACS plots show binding of 20 nM AF-CXCL14 to the three monocyte subsets (red histograms). Grey histograms represent cells labelled with AF-muCCL1 as negative control.

5.4 CXCL14 Target Cells in Mice

Previous work from our group already established the presence of a target population of CXCL14 within the skin compartment in humans (Schaerli *et al.*, 2005). Considering the difficulties associated with obtaining regular skin tissue from healthy human individuals, it was decided to identify CXCL14 target cells in mice and characterise them further. This approach would have the obvious advantage of frequent access to healthy murine tissues, as well as minimising variation between experimental animals. Additionally, working with mice allows examination of multiple peripheral tissues, both epithelial tissues and various internal organs, as well as access to genetically modified animals and disease models for future studies.

5.4.1 CXCL14 Target Cells in Murine Splenocytes

In order to identify target cells of CXCL14 within murine tissues, single cell suspensions from murine spleens were assessed for their capacity to migrate towards CXCL14 using transwell migration assays. Human CXCL12 was used as a positive control as it is well established that immune cell subsets in the spleen express CXCR4. Moreover, CXCL12 is very highly conserved between mouse and human, like CXCL14. Following migration, cells were stained with fluorochrome-conjugated mAbs directed against CD3, B220, NK1.1, CD11b and Ly6C to allow identification of T cells, B cells and two subsets of monocytes, depending on the expression levels of Ly6C. Classical monocytes in mice express high levels of Ly6C, whereas non-classical monocytes show lower expression levels (Ziegler-Heitbrock *et al.*, 2010; Yona *et al.*, 2013). **Figure 5.3a** shows the gating strategy to identify T cells, B cells and monocytes. **Figure 5.3b** shows migration responses towards human CXCL14. Of the cell subsets tested, monocytes showed the highest migration towards CXCL14, peaking at 3 µM. Both $Ly6C^{hi}$ and $Ly6C^{lo}$ monocytes responded to CXCL14 ($Ly6C^{hi}$ cells showed 9.7 ± 10.6% and $Ly6C^{lo}$ cells showed 9.3 ± 12.4% migration towards 3 µM CXCL14). T and B cells showed minimal responses towards CXCL14, in contrast to their responses towards CXCL12 which was used as positive control. Notably, there was substantial experimental variation which was evident from the migration to buffer alone condition and could be attributed to experiment-to-experiment variation. **Figure 8.2** in the Appendix shows traces of each experimental replicate to demonstrate the variability. Binding with AF-CXCL14 revealed

that Ly6C^{lo} cells showed the highest expression of CXCL14 receptor(s) on their surface (**Figure 5.3c**). In full agreement with their migratory potential towards CXCL14, B cells showed the lowest binding to AF-CXCL14.

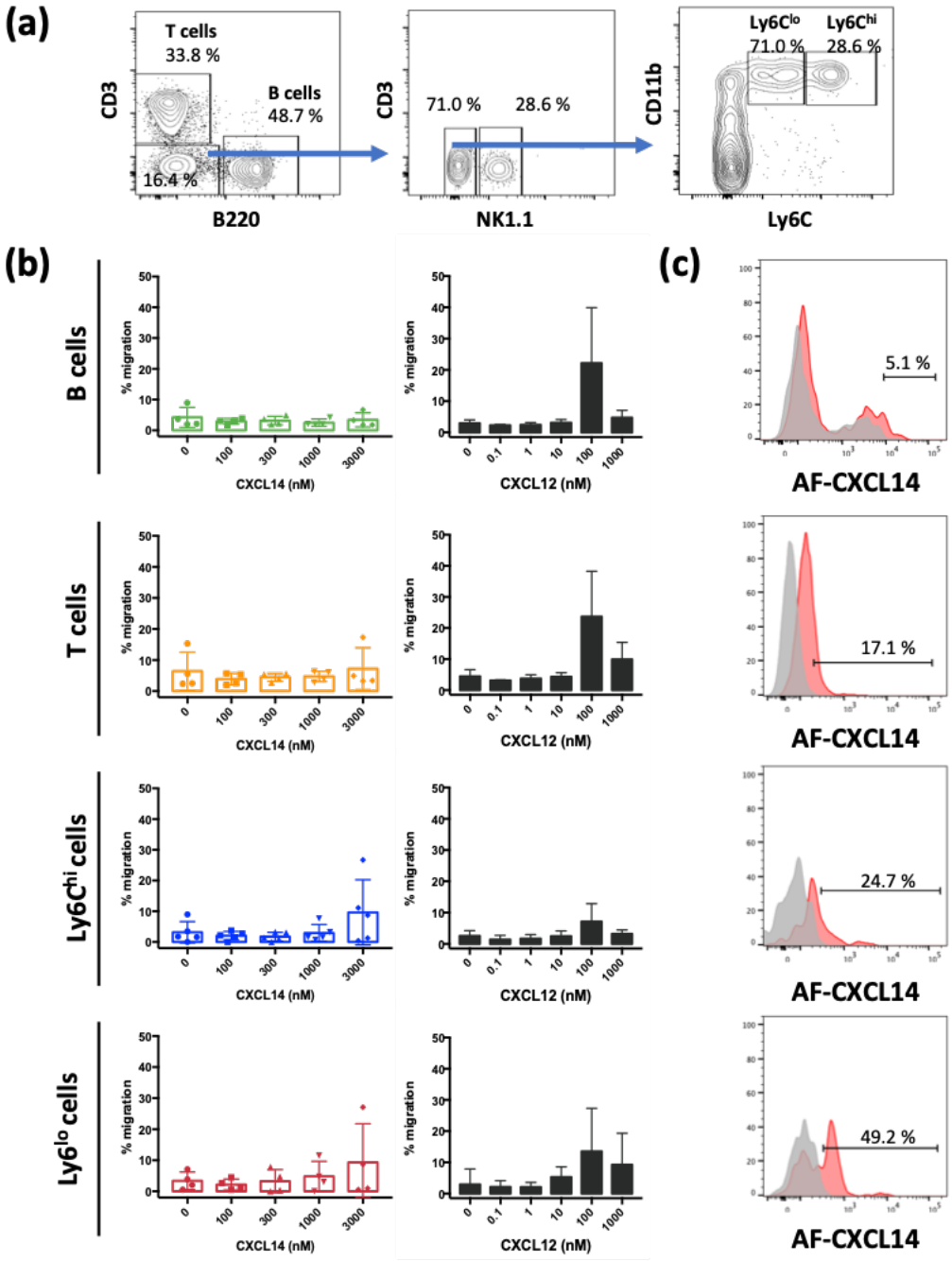


Figure 5.3. Murine monocytes are the major targets for CXCL14 among splenocytes. Migratory responses of murine splenocytes towards CXCL14 were tested by transwell migration. **(a)** Gating strategy for detection of T cells, B cells and monocytes within splenocytes, following exclusion of debris, cell aggregates and dead cells. **(a)** Migration of B cells (identified by $B220^+$ staining), T cells (identified by $CD3^+$ staining) and Monocytes (identified by Ly6C staining) towards CXCL14 (left panels) or CXCL12 (right panels). Data are mean + SD of 4-5 independent experiments. **(b)** Binding of 20 nM AF-CXCL14 to each cell subset (red histograms). Grey histograms represent cells labelled with AF-muCCL1 as negative control. Staining was performed in the presence of an Fc blocker. Plots are representative of 5 experiments. The percentage of cells which are AF-$CXCL14^+$ is indicated.

5.4.2 Identification of CXCL14 Target Cells in Murine Tissues

Unpublished work from our group revealed the presence of several subsets of CXCL14 target cells in human skin. Among tissue immune cells, which were collectively identified by CD45 expression, $CD14^+$ DCs showed very high binding of AF-CXCL14 but failed to show migration. Additionally, a fraction (approx. 25%) skin resident macrophages showed binding of AF-CXCL14. Of note, among skin tissue cells, which are CD45 negative, AF-CXCL14 clearly stained a subpopulation of potential CXCL14 target cells, suggesting that CXCL14 may also act on tissue cells (Collins, 2016). CXCL14 is very highly expressed in a variety of epithelial tissues and internal organs in mice, including the small intestine, colon, skin, lung, kidney and heart (Meuter and Moser, 2008).

Considering the expression of CXCL14 in such a variety of sites, this work aimed to detect the presence of CXCL14 target cells in different murine tissues. Single cell suspensions were isolated from various digested tissues, including skin, colon, liver, heart, kidney and lung. Cells were then stained with fluorochrome-conjugated mAbs and CXCL14 target cells were identified, within the $CD45^-$ compartment of live cells, based on AF-CXCL14 expression. Representative plots of skin staining show the gating strategy used for all tissues (**Figure 5.4a**). Gating of AF-$CXCL14^+$ was set based on FMO control. The percentage of AF-CXCL14 cells within the $CD45^-$ tissue compartment was compared to the percentage of cells stained with murine $CCL1^{AF647}$ (AF-muCCL1), as previous work in our group showed that this labelled chemokine is a more reliable negative control than unstained cells (**Figure 5.4b**) (Collins, 2016). Among the tissues examined, skin showed the most significant results (15.6 ± 6.6% AF-$CXCL14^+$ cells compared to 0.91 ± 0.63% AF-$muCCL1^+$). Even though Meuter et al. showed no expression of CXCL14 mRNA in liver tissue (Meuter and Moser, 2008), these results showed a clear presence of AF-CXCL14 binding cells within the $CD45^-$ compartment of liver tissue. Both heart and kidney showed medium expression of CXCL14 in the past, which agrees with the presence of CXCL14 target cells. Although lung tissue showed high expression of CXCL14 in previous studies (Meuter and Moser, 2008), no AF-$CXCL14^+$ cells could be detected above background. These results thus clearly show that there is a population of CXCL14 receptor-expressing cells within the $CD45^-$ compartment in various murine tissues. I decided to name these cells TR14 cells (Tissue Resident CXCL14 binding cells) to indicate

the fact that they are characterised by AF-CXCL14 staining and that they are primarily tissue-resident, i.e. non-migratory.

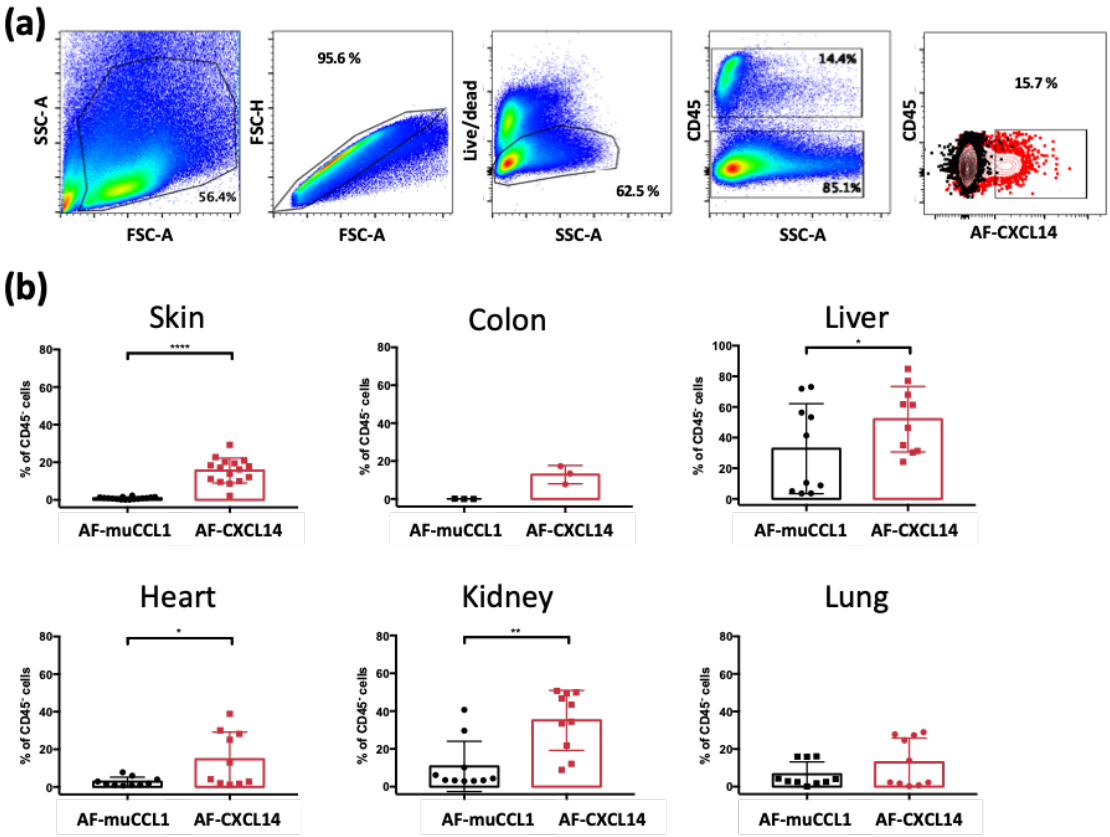


Figure 5.4. Tissue Resident CXCL14 binding cells (TR14 cells) are present in different murine tissues.

(a) Different murine tissues were processed, and single cell suspension was assessed for presence of CXCL14 target cells. Live single cells were gated on by excluding debris (first panel), cell aggregates (second panel) and dead cells (third panel). Target cells were identified by staining with 20 nM AF-CXCL14 (red staining) and compared to staining with AF-muCCL1 (black staining). AF-CXCL14^{+} gate was set based on AF-CXCL14 FMO. Plots are from skin tissue and are representative of 8 experiments using a total of 32 mice. **(b)** Graphs represent target cells as a percentage of CD45^{-} cells. Data are mean + SD of 3-8 experiments with each data point depicting individual biological repeats. *p<0.05, **p<0.01, **** P <0.0001, Wilcoxon test or Paired T test based on normality of the data, assessed by D'Agostino & Pearson normality test.

5.4.3 RNA Sequencing Analysis of TR14 Cells in Skin and Colon

The presence of CXCL14 target cells was further investigated in tissues with prominent presence TR14 cells, including skin and colon. Work from previous members of our group (Dr Paul Collins and Dr Michelle McCully) was aiming to translate the work already done with human skin to the mouse system, and hypothesised that due to the high expression of CXCL14 in these tissues, it is very likely that CXCL14 target cells can also be detected in the mouse. Data collected showed that mouse skin and colon contained our cells of interest and they therefore sought to isolate these cells with the aim to examine their gene signature by RNA sequencing (RNA seq). Furthermore, they aimed to isolate additional populations, including macrophages and tissue cells in order to compare gene expression among all populations. Other tissues were not examined at that point. Initially, skin and colon tissues were digested and labelled with fluorochrome-conjugated mAbs, including CD45, CD11b, CCR2, Lineage markers, F4/80, CD64 and Ly6C. Following exclusion of debris, cell aggregates and dead cells, different cell populations were identified and FACS sorted, as shown in **Figure 5.5.** The gating strategy shown is representative of two independent experiments. Among the $CD45^+$ cells, skin macrophages were identified as $CD45^+CD11b^+CCR2^-CD64^+$, within both skin and colon cells. Skin and colon TR14 cells were identified as $CD45^-CXCL14^+F4/80^+$, whereas skin tissue cells were sorted based on $CD45^-CXCL14^-F4/80^-$ expression. Two independent FACS sorts were performed, each containing two samples of skin/colon TR14 cells and one sample of skin/colon macrophages and skin tissue.

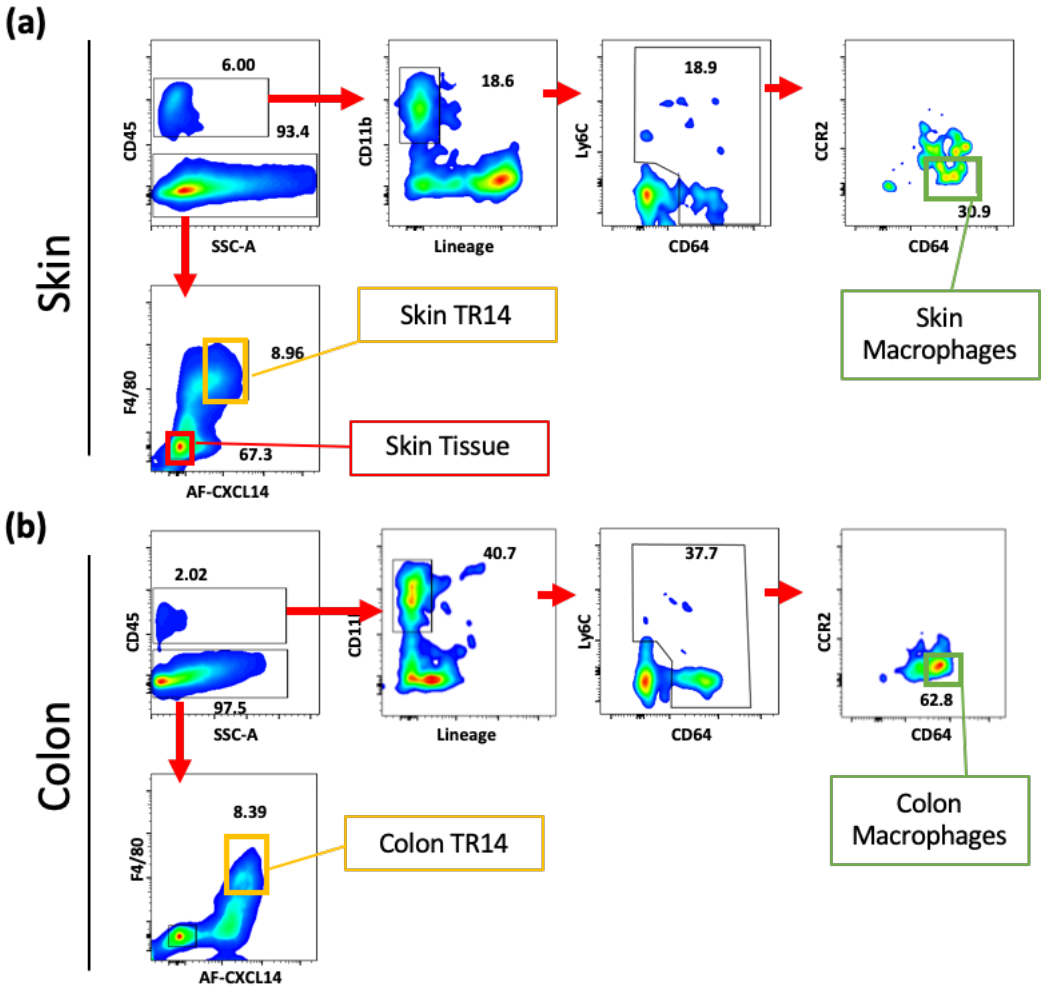


Figure 5.5. Gating strategy to identify immune cell populations isolated from murine skin and colon for RNA sequencing.

Mouse skin and colon were digested, and single cell suspension was stained with cell surface markers and sorted by FACS. Cell populations of interest that were selected for RNA sequencing analysis include Skin/Colon macrophages (green gate - $CD45^{+}CD11b^{+}CCR2^{-}CD64^{+}$), Skin/Colon TR14 cells (yellow gate - $CD45^{-}CXCL14^{+}F4/80^{+}$) and Skin tissue cells (red gate - $CD45^{-}CXCL14^{-}F4/80^{-}$). Sorted cells were resuspended in Buffer RLT Plus and RNA was then extracted for shipping to the Sequencing facility in Cleveland, Ohio. This work was done by Dr Paul Collins.

RNA from these samples was isolated and next-generation sequencing of mRNA was performed by the Institute for Computational Biology at Case Western Reserve University, Cleveland, Ohio. Analysis of the RNA seq data was performed by Dr You Zhou and Dr Robert Andrews locally at Cardiff University, Division of Infection and Immunity. Gene expression values were normalised and **Figure 5.6a** shows the top 2000 most highly abundant genes in each sample. Counts below 100 were excluded as they were considered background expression. This heatmap shows the differences in gene expression between our different sorted samples. The dendrogram clearly demonstrates that based on their most highly expressed genes, skin and colon TR14 cells have a more similar gene expression profile, while skin and colon macrophages also cluster together. Additionally, differences between different sorting experiments are visible among the TR14 cell samples.

This preliminary analysis of the gene expression profile of TR14 cells included comparison of our sorted cell types with publicly available databases. For this comparison, the GSE109125 Dataset (SRA - SRP128986) from the ImmGen database was used, which contained RNA seq data for 98 purified immune cell populations representing all lineages. Following exclusion of <100 gene counts, the ENSEMBL 97 database was used to map the gene numbers to gene names. The sorted cell samples were merged with the database and subjected to TMM normalisation, followed by batch adjustment, to account for data deriving from different sources. Normalised data were plotted in a Multidimensional Scaling plot (**Figure 5.6b)**. Data points within this plot with similar gene signatures are positioned together, whereas more dissimilar samples are further apart. This analysis was thus done in an effort to identify whether our cells of interest were more similar to other known cell subsets. Circled clusters of cells with labels in bold represent our sorted samples. Skin and colon macrophages clustered closer to macrophages from the public database, which served as an internal control. Skin and colon TR14 cell samples clustered further away from the sorted macrophages and other immune cell types, but closer to skin tissue cells as well as different populations of stromal cells. In particular, skin and colon TR14 cells were more similar to subcutaneous lymph node fibroblastic reticular cells and subcutaneous lymph node blood endothelial cells from the public database, while thymic medullary epithelial cells

clustered slightly further away from TR14 cells. Examining these subsets in more detail, subcutaneous lymph node fibroblastic reticular cells were sorted based on the following markers: $CD45^{-}$ $CD31^{-}$ $CD35^{-}$ $MadCAM^{-}$ $PDPN^{+}$ $CD140^{+}$, whereas subcutaneous lymph node blood endothelial cells comprised of two separate cell types. One group of cells consisted of two samples of subcutaneous lymph node lymphatic endothelial cells that were sorted based on $CD45^{-}$ $CD31^{+}$ $PDPN^{+}$ expression and were located at the top of this cluster. The other group of cells consisted of three samples of subcutaneous lymph node blood endothelial cells, that were sorted based on $CD45^{-}$ $CD31^{+}$ $PDPN^{-}$ $PNAd^{+}$. Collectively, these data show that among the samples sorted from skin and colon, TR14 cells are more similar to skin tissue cells than either skin or colon macrophages. Moreover, skin and colon TR14 cells share similarities with stromal cells, rather than other known immune cell types.

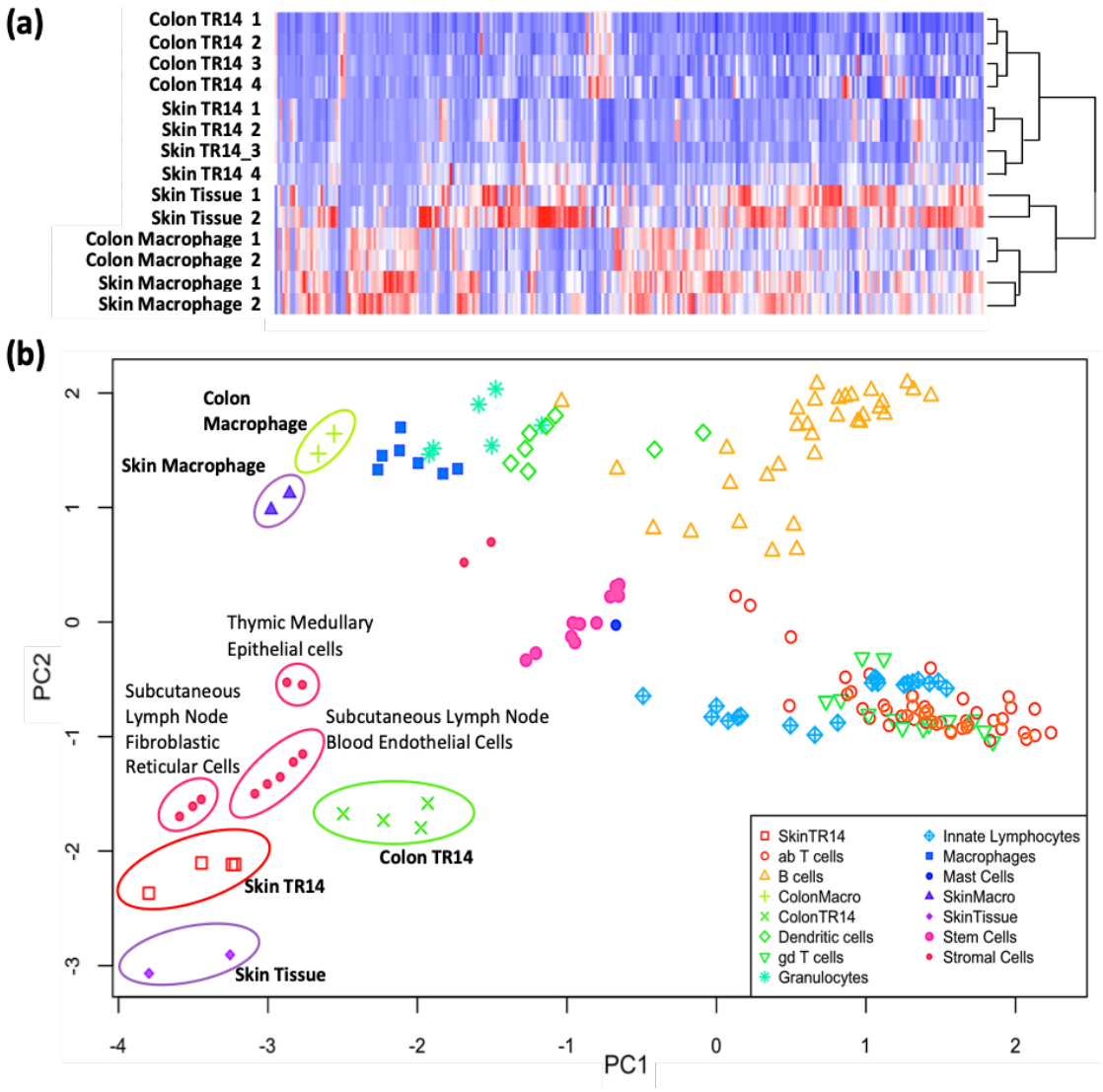


Figure 5.6. RNA sequencing analysis of sorted TR14 cells.

(a) Heatmap of 2000 most highly expressed genes of each sample of sorted tissue cells. Gene counts blow 100 were excluded from this analysis. **(b)** Multidimensional Scaling Plot (MDS) of 14 sorted tissue samples from our analysis and 157 mouse blood and tissue samples sourced from the public repository Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) – database used is GSE109125. Following exclusion of gene counts below 100, genes were mapped to gene numbers with the use of the ENSEMBL 97 database (GRCm38.p6). Samples from our sorting and the public database were then merged and TMM normalisation was applied. Batch effect was corrected using Voom package in R. MDS plot was generated using Limma package. This work was done by Dr You Zhou.

Further analysis of the RNA seq data of these samples aimed to assess the chemokine and chemokine receptor gene expression of each cell population, which is shown in **Figure 5.7**. Chemokines and receptors were grouped based on their structural characteristics, namely the position of the conserved cysteine residue. Expression values below 100 were excluded (blank), as they were considered too low. Additionally, expression of CXCL16 by colon macrophages was also excluded as due to its high value (22195.3 - highlighted in red) (see figure below), it skewed the colouring of all other values. Clustering of these samples based on chemokine and chemokine receptor gene expression revealed that based on the expression of these genes, skin TR14 cells were more related to skin tissue cells than colon TR14 cells. This preliminary analysis provided many indications about the migratory capacity of our cells of interest, though gene expression was not further confirmed. Assessment of chemokine and chemokine receptor gene expression by skin tissue cells could not be evaluated in depth as little is known in the field regarding their gene expression. Certain prominent gene expression values of macrophages could be distinguished, as they could be compared to our existing knowledge of chemokine receptor gene expression. For instance, CXCR4 and CX3CR1 are known to be highly expressed by macrophages. Notably, colon macrophages have been described to express higher levels of CX3CR1 than other macrophages (Yona *et al.*, 2013; Bain and Schridde, 2018), which was consistent with our data. Additionally, CCR1, CCR2 and CCR5 were also expressed on macrophages (Murphy *et al.*, 2000). However, certain expression values were unexpected, such as CCR7, which is responsible for migration to lymphoid organs and is thus not expected to be expressed by resident cells. High expression of ACKR5 by colon and skin macrophages was a novel finding as it has not been reported in the literature. Regarding chemokine expression, the skin homing chemokine CCL27 (Bachelerie *et al.*, 2013) was expressed by skin tissue cells and skin TR14 cells, whereas the gut-related chemokine CCL28 was expressed by colon TR14 cells. An interesting observation was that although the membrane-bound chemokine CXCL16 was expressed by all populations, its receptor CXCR6 was not expressed by any. Finally, although it is hard to make a general conclusion from this analysis, it highlights chemokine and chemokine receptor genes that are similarly expressed among the sorted cell subsets in our analysis. Although macrophages show differences depending on tissue, they are more similar than skin tissue cells or TR14

cells. Moreover, skin TR14 cells share more similarities with skin tissue cells than colon TR14 cells or macrophages.

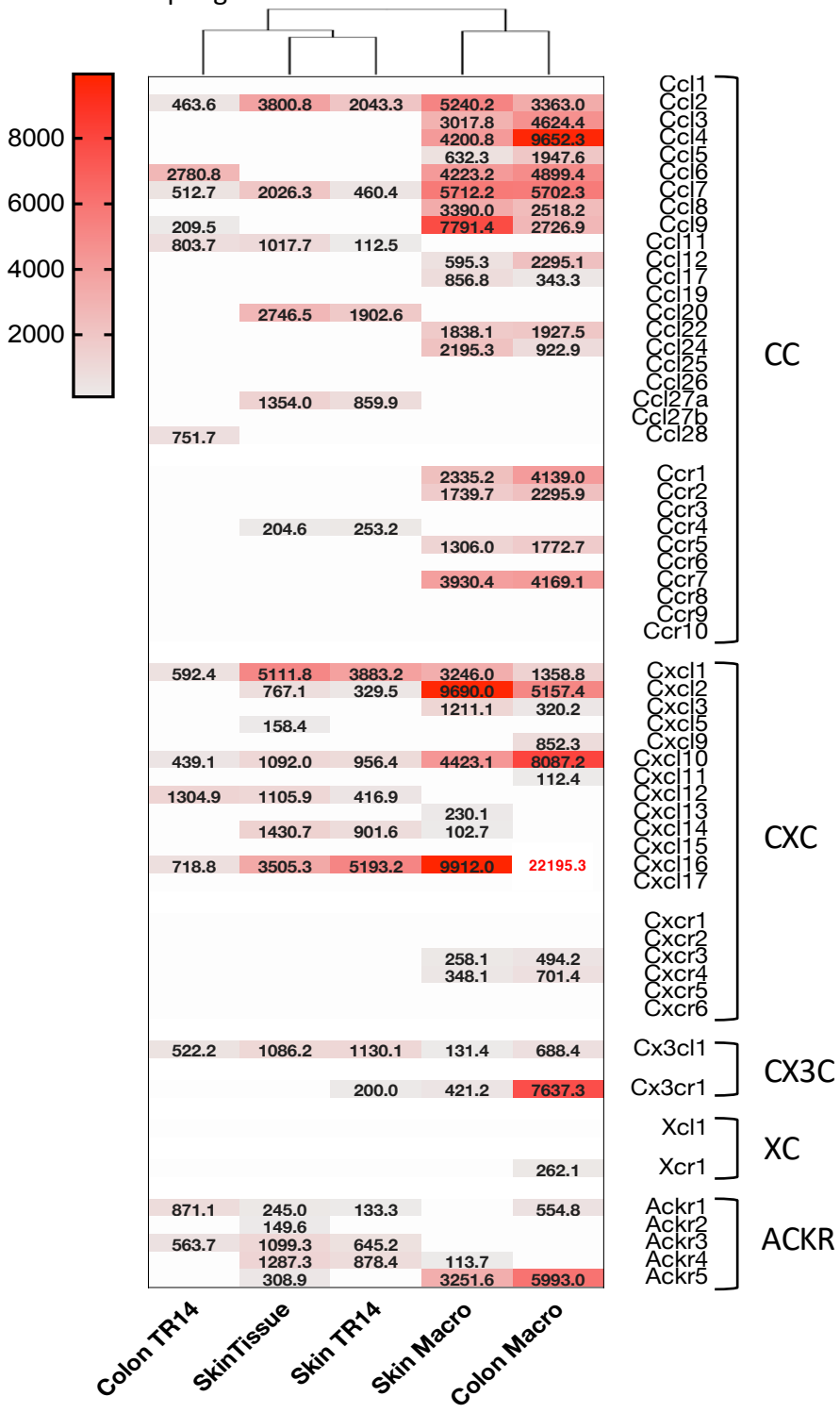


Figure 5.7. Chemokine and chemokine receptor analysis of sorted samples from murine skin and colon.

Gene counts were normalised using Deseq2 and heatmap of chemokine-related genes was plotted. Chemokines and chemokine receptors were categorised based on the relative position of the first two NH_2-terminal cysteine residues, and atypical chemokine receptors.

F4/80 gene expression analysis revealed surprising results. Although it was used as a cell surface marker when sorting TR14 cell samples for RNA seq analysis (**Figure 5.5**), minimal expression was seen at the gene level. In particular, **Table 5.2** shows the normalised F4/80 gene expression values for each subset, where skin TR14 cells showed mean value of 20.5 ± 32.3, compared to skin macrophages which showed mean value of 12,903 ± 2,600. Considering that normalised gene counts below 100 were considered too low throughout this analysis, these data revealed an absence of F4/80 expression at the gene level. These discrepancies in F4/80 gene expression among diverse F4/80$^+$ cells required further investigations.

Table 5.2. Normalised gene counts from RNA seq analysis show expression of the marker F4/80.

	Skin TR14 cells				Skin Tissue		Skin Macrophages		Colon Macrophages		Colon TR14 cells			
F4/80	0	0	73	9	0	0	14741	11064	18943	20815	4	4	0	0

The next logical step in this analysis was to test whether the F4/80 staining was indeed non-specific, as the RNA seq data suggested. Isolated cells from skin and colon samples were digested, and stained with fluorochrome-conjugated mAbs, including different fluorochrome-conjugated F4/80 antibodies of the same clone (F4/80-APC-Cy7 and F4/80-BV421). Representative plots of 3-4 independent experiments are shown in **Figure 5.8.** Live cells were gated based on CD45 expression. Gating on CD45$^+$CX3CR1$^+$ cells allowed identification of skin and colon macrophages, which were used as a positive control population, as they were expected to express F4/80 on the cell surface. Notably, skin macrophages showed much lower expression of CX3CR1 than colon macrophages, which was consistent with the literature (Bain *et al.*, 2013; Yona *et al.*, 2013). Staining of CD45$^+$ cells revealed minimal differences between the two differently fluorochrome-conjugated F4/80 antibodies. Staining with F4/80 - APC-Cy7 was positive

in both colon and skin CD45⁻ cells, in correlation with AF-CXCL14 staining. Lower levels of positivity were observed in skin cells compared to previous experiments which could be attributed to fixation with 2% formaldehyde. Gating of AF-CXCL14 positivity was set based on AF-muCCL1 staining. However, staining with F4/80 – BV421 did not show the same results. In fact, the BV421-conjugated F4/80 antibody failed to stain TR14 cells mouse skin and colon, demonstrating that staining with F4/80 – APC-Cy7 was indeed non-specific. These data revealed a fluorochrome-specific issue, rather than clone-dependent. Notably, no other antibody used throughout this work was labelled with APC-Cy7.

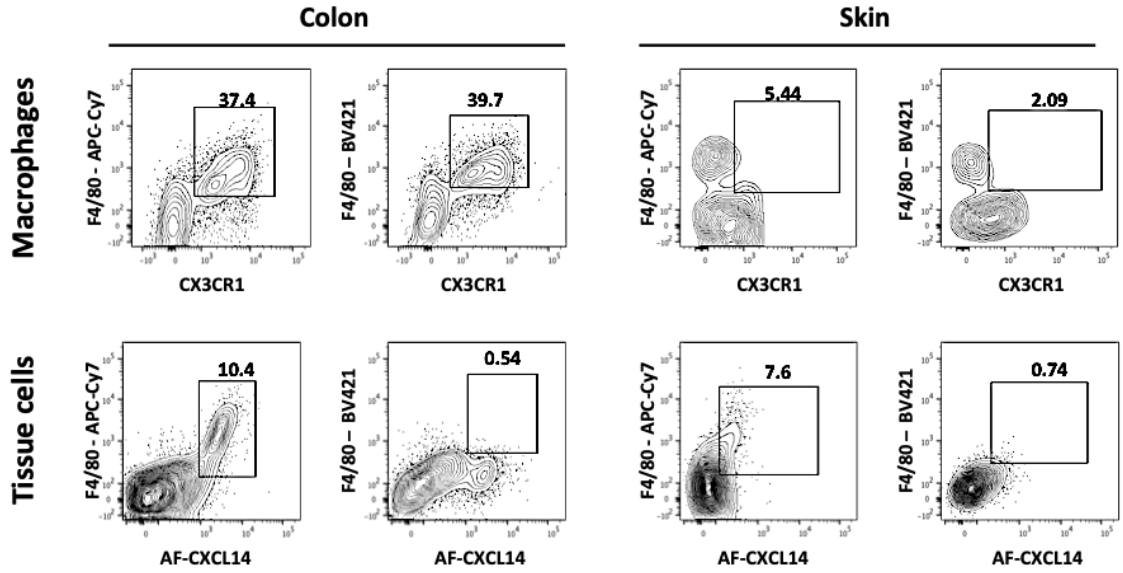


Figure 5.8. TR14 cells do not express F4/80 on their cell surface.
Cell surface staining of F4/80 was assessed by flow cytometry. Single cell suspension from murine skin and colon were stained with fluorescently labelled antibodies and staining was assessed by FACS Canto II. Two different fluorochrome-conjugated F4/80 antibodies were used to assess staining. Gates were set based on FMO controls for F4/80 and CX3CR1 gates and AF-muCCL1 staining for AF-CXCL14 gates. Representative FACS plots are shown from 3-4 independent experiments.

5.4.4 Phenotypic and Morphological Characterisation of TR14 Cells

In addition to characterisation of TR14 cells based on their gene signature using RNA seq analysis, it was also important to phenotypically define these cells, as well as examine their morphology. A selection of cell surface markers was used to attempt to further characterise these cells, as shown in **Figure 5.9a**. Taking into account our evidence of non-specific staining using F4/80-APC-Cy7, each marker that was tested by fluorochrome-conjugated antibody by flow cytometry was compared to the normalised gene expression values obtained from the RNA seq analysis (**Figure 5.9b**). Although gates on these plots were set based on FMO staining (grey histogram), staining of $CD45^{-}$AF-$CXCL14^{-}$ cells (tissue cells) was included as control (blue histogram). Of interest, staining with CXCR4 showed high positivity of TR14 cells (71.3% based on FMO staining), although tissue cells also showed some positivity. Nevertheless, RNA seq revealed no gene expression (6.53), compared to higher values observed in colon macrophages (701.4) and skin macrophages (348.1). Staining of the chemokine receptor CCR3 also caused some issues. As shown in **Figure 5.9b**, gene expression values were below detection, yet staining with either a PE-conjugated or FITC-conjugated antibody of the same clone showed high expression of cell surface CCR3 (data not shown). In an effort to elucidate the cause of cell surface staining in the absence of gene expression, skin and colon cells were incubated with murine CCL11 (muCCL11) in order to induce internalisation of its putative receptor on the cell surface. However, incubation with chemokine did not induce reduction in CCR3 cell surface staining. It should be noted that there is no evidence of chemokine receptor internalisation on tissue cells in the literature. Ultimately, a PE-conjugated CCR3 antibody of a different clone and supplier was used to stain both skin and colon TR14 cells, as well as $CD45^{+}SSC^{hi}$ spleen cells as a positive control (**Figure 8.3**). Staining with this antibody following incubation with muCCL11 reduced MFI values from 136 to 97 on $CD45^{+}SSC^{hi}$ cells indicating lower levels of receptor internalisation. Although CCR3 staining of TR14 cells decreased when using this antibody, cell surface expression did not further decrease following chemokine incubation. Overall, these data show that certain markers show discrepancies in cell surface expression and gene expression data. Specific markers showed consistently increased expression at both the gene and cell surface level. These include EpCAM,

CD34, CD31, Sca-1 and CX3CR1, as their cell surface expression correlated with the gene expression. EpCAM and CD31 are markers associated with cell adhesion, while CD34 and Sca-1 are associated with haematopoietic stem cells (Sidney *et al.*, 2014). CX3CR1 is expressed by various immune cell subsets, most notably by a subset of monocytes in blood and macrophages in the gut (Imai *et al.*, 1997; Geissmann, Jung and Littman, 2003; Ishida, Gao and Murphy, 2008).

Following phenotypic characterisation of TR14 cells, morphological analysis of these cells was also carried out. To achieve that, single cell suspensions of digested colon and skin tissue were stained with CD45 and AF-CXCL14 or AF-muCCL1, and FACS sorted based on their expression of these markers. In particular, skin and colon TR14 cells, as well as skin and colon $CD45^{-}AF\text{-}CXCL14^{-}$ cells (skin tissue cells) were examined in more detail. Cytospins of these sorted populations were stained with May-Grünwald and Giemsa stain to analyse their morphology, as shown in **Figure 5.9c**. As opposed to $CD45^{-}AF\text{-}CXCL14^{-}$ cells, TR14 cells appeared to have more immature-like features, including a low nucleus-to-cytoplasm ratio. Images of these cells showed that skin and colon TR14 cells appeared small and no granulation was visible, although cell debris was noticeable in the background. This staining thus supported the notion that TR14 cells are not immune cells, based on the differences in the morphological characteristics with key immune cell subsets. Lack of granulation in the cytoplasm excluded similarities eosinophils and mast cells. Additionally, lack of dendrites or vacuolar cytoplasm indicated that they are different from DCs and macrophages, respectively. Although lymphocytes also show a low nucleus-to-cytoplasm ration, TR14 cells lacked key lymphocyte markers, therefore similarities to lymphocytes could also be excluded. Notably, a low nucleus-to-cytoplasm ratio is also associated with progenitor cells, which would correlate with the expression of CD34 and Sca-1 by TR14 cells (Kuehnle and Goodell, 2002; Mass *et al.*, 2016; Yamane, 2018).

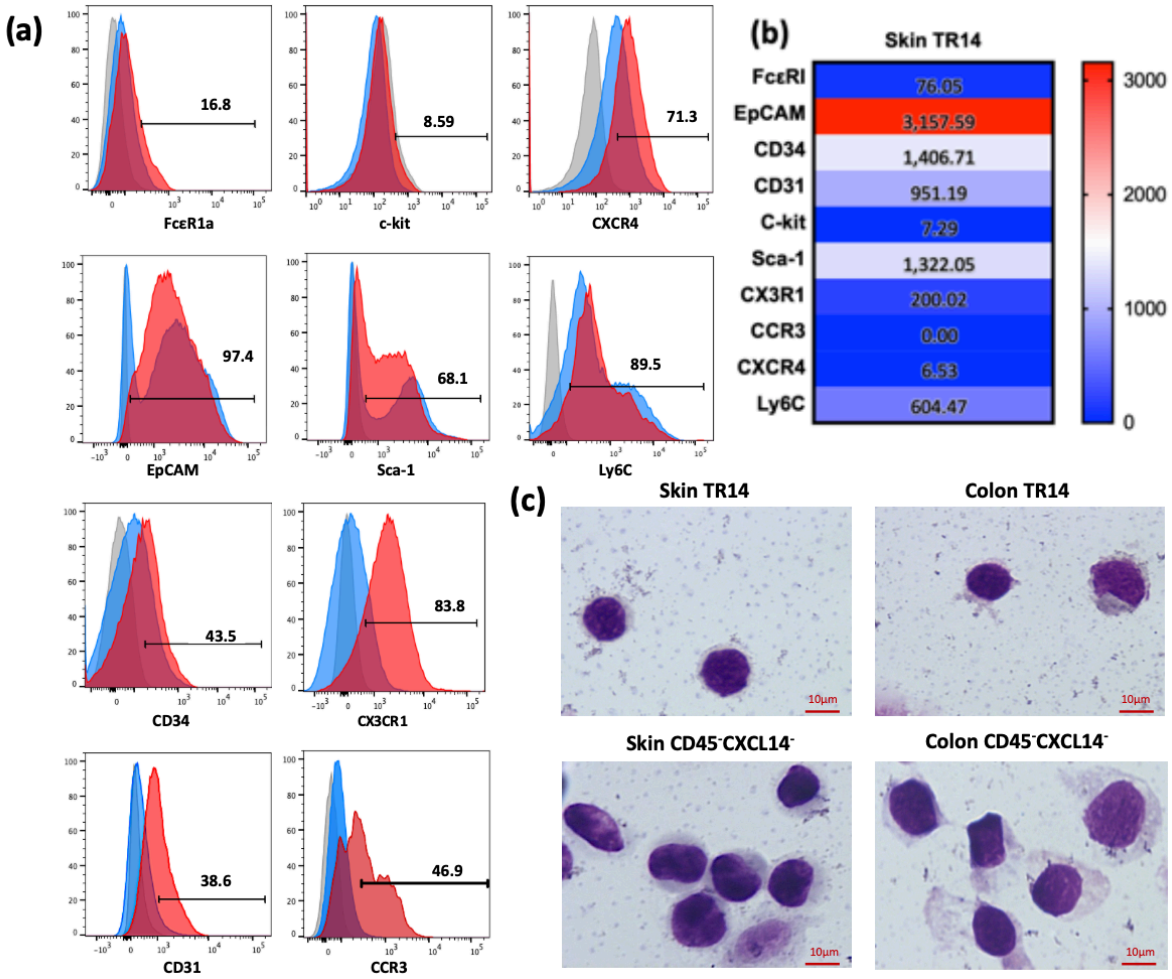


Figure 5.9. Phenotypic and morphological characterisation of TR14 cells.

(a) Selected cell surface marker expression was assessed by flow cytometry. Grey histograms represent FMO staining of each cell surface marker, red histograms show staining of TR14 cells and blue histograms show staining of CD45$^-$CXCL14$^-$ cells. Percentages show positivity for each marker based on staining by FMO control. **(b)** Heatmap of Deseq2 normalised gene counts of each marker assessed by flow cytometry. Numbers represent mean across 4 TR14 cell samples. **(c)** Morphological analysis of sorted populations. Single cells from indicated murine tissues were stained and FACS sorted. Cytospins of sorted cells were stained with May-Grünwald and Giemsa stain to assess morphology. Analysis of TR14 cells and CD45$^-$CXCL14$^-$ cells from skin and colon are shown. Images were captured with a Zeiss Axioscan microscope using x63 magnification.

5.4.5 Enzymatic Digestion Affects Assessment of Functionality of TR14 Cells

Data presented so far showed that TR14 cells are present in multiple murine tissues and share similarities with stromal cells, based on analysis of our RNA seq data. Additionally, they can be defined by certain cell surface markers, including EpCAM, CD34, CD31, Sca-1, CX3CR1 and Ly6C. Their morphology appears more immature-like, potentially similar to that of precursor cell types. Nevertheless, one of the most important aims of this project was to demonstrate that these cells are functionally active. Functionality could be tested by examining TR14 cells in *in vitro* chemotaxis and Ca^{2+} mobilisation assays.

Throughout this project, multiple attempts of optimising migration of TR14 cells towards CXCL14 were made, with unfortunately no positive outcome (data not shown). Initially, different digestion protocols were tested on skin tissue cells, including overnight dispase digestion, compared to digested cells that were rested overnight at 4°C. Overnight incubation aimed to let cells recuperate and allow possible cell-surface proteins that were cleaved due to enzymatic digestion to be re-expressed at the cell surface. Throughout all optimisation experiments, migration of $CD45^+$ cells towards CXCL12 was used as a positive control in agreement with the expression of CXCR4 on their cell surface. Moreover, coating of plates with collagen was tested to provide more physiological conditions and facilitate migration. Both 5 µm and 8 µm pore size plates were tested, but the background migration in 8 µm plates was very high. When 8 µm pore plates were used, there was some specific migration of $CD45^+F4/80^+$ cells towards 100 nM CXCL12, which suggested that addition of collagen facilitated migration of macrophages (data not shown). Therefore, collagen coating of the transwell plates was used in all future experiments.

Following multiple experiments showing no or low levels of migration, a different approach was tested. Magnetic isolation prior to migration assay was tested as I hypothesised that the transwell pores were getting blocked by tissue cells, thereby hindering migration of $CD45^+$ cells. CD45 magnetic isolation was performed on freshly digested cells, which showed that $Ly6C^{hi}$ cells within the $CD45^+$ tissue compartment showed migration towards 3 µM CXCL14 (6% compared to 0% background). This finding confirmed the hypothesis that the pores of the transwell were blocked by tissue cells. Nevertheless, TR14 cells, which are by definition $CD45^-$, showed no migration (data not

shown). A different approach was also investigated, where pieces of separated dermis and epidermis were incubated with chemokines overnight, followed by quantification of emigrant cells by flow cytometry. Results of this experiment showed that $CD45^{+}F4/80^{+}$ cells from the epidermis showed increased migration towards 100 nM CXCL12. Langerhans cells would be the only cells in the epidermis that are known to express F4/80, although additional markers for their precise identification were not included (Schuler and Steinman, 1985). Notably, $CD45^{+}F4/80^{+}$ cells from the dermis showed no specific migration, which is consistent with the notion that resident macrophages are firmly positioned within the tissue and would not be expected to migrate out of the tissue. However, some monocytes within the dermis are also expected to express F4/80, which presumably did not migrate (Jakubzick *et al.*, 2013). TR14 cells from the epidermis showed slightly higher migration towards 100 nM CXCL12, although variation between experimental repeats was high.

Collectively, the lack of migration after multiple attempts led me to hypothesise that treatment with proteolytic enzymes in order to isolate the cells could also be negatively affecting the ability of these cells to migrate. In order to address this hypothesis, murine spleens were processed in the same way as skin, using the same enzymatic digestion. Spleens were selected as a control tissue since my previous results demonstrated that splenocytes showed clear migration towards both CXCL14 and CXCL12. Migration of single cell suspension towards CXCL14 and CXCL12 was assessed using transwell migration (**Figure 5.10**). Migrated cells were stained with fluorochrome-conjugated mAbs against CD3, CD11b and Ly6C, that allowed identification of T cells and monocyte subsets. Both $Ly6C^{lo}$ and $Ly6C^{hi}$ cells showed decreased migration towards 3 µM CXCL14 (3.95 ± 1.39% migration of non-digested cells compared to 1.72 ± 0.3% of digested cells). T cells also showed decreased migration towards 100 nM CXCL12 following digestion (2.81 ± 1.92% migration of non-digested cells compared to 1.37 ± 0.67% of digested cells. Although the results from this experiment were not significant, they indicated that enzymatic digestion may play a role but may not be the only reason for lack of migratory responses observed in skin cells.

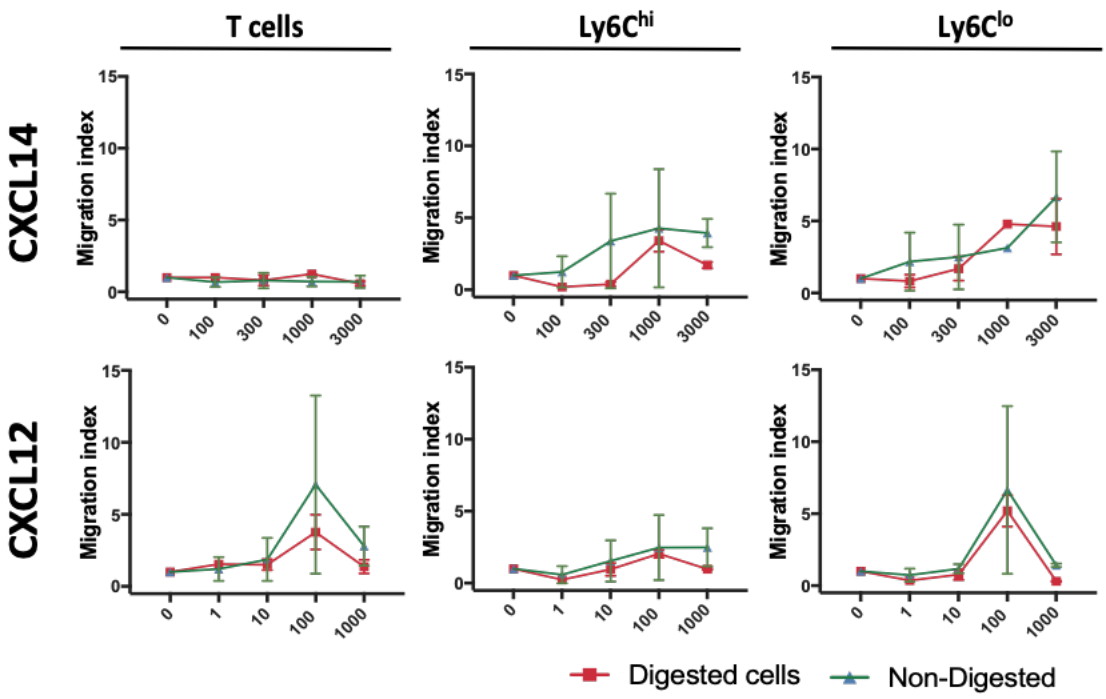


Figure 5.10. Enzymatic digestion of tissues causes decrease in migration of immune cells.

Murine spleens were digested following the digestion protocol used for murine skin. Migratory responses of single cells following enzymatic digestion towards CXCL4 and CXCL12 was assessed by transwell migration. T cells were identified based on CD3 expression, and monocytes based on Ly6C expression. Data shown are mean + SEM from 2 independent experiments.

5.5 Discussion

Work in this Chapter was aiming to define the CXCL14 target cells within murine tissues. In addition to the standard prototype targets for chemokines which are immune cells, this Chapter provides evidence for the existence of an as yet ill-defined population of cells in murine tissue that does not express the prototypical immune cell marker CD45. To the best of my knowledge, there is no immune cell subset known that does not express CD45. Human skin TR14 cells were found to be abundant, representing up to 20% of $CD45^-$ cells, thus constituting a significant compartment in healthy tissue. Analysis and characterisation of these cells could reveal more information regarding the physiology of CXCL14, and what its function is in epithelial tissues such as skin. These cells were first identified in both the dermis and epidermis layers of human skin (Collins, 2016). Although present in tissue emigrant cells, higher numbers were detected following further digestion of the tissue. They were therefore assumed to be tissue resident cells and named TR14 cells, due to their increased detection following proteolytic digestion of the tissue and absence from peripheral blood. The aim of examining the mouse system was to try to translate our findings from human to mouse, to allow phenotypic and functional characterisation of these cells in a variety of healthy tissues, since CXCL14 is an epithelial chemokine expressed in numerous peripheral tissues and internal organs. TR14 cells, defined by their binding of AF-CXCL14 and lack of CD45 expression, were identified in murine tissues such as the skin, colon, liver, heart, kidney and lung at various levels. Characterisation of these cells involved both gene expression, phenotypic and morphological examination. Preliminary analysis of the transcriptome of mouse skin and colon TR14 cells revealed that they share similarities with stromal cells. However, investigation of the cell surface markers expressed by TR14 cells was problematic due to evidence of non-specific binding of antibodies and discrepancies between gene expression and surface protein expression. Finally, attempts to demonstrate the functionality of these cells by examining their migratory potential by transwell migration have not been fruitful so far and will need to be further examined with priority.

Since its discovery two decades ago (Hromas *et al.*, 1999; Frederick *et al.*, 2000; Kurth *et al.*, 2001) a whole host of functions has been described for CXCL14, ranging from

induction of chemotaxis in various immune cell subsets including B cells, monocytes, neutrophils and DC precursors (Cao *et al.*, 2000; Sleeman *et al.*, 2000; Kurth *et al.*, 2001; Shellenberger *et al.*, 2004; Starnes *et al.*, 2006), to broad spectrum antimicrobial activity (Maerki *et al.*, 2009; Dai *et al.*, 2015). As well as being produced by tissue cells including fibroblasts, keratinocytes and endothelial cells, CXCL14 has been reported to show activity on tissues, including endothelial cells and fibroblasts (Cao *et al.*, 2000; Frederick *et al.*, 2000; Kurth *et al.*, 2001; Shellenberger *et al.*, 2004; Schaerli *et al.*, 2005; Mokhtar *et al.*, 2009). Previous work from our group has focused on the identification of CXCL14 target cells in peripheral blood and revealed that monocytes are the major targets of CXCL14 among blood leukocytes (Schaerli *et al.*, 2005). In particular, classical ($CD14^{++}CD16^{-}$) monocytes displayed the strongest response, while intermediate ($CD14^{++}CD16^{+}$) monocytes displayed a weaker response, which was also confirmed in the current work. The three monocyte subsets exhibit unique genotypic and phenotypic profiles (Wong *et al.*, 2011), but their functional differences are yet to be elucidated. Classical monocytes express the inflammatory chemokine receptors CXCR1, CXCR2 and CCR2, which led to the suggestion that this subset exits circulation to enter inflamed tissues where they differentiate into macrophages or DCs (Geissmann, Jung and Littman, 2003). In contrast, it has been proposed that non-classical monocytes are excluded from sites of inflammation and may remain in the bloodstream, operating as a blood-resident macrophage population (Cros *et al.*, 2010). More recently, it was shown that classical monocytes likely give rise to the intermediate monocytes, followed by non-classical subsets (McGovern *et al.*, 2014). CXCL14 responsiveness data thus suggest that classical monocytes lose their ability to respond to CXCL14 as they move along the developmental pathway. This, in turn, correlates with the requirement of classical monocytes to respond effectively to cues to enter tissues, including CXCL14. Geissmann *et al.* also showed that like its human counterpart, the murine monocyte compartment is heterogeneous with subsets defined by Ly6C expression (Geissmann, Jung and Littman, 2003). $Ly6C^{hi}$ monocytes were found to enter the tissue both under inflammatory and steady state conditions, therefore they were described as “classical” monocytes. $Ly6C^{lo}$ monocytes were shown to never enter tissues, but rather patrol the vasculature and scavenge necrotic endothelial cells instead of acting as a circulating intermediate (Carlin *et al.*, 2013). Considering the evidence that classical monocytes are

the primary targets of CXCL14 in human blood, it was postulated that murine monocytes would also be the main targets of CXCL14. Investigation of splenocyte migration showed that indeed, monocytes primarily migrated towards CXCL14. In contrast to the human monocyte findings, both Ly6C subsets responded to CXCL14. Migratory responses of $Ly6C^{lo}$ monocytes correlated well with binding of AF-CXCL14, whereas $Ly6C^{hi}$ showed less binding of AF-CXCL14.

Mouse and human CXCL14 differ by only two amino acid substitutions but experiments performed during this work reassured us that this reagent, although of human origin, could also be used in the mouse system. Continuing on from previous work of our group, which described for the first time a population of cells extracted from human skin that expressed the myeloid markers CD1a and variable levels of CD14, but were negative for the pan-leukocyte marker CD45 (TR14 cells) (Collins, 2016), these cells were also detected in murine skin. Additionally, TR14 cells were also found at other sites, some of which have been shown to express high levels of CXCL14, including intestine, kidney and lung (Meuter *et al.*, 2007). However, although CXCL14 has been shown to be expressed in the brain, TR14 cells were not detectable in the brain (data not shown). High expression of CXCL14 was also seen in placenta, however it was not examined for TR14 cell presence. There are reports showing that CXCL14 can interact with negatively charged glycosaminoglycans (Penk *et al.*, 2019), therefore at present we cannot exclude the possibility that many of these TR14 cells actually do not express a functional CXCL14 receptor, but rather bind the AF-CXCL14 non-specifically due to interaction with negatively charged glycosaminoglycans. In order to exclude this possibility, experiments in the future should aim to cleave polysaccharides present on the surface of the cells (e.g. using heparinases) so that any non-specific interaction is inhibited. Phenotypic characterisation of skin TR14 cells was troublesome due to experimental difficulties. Gene expression data were valuable as a guide of cell surface marker expression, although one should be mindful of potential differences between gene and protein expression. **Table 5.3** shows a summary of cell surface markers on TR14 cells tested by flow cytometry and the equivalent gene expression values for each marker. Cell surface expression was assessed by antibody staining detected by flow cytometry and gating on positive populations was set based on FMO controls for each marker. There are reports

of enzymatic digestion causing both higher and lower cell surface expression of certain markers (Autengruber *et al.*, 2012), which could also explain some of the differences between gene and cell surface expression. Variable turnover of cell surface proteins on TR14 cells may also explain in part the observed discrepancies between gene and protein expression. In order to confidently address this issue, one would require a positive control population or cell line expressing each of the markers in question, which would be costly and time consuming. In order to draw conclusions from this preliminary analysis to identify markers that define TR14 cells, one should consider both cell surface and gene expression values. Certain expressed markers that correlate between the two are EpCAM, CD34, CD31, sca-1, CX3CR1, Ly6C and CD115. Concurrent expression of these markers has not been associated with single types of well characterised cells, to my knowledge. However, cells associated with some of these markers include stromal cells, tissue stem cells and monocyte/macrophages. Given our continued interest in chemokine biology, the chemokine and chemokine receptor gene expression profiling were performed, so as to define the migratory potential of TR14 cells. Although this analysis provided some valuable information of the level of expression of these genes, their expression was not validated, due to time limitations. Interestingly, skin TR14 cells express *Cxcl14*, which considering that they also express the CXCL14 binding proteins, suggests an autocrine mode of action. Nevertheless, expression levels were not as high as skin tissue cells, which are the major sources of CXCL14 (Frederick *et al.*, 2000; Kurth *et al.*, 2001; Schaerli *et al.*, 2005). In particular, previously published work from our group has shown that murine CXCL14 is highly expressed within the epidermis and the dermis, where CXCL14-producing endothelial cells associated with blood vessels (Meuter *et al.*, 2007).

Gene expression analysis was performed in an effort to define skin and colon TR14 cell subsets. Tissue cells and macrophage populations were also analysed in order to investigate their phenotypic and functional relatedness to TR14 cells. Comparison of gene expression of all populations showed that TR14 cells share more similarities with TR14 cell-depleted skin tissue cells than macrophages. The finding that TR14 cells are related to stromal cells was exciting as non-haematopoietic stromal cells play an important role in the immune system both from a structural point of view by providing

physical support of tissues, as well as continuously interacting with immune cells and releasing growth factors, adhesion molecules and cytokines (Roozendaal and Mebius, 2011; Crowley, Buckley and Clark, 2018). A brief analysis of pathways that are upregulated or downregulated in skin TR14 cells in comparison to stromal cells was also performed by our collaborator Dr You Zhou (data not shown). This analysis using the Qiagen software Ingenuity Pathway Analysis (IPA) did not reveal distinct information that can directly be applied to an experimental setting. In the future, a more in-depth inspection of IPA analysis results would help identify certain gene sets that are important in the function of these cells. In particular, examination of pathways related to migration, antigen presentation as well as phagocytic activity would be of interest. CXCL14 expression is constitutive throughout development, therefore pathways relating to that should be examined.

Table 5.3. Cell surface and gene expression of selected markers by mouse skin TR14 cells.

Marker	Cell expression/ Functionality	Cell surface expression (Flow cytometry)	Gene expression (RNA seq analysis)
CD45	Pan immune cell marker	-[a]	-[b]
F4/80	Macrophage marker	-	-
FcεR1a	Eosinophils, mast cells, basophils/IgE receptor	+/-	-
EpCAM	Epithelial cells/Cell adhesion	++	++
CD34	HSC[c] marker, stromal cells/Cell adhesion	+	++
CD31	Endothelial cells, leukocytes/Cell adhesion	+	+
c-kit	HSC, MPP, CMP marker/ SCF[c] receptor	-	-
sca-1	HSC[c] marker	++	+
CX3CR1	Macrophage, monocyte marker/CX3CL1 receptor	++	+/-
CCR3	Eosinophil, basophil marker	+	-
CXCR4	Haematopoietic cell marker/CXCL12 receptor	++	-
Ly6C	Monocyte, macrophage marker	++	++
CD11b	Leukocyte marker (mainly macrophage/monocyte)	-	+
CD11c	Leukocyte marker (mainly DC)	low	-
MHC II	Various cell expression/Antigen presentation	-	++
CD115	Macrophage, monocyte marker/CSF1 receptor	+/-	+

[a] Surface expression >50% was annotated high (++), <50% was annotated positive (+), <20% was annotated +/- and below 10% was considered negative (-).
[b] Normalised gene expression values below 100 were considered negative (-), <500 are annotated +/-, 500< and <1000 are annotated (+) and >1000 are annotated (++).
[c] Abbreviations: HSC; Haematopoietic stem cell, MPP; multipotent progenitor, CMP; common myeloid progenitor, SCF; stem cell factor, CSF1; colony stimulating factor 1.

Although a vital part of this work, up to now, murine skin TR14 cells have not displayed migratory responses towards CXCL14. Human skin $CD14^+$ DC showed very high binding of AF-CXCL14 and are therefore considered CXCL14 target cells. However, they did not demonstrate migratory responses towards CXCL14 throughout Dr Paul Collin's work, in contrast to human skin TR14 cells (Collins, 2016). Previous work from Schaerli *et al.* used an *in vitro* tissue model to show that human epidermal equivalents were capable of inducing the differentiation of $CD14^+$ monocytes into Langerhans-like cells. It was thus proposed that CXCL14 has the important role of guiding $CD14^+$ monocytes from blood to distinct epidermal niches, where they could differentiate into Langerhans cells (LCs) in response to local growth and differentiation factors (Schaerli *et al.*, 2005). During that work, skin tissue emigrant $CD14^+CD1a^-$ mononuclear cells were shown to migrate towards CXCL14. However, that work also highlighted that mild protease treatment used for rapid recovery of mononuclear cells from dermatome-excised skin tissue destroyed responsiveness to CXCL14 while leaving migration responses to the prototype monocyte chemokine CCL2 intact. Schaerli *et al.* used collagenase treatment, whereas digestion of murine skin was performed by incubation with dispase, DNase and liberase (see Chapter 2 for further details), where liberase contains a blend of collagenases. Preliminary experiments testing the effect of our current digestion protocol in mouse spleen did not reveal a striking inhibition of migration. Furthermore, functional characterisation of cells by transwell migration is problematic due to presence of tissue cells that are blocking the pores. A different approach was also tested, namely incubation of tissue with chemokines, followed by assessment of emigrant cells. These were preliminary experiments to assess whether this technique could be used in the future to selectively enrich for CXCL14 target cells. Therefore, in depth phenotypic analysis was not performed in the emigrant cells. However, these experiments revealed certain drawbacks associated with this technique. Firstly, if is not clear how strongly embedded within the tissue TR14 cells are, therefore they might require enzymatic digestion to isolate them, which is a common procedure for isolating non-migratory/resident macrophages and tissue cells. Secondly, the longer cells stay in culture, the higher the likelihood of them differentiating. Therefore, a different approach should be examined. Specifically, calcium mobilisation is an alternative method that could be applied in this work. In this case, measurement of calcium

mobilisation using confocal microscopy would be the method of choice since it requires low numbers of cells and can be done quickly with primary tissue cells. Nonetheless, it would be best to enrich TR14 cells using magnetic bead selection for markers discussed above before carrying out confocal microscopy studies. These cells could also be sorted by FACS based on a marker that distinguishes them from other tissue cells, to allow clear definition of their functionality in both mouse and human.

In conclusion, my studies of TR14 cells have certainly advanced our understanding of these cells. However, real progress has been hampered by many technical difficulties. Future work will involve more thorough characterisation of TR14 cells in human skin, since less enzymatic digestion is required. For instance, RNA seq work with human TR14 cells from human skin could be compared with our gene expression data from mouse TR14 cells. Additionally, skin resident immune and non-immune populations should also be isolated and analysed for direct and detailed phenotypic comparison to my data from TR14 cells from mice. Finally, as discussed above, establishing the functionality of these cells is of utmost importance. Ultimately, our phenotypic and functional data need to be verified by the endogenous CXCL14 receptor(s) whose identification remains a corner stone in chemokine research.

Chapter 6: General Discussion

6.1 Summary

CXCL14, one of the 48 members of the chemokine superfamily, is the least understood chemokine to date. In my PhD project, I have sought to expand our current understanding regarding the activity and target cells of CXCL14 by revealing novel functions for CXCL14, most notably its ability to interact with and influence the activity of other chemokines and their receptors. In continuation from our previous work demonstrating strong synergism between the two evolutionarily conserved chemokines, CXCL14 and CXCL12, I have sought to examine the structure-function relationship of CXCL14. I have demonstrated that CXCL14 can also synergise with other homeostatic chemokines, in particular CCL19, CCL21 and CXCL13. Previous work on CXCL14 function identified roles in a variety of processes, including killing of microorganisms (Maerki *et al.*, 2009; Dai *et al.*, 2015) and both tumour progression and suppression (Schwarze *et al.*, 2005; Ozawa *et al.*, 2006; Peterson *et al.*, 2006; Wente *et al.*, 2008; Song *et al.*, 2010; Tessema *et al.*, 2010). CXCL14 has also been related to non-immune functions including regulation of body weight and glucose metabolism (Tanegashima *et al.*, 2010; Hara and Tanegashima, 2012). CXCL14 is constitutively expressed in a number of peripheral tissues, including skin, gut, kidney, brain and placenta (Hromas *et al.*, 1999; Cao *et al.*, 2000; Frederick *et al.*, 2000; Kurth *et al.*, 2001; Meuter and Moser, 2008), and is postulated to play a vital role in regulating immune surveillance in these tissues. Partly due to lack of a specific receptor, the precise identity of CXCL14 target cells has not been well defined. Earlier, and often contradictory reports, have indicated that CXCL14 target cells include monocytes, B cells, neutrophils, immature DCs, activated blood and uterine NK cells (Cao *et al.*, 2000; Kurth *et al.*, 2001; Shellenberger *et al.*, 2004; Starnes *et al.*, 2006; Mokhtar *et al.*, 2009; Salogni *et al.*, 2009). Our group has had an invested interest in the immune surveillance of skin during homeostasis. Recent work led to the identification a novel subset of “tissue cells” within the human skin, with the aid of an Alexa Fluor 647-conjugated CXCL14 reagent to detect expression of CXCL14 receptors and transwell migration assays to assess cell migration (Collins, 2016). To facilitate functional studies of these novel CXCL14-binding cells, I have

turned to mice, whose tissues and organs are in ready supply. These studies included phenotypic, transcriptomic and functional analysis with cells freshly isolated from murine skin and other tissues/organs.

6.2 CXCL14 Synergism with Other Chemokines

Although the receptor via which CXCL14 induces migration of its target cells remains unknown, our group has previously reported that CXCL14 interacts with the chemokine receptor CXCR4 (Collins *et al.*, 2017). As far as we know this interaction does not induce activation of signalling cascades although CXCL14 considerably modulates the activity of the CXCR4 ligand CXCL12. The current model proposes that CXCL14 is a positive allosteric modulator of CXCR4, where CXCL14 shifts the balance of the CXCR4 conformational states to those recognised by its functional ligand CXCL12. Mechanisms of chemokine synergy described previously by other laboratories include 1) heterodimerisation of chemokine receptors on the cell surface, which are activated by their respective ligands simultaneously or sequentially; 2) chemokine heterocomplex formation that can activate a single receptor; and 3) synergism of chemokine receptors leading to amplification of receptor signalling events (Gouwy *et al.*, 2012; Proudfoot and Uguccioni, 2016) and **Figure 1.8**. Our group's model therefore represents a novel mechanism of chemokine synergy, as there is no published evidence of a chemokine acting as a positive allosteric modulator, i.e. enhancing the potency of another chemokine through binding to its receptor in a non-signalling manner. Interaction of CXCL14 with CXCR4 has also been reported by others, who have shown that CXCL14 can bind CXCR4 with high affinity but does not trigger downstream signalling events (Tanegashima, Suzuki, *et al.*, 2013; Otte *et al.*, 2014). Interestingly, while one group reported that CXCL14 is an inhibitor of CXCR4-mediated cells responses (Tanegashima, Suzuki, *et al.*, 2013; Tanegashima, Tsuji, *et al.*, 2013), another group failed to confirm these findings (Otte *et al.*, 2014). My current work complements these findings by demonstrating that CXCL14 can synergise with chemokines other than CXCL12. In particular, CXCL14 synergised strongly with homeostatic chemokines, including the CCR7 ligands CCL19 and CCL21 and the CXCR5 ligand CXCL13. Synergism with the inflammatory chemokines such as CCL5, CXCL10 and CXCL11, was less striking. Synergism with all chemokines tested was only observed with respect to the induction

of calcium mobilisation, while CXCL10 and CXCL11 synergised with CXCL14 in the induction of migratory responses. Since the primary function of chemokines is the control of immune cell migration, our clear chemotaxis results with primary and receptor-transfected cells strongly suggests that to date, chemokine-synergism should be regarded as the main function of CXCL14.

The mechanism of action of the synergistic interaction between CXCL14 and the lymphoid tissue-homing chemokines CCL19, CCL21 and CXCL13 is unclear. However, based on our work with the CXCL12/CXCR4 system (Collins *et al.*, 2017), I hypothesise that CXCL14 is a positive allosteric modulator of CCR7 and CXCR5. This hypothesis could be confirmed by testing the interaction of CXCL14 and these receptors by FRET analysis. In collaboration with a group at the Luxembourg Institute of Health, we are currently in the process of assessing the effect of CXCL14 on β-arrestin recruitment in various chemokine receptor-transfected cells. Data collected so far has shown that CXCL14 does not induce β-arrestin recruitment in any of the cell lines tested. The next step of this analysis would be to focus on the effect of CXCL14 combined with chemokines that have shown synergism *in vitro*, namely CXCL12, CXCL13, CCL19/CCL21, CXCL10 and CCL5. Additionally, competition of binding of these chemokines to their respective receptors would provide more insight into the synergistic mechanism of CXCL14 with these chemokines. For instance, tests could employ fluorescently labelled chemokines (similar to AF-CXCL14) to examine binding to their respective receptor, followed by assessment of competition by addition of CXCL14. One should also consider the reported interaction of ACKRs with chemokines that CXCL14 is shown to synergise with. For instance, ACKR1 binds CCL5 and CXCL11 (Vacchini, Locati and Borroni, 2016) therefore CXCL14 could potentially interact with ACKR5, in addition to CCR5 and CXCR3. Furthermore, ACKR3 is a known receptor for CXCL12 (Murphy and Heusinkveld, 2018), thus we should examine the possibility that CXCL14 is a positive allosteric modulator of this receptor as well. Finally, ACKR4 binds CCL19, CCL21 and CXCL12, and is expressed by thymic epithelial cells, bronchial cells and keratinocytes (Lu and Cyster, 2019). These are sites where CXCL14 is expressed constitutively, therefore the potential synergistic interaction of CXCL14 with the ACKR4 system should be investigated.

In this PhD thesis, I aimed to confirm the synergistic interaction of CXCL14 and CXCL12 *in vivo*, using the peritoneal cavity model. Unfortunately, this approach showed limited success, potentially due to truncation of CXCL12 by CD26, since no migration was observed by the expected leukocyte sunsets, such as T and B cells. In the future, this experiment should be repeated using an orally administered CD26 inhibitor, which has been used in the past to reverse the inhibitory effect on CXCL12 activity (Janssens *et al.*, 2017). Synergy between chemokines in the recruitment of immune cells was previously demonstrated *in vivo* using the air pouch model (Schiraldi *et al.*, 2012). In this model, sterile air is administered by subcutaneous injection into the back of the mouse, thereby mimicking the synovial cavity in the absence of inflammatory stimuli (Sin *et al.*, 1986). In addition, the intra-articular model has also been used to demonstrate the *in vivo* activity of CXCL12 and the role of CD26 in the regulation of this response (Janssens *et al.*, 2017).

The physiological relevance of the reported synergistic activity of CXCL14 with other homeostatic and inflammatory chemokines is a very interesting finding that may be relevant to both tissue homeostasis and disease. As CXCL14 expression was not detected in secondary lymphoid organs (Meuter and Moser, 2008), it seems unlikely that CXCL14 synergises with these lymphoid tissue-homing chemokines to control the homeostatic migration of T cells, B cells and mature DCs within these organs. However, CXCL14 has been implicated in inflammatory processes, including lesions in the joint, characteristic of the autoimmune disease rheumatoid arthritis (Chen *et al.*, 2010). Ectopic (or tertiary) lymphoid organs frequently develop in inflamed tissues as a result of autoimmune responses, such as rheumatoid arthritis. Lymphoid tissue associated chemokines, such as CXCL13, CCL19 and CCL21 are all expressed in these ectopic lymphoid structures (Hjelmström *et al.*, 2000; Pitzalis *et al.*, 2014). It can therefore be postulated that CXCL14 synergism with these homeostatic chemokines may boost recruitment of inflammatory cells. In doing so, the homeostatic chemokine CXCL14 would paradoxically be enhancing the recruitment of immune effector cells to sites of inflammation. In fact, increased CXCL14 levels have been observed in the joint in a murine model of collagen-induced arthritis, while transgenic mice over-expressing CXCL14 developed a more severe arthritis than wild type controls (Chen *et al.*, 2010). Recruitment of lymphocytes and

mature DC should be evaluated *in vivo* in models already discussed. If that work confirms my hypothesis, CXCL14 could represent a novel target for treatment of rheumatoid arthritis, and potentially other chronic inflammatory conditions characterised by the presence of ectopic lymphoid organs.

6.3 CXCL14 Structure-Function Relationship

Studies attempting to understand the receptor-chemokine interaction by solving GPCR structures have provided new insights to ligand binding models, binding locations, kinetics and associated ligand pharmacology (Cooke *et al.*, 2015). Combining this information will help with the development of more efficacious drugs. The interaction between chemokines and their receptors has historically been described as a two-step process involving two recognition sites as initially discussed by Crump, 1997 (Burg *et al.*, 2015; Qin *et al.*, 2015; Kufareva *et al.*, 2017). Crystal structures of chemokine receptors with chemokines or small molecules have confirmed this model but also showed an additional interaction interface (Qin *et al.*, 2015; Kufareva *et al.*, 2017). Although these studies enhanced our understanding of chemokine-receptor interaction, they lacked structural information about the distal N terminus of the receptor. Radiolytic footprinting experiments using the ACKR3:CXCL12 complex recently demonstrated an additional point of contact during the chemokine-receptor interaction in the receptor's distal N-terminus (Gustavsson *et al.*, 2017). Mutagenesis studies have also been employed to show that the receptor N terminus primarily contributes to chemokine binding, whereas interactions in the receptor binding pockets are important for both binding affinity and receptor activation (Allen, Crown and Handel, 2007; Scholten *et al.*, 2012). Further studies utilising nuclear magnetic resonance spectroscopy demonstrated that soluble peptides corresponding to receptor N termini can interact with chemokines (Ziarek *et al.*, 2017). Very recently, the crystal structure of human CCR7 with an intracellular allosteric antagonist was solved, which provided evidence of promising hotspots for targeting chemokine receptors with small molecular weight compounds (Jaeger *et al.*, 2019). Collectively, these studies illustrate the complex nature of studies investigating the structure-function relationship of chemokines and ultimately show that a combination of experimental approaches is required to fully explain the nature of this interaction. Progress on studying CXCL14 and its subdomains has been greatly

hampered by the failure to identify its cognate receptor. CXCL14 is the only chemokine that contains such a short N-terminus and it can be postulated that this feature is the primary reason why CXCL14 is a low-potency chemokine. Moreover, CXCL14 contains an additional five amino acid insertion, not seen in any other CXC chemokine. Preliminary structure-function studies of CXCL14 performed by our group revealed that amino-terminal extensions of CXCL14 did not improve its potency, highlighting that the exceptionally short N-terminus plays a vital role in the function of CXCL14 (Schaerli *et al.*, 2005).

Based on the strong synergistic activity of CXCL14 and the CXCR4 system our group reported (Collins *et al.*, 2017), this PhD thesis aimed to examine whether modification of the N-terminus of CXCL14 could render it a CXCR4 agonist. This work demonstrates that replacing the CXCL14 N-terminus with that of CXCL12 converts the chemokine into a partial CXCR4 ligand. This is a novel finding regarding CXCL14 activity and should be investigated further to fully elucidate the nature of this interaction by additional functional experiments, including β-arrestin recruitment, cAMP activation, actin polymerisation and ERK activation. Moreover, FRET analysis could reveal whether CXCL14-12 hybrids induce formation of CXCR4 clusters on the cell surface, akin to our observations of CXCL14 and CXCR4. Similar approaches to those employed for investigation of CXCR4 peptides and CXCL12 (Kofuku *et al.*, 2009; Ziarek *et al.*, 2017) could be utilised to provide a more detailed model of interaction of CXCL14-12 hybrids with CXCR4. Studies should also be done using *in vivo* models of immune cell migration as a proof of concept of the observed synergism. Considering that CXCL12 also interacts with ACKR3, interaction CXCL14 hybrids with ACKR3 should also be examined. However, larger amount of synthetic material needs to be available in order to properly investigate the reduced activities of hybrid CXCL14 proteins.

The activity of CXCL14 as a PAM of CXCR4 also extends to HIV infection. Previous work in our research group showed that CXCL14 unexpectedly enhanced HIV-1 infection, possibly by promoting conformational changes in CXCR4 aggregates that render the cells more susceptible to infection (Collins *et al.*, 2017). Assessment of the role of CXCL14-12 hybrids in HIV-1 infection would be valuable to determine whether the CXCL14 globular core, as opposed to the N-terminal region, is responsible for enhanced HIV-1 infection.

CXCL14 is also known to have anti-microbial capacities, as it shares various structural features with non-chemokine anti-microbial peptides. These include a core structure consisting of three anti-parallel β-strands, similar to β-defensin, as well as a C-terminal α-helix that is reminiscent of LL-37 (Wolf and Moser, 2012). Dai et al showed that the N-terminal region of CXCL14 is responsible for mediating the antimicrobial properties, whereas the globular core is responsible for the chemotactic activity (Dai *et al.*, 2015). It would therefore be interesting to see whether CXCL14-12 hybrids would maintain the ability to function as anti-microbial peptides.

6.4 TR14 Cells

Epithelial tissues such as the skin are constantly exposed to a large variety of environmental hazards that include UV irradiation, toxins, and most importantly, a myriad of commensal and pathogenic microbes. Maintenance and restoration of tissue homeostasis is a long-lasting and vital process. Chemokines and their receptors, along with adhesion molecules are involved in the orchestration of immune surveillance, as well as controlling responses to infections, injury and transformed cells (Schaerli *et al.*, 2004; McCully and Moser, 2011; McCully, Kouzeli and Moser, 2018). Key to local immune surveillance of virtually all tissues are tissue resident cells, including the members of the mononuclear phagocyte system (MPS), such as tissue macrophages and DCs as well as resident T cells and stromal cells, including fibroblasts, fibrocytes and structural cells of the blood and lymph vessels. Cells of the MPS have been studied in detail and shown to be the first line of defence against invading pathogens, due to their antigen presenting capacity and cytokine production that influence the activity of neighbouring cells (Banchereau and Steinman, 1998; Davies, Jenkins, *et al.*, 2013; Haniffa, Gunawan and Jardine, 2015). Stromal cells in turn also play an important role in tissue homeostasis, as they have a supporting effect through the production of extracellular matrix but they also present antigens, produce cytokines and growth factors (Haniffa, Collin, *et al.*, 2009; Roozendaal and Mebius, 2011; Crowley, Buckley and Clark, 2018). It is evident that the immune surveillance system in healthy peripheral tissues is continuously active and highly complex. Work from our group on CXCL14 has led us to the discovery of a novel type of cells in peripheral tissues characterised by AF-CXCL14 binding, that we have tentatively called TR14 cells (tissue resident CXCL14

binding cells). Up to this date, our findings regarding TR14 cells in mice and humans demonstrate diverging features of these cells and will therefore be discussed separately. Besides TR14, skin tissue also contains subsets of AF-CXCL14 binding myeloid immune cells.

6.4.1 Human TR14 Cells

Human TR14 cells were identified as a potential addition to the MPS present in tissues, in the form of a novel subset of myeloid cells present in healthy human skin (Collins, 2016). At this point, we have no information regarding the origin or function. They were identified based on the expression of CXCL14 receptors (as indicated by binding of AF-CXCL14), as well as their migratory responses towards CXCL14. TR14 cells were identified in both the dermis and epidermis layers of human skin. Although present among tissue emigrant cells, higher numbers (up to 20% of $CD45^-$ cells) were detected following further enzymatic digestion of the tissue. Therefore, they were assumed to be tissue resident cells due to their increased detection following proteolytic digestion of the tissue and absence from peripheral blood. They were further defined by their co-expression of the myeloid markers CD1a and CD14, as well as the lack of expression of the protein tyrosine phosphatase and pan-leukocyte marker, CD45. CD45 is widely used to distinguish immune cells from tissue cells, therefore it is postulated that these cells are of non-haematopoietic origin. There are numerous studies investigating the immune cell subsets in human skin where researchers have excluded the $CD45^{dim}$ and/or $CD45^-$ cells from their analyses (Haniffa, Ginhoux, *et al.*, 2009; McGovern *et al.*, 2014). Human skin TR14 cells were identified as $CD14^{dim}$, therefore it could be speculated that due to shared ability to respond to CXCL14, these cells are derived from $CD14^+$ monocytes. However, there is currently no evidence to support this. In order to determine whether human TR14 cells are a novel subset of the mononuclear phagocyte system (MPS), further phenotypic and functional characterisation is required. Studies in human skin should focus on the morphology and localisation of these cells within the tissue. I have already begun optimisation experiments for the use of AF-CXCL14 in immunofluorescence in PBMC, that in a next step could be applied to define the location of TR14 cells within the tissue. Alternatively, biotinylated CXCL14 could be used for this purpose. The study of cell surface marker expression by TR14 cells should include, in

addition to $CD1a^+$ and $CD14^+$ DCs, macrophages, epidermal LCs, and peripheral blood monocytes (Haniffa, Collin, *et al.*, 2009; Ziegler-Heitbrock *et al.*, 2010). Markers explored should include particular markers related to migration (chemokine receptors and adhesion molecules), antigen presentation (MHC class II and co-stimulatory molecules e.g. CD40 and CD80/86) and responses to pathogens (TLRs and other pattern-recognition receptors). The functional analysis *in vitro* should include calcium mobilisation in response to CXCL14, as well as examine the phagocytosis properties of TR14 cells, for instance by assessing their ability to phagocytose fluorescently labelled substrates. Furthermore, experiments measuring responses to pathogens, including the production of cytokines (IL-1β, IL-6, TGF-β and TNF-α), chemokines (CXCL8, CXCL10 CXCL14 and CCL1-CCL5) and anti-microbial molecules (defensins, cathelicidins and NOS/ROS) should be performed. Finally, efforts should be focused on determining the transcriptome of human skin TR14 cells by single cell RNA sequencing. CXCL14 target populations within the $CD45^+$ compartment of skin should also be sorted for transcriptomics analysis, along with known tissue cell types (e.g. fibroblasts, endothelial cells and keratinocytes). These data should then be compared to publicly available databases of known cell types. In particular, differentially expressed genes and associated pathways of potentially functional significance should be examined (adhesion/migration receptors, antigen presentation and co-stimulatory molecules, intracellular and cell surface PAMP/DAMP receptors, phagocytic receptors). Results from transcriptome analyses, in combination with phenotypic and functional analyses will guide the identification and characterisation CXCL14 target cells and ultimately, the physiological role of CXCL14 in human skin.

6.4.2 Mouse TR14 Cells

The very high sequence conservation of CXCL14 across different species suggests that cross-species functional conservation is likely (Wolf and Moser, 2012). My work so far has shown that mouse monocytes, in accordance with human monocytes, also respond to CXCL14. This PhD thesis demonstrates that mouse monocytes also bind AF-CXCL14, in experiments using human CXCL14 derived staining reagents. Mouse and human CXCL14 only differ by two amino acid residues, and I have provided convincing evidence this reagent can be reliably applied to identifying and studying CXCL14 target cells in

mice. CXCL14 is highly expressed within the skin and other murine tissues, including the lung, brain and gut under steady-state conditions (Meuter and Moser, 2008). So far, the majority of published work has focused on the immune cells present within those tissues. My work aimed to translate our findings of human skin TR14 cells to mice, as working with mice allows continuous access to a broader range of healthy tissues. As with their human counterparts, mouse TR14 cells were found to be abundant in the skin and were also identified in other high CXCL14 expressing tissues, including the lung and kidney. CXCL14 is particularly positively charged, therefore at present we cannot exclude the possibility that many of these TR14 cells do not express a functional CXCL14 receptor, but rather bind the AF-CXCL14 non-specifically due to interaction with negatively charged glycosaminoglycans. In order to exclude this possibility, experiments in the future should aim to cleave polysaccharides present on the surface of the cells (e.g. using heparinases) so that any non-specific interaction is inhibited. Murine CXCL14 is also highly expressed in the brain, placenta, ovary and muscle (Sleeman *et al.*, 2000; Meuter and Moser, 2008). Of these tissues, brain was assessed for presence of TR14 cells, but staining did not reveal a distinct population. Future work should examine other sites where CXCL14 is highly expressed in order to define the distribution of TR14 cells around the body, such as the placenta, ovary and muscle. Although human and mouse TR14 cells were identified based on their expression of CXCL14 receptors, assessed by binding of AF-CXCL14 and lack of CD45 expression, our work so far has shown that there are differences between mouse and human TR14 cells. Human skin TR14 cells show similarities with members of the MPS and strong migratory responses towards CXCL14. Our data regarding mouse TR14 cells up to now do not provide a clear picture about the potential origin of these cells.

Although phenotyping analysis of skin TR14 cells revealed expression of a variety of markers, their combinatorial expression has not been associated with single types of well characterised cells. Transcriptomic analysis comparing sorted TR14 cells from skin and colon to publicly available databases revealed similarities of skin TR14 cells with stromal cells, based on the distance between TR14 cells and stromal cells in a multidimensional scaling plot. Stromal cells and their functionality have been widely studied in primary and secondary lymphoid organs, whose cellular composition is largely

divided into a haematopoietic or a nonhaematopoietic compartment. Cells of the haematopoietic compartment express CD45 on the cell surface, in contrast to nonhaematopoietic cells that do not. The same distinction exists in peripheral tissues, including the skin. Epithelial tissues like the skin continuously perform a variety of protective functions, including prevention of insults by invading pathogens (Fuchs, 2007). This is achieved by a variety of mechanisms that ultimately maintain tissue homeostasis, including the continuous communication among immune, epithelial, stromal and stem cells (Blanpain and Fuchs, 2009). Stromal cell research has progressed over the last decades in terms of their function, interaction with other cells and involvement in diseases and there is even evidence of antigen presentation by stromal cells (Roozendaal and Mebius, 2011; Nowarski, Jackson and Flavell, 2017). Research on CXCL14 activity on tissue cells thus far has been mainly focused to studies in cancer using cell lines. Therefore, the finding that CXCL14 target cells within the tissue are related to stromal cells is exciting and novel. Stromal cells analysed during this analysis by multidimensional scaling included follicular dendritic cells, blood endothelial cells and lymphatic endothelial cells. Future work should also compare skin TR14 cells with skin-related stromal cells, including fibroblasts. Gene expression data of skin TR14 cells should be compared to public databases comprising of gene expression data of skin stromal cells, as well as epithelial cells. Additionally, skin stromal cells could be identified by flow cytometry to examine their specific binding of AF-CXCL14. There is also evidence that haematopoietic precursor cells lack expression of CD45 that they acquire later on during development into haematopoietic cell subsets and erythroid cells (Yamane, 2018). CXCL14, along with CXCL12, have been shown to be constitutively expressed in developing organs at adjacent but non-overlapping sites (García-Andreś and Torres, 2010; Gordon *et al.*, 2011; Ojeda, Munjaal and Lwigale, 2013; Nassari *et al.*, 2017). It would therefore be important to examine the presence and function of TR14 cells during embryonic development. Immune surveillance and specifically aging, are governed by homeostatic chemokines with tissue-specific expression profiles that retain immune surveillance cells. It would be interesting to see whether the observed TR14 cell niches are retained in the tissue in later life.

Up to this point, work conducted using the CXCL14 knockout (CXCL14-KO) mice has failed to enhance our understanding of its physiological functions. Our group and others have previously reported a severe breeding defect in these mice (Meuter *et al.*, 2007; Nara *et al.*, 2007; Tanegashima *et al.*, 2010). In viable CXCL14-KO mice no immune phenotype was detected, with macrophage and DC populations in healthy epithelial tissue. Additionally, recruitment of cells to inflamed peritoneum and skin wound healing following mechanical injury all appeared to be unimpaired (Meuter *et al.*, 2007). CXCL14-KO mice reaching adulthood have 7-11% lower body weight than their wild-type or $CXCL14^{+/-}$ littermates (Meuter and Moser, 2008; Tanegashima *et al.*, 2010). Additionally, adult CXCL14-KO mice on a high fat diet revealed reduced numbers of adipose tissue macrophages (Nara *et al.*, 2007). Collectively, these reports suggest that CXCL14 and its target cells may be playing a central role in tissue health and metabolism in mice. The presence and function of TR14 cells in various tissues in CXCL14-KO mice need to be further examined, with similar approaches as discussed above. The absence of TR14 cells would provide the first conclusive evidence for CXCL14 playing an essential role in the maintenance of tissue homeostasis, thus having profound implications for mucosal immunity in both mice and humans. Moreover, given that phenotypic analysis of mouse skin TR14 cells showed that they express CX3CR1, TR14 cell tissue localisation in mice lacking CX3CR1 could be examined (Jung et al., 2000). Additionally, $CX3CR1^{GFP/GFP}$ mice can be used to analyse the origin and tissue distribution of skin TR14 cells, which will express GFP under the control of the CX3CR1 promoter. In addition, the functionality of TR14 cells under inflammatory conditions should be investigated Additional sites should also be examined for presence of TR14 cells and other inflammation models involving other tissues should be tested. For instance, recruitment of inflammatory monocytes to the peritoneum has been tested in the past, following administration of zymosan or thioglycolate (Davies, Rosas, *et al.*, 2013). The overall aim of these studies should be to define the functionality of TR14 cells in tissues under healthy and inflammatory conditions.

6.5 Conclusion

Although knowledge within the field of chemokines is continuously expanding, CXCL14 remains as one of the least understood members of this family of functionally and

structurally related proteins. Since its discovery 17 years ago, our knowledge of CXCL14 functions has considerably increased. There are still many questions remaining regarding the role of CXCL14 in tissues and the functions of its target cells. However, we now have more insight regarding its target cells in tissues, as well as its ability to influence the activity of other chemokines. Based on my own findings and the previous work in our group, I postulate that low levels of CXCL14 in one end of a chemokine gradient in tissues mediate synergistic activity, whereas higher concentrations at the other end of a chemokine gradient convey their function on distinct target cells by directly interacting with specific CXCL14 receptors. For instance, in ectopic lymphoid structures, low levels of CXCL14 could induce recruitment of inflammatory cells by synergising with lymphoid tissue associated chemokines. Alternatively, in healthy tissues CXCL14 could synergise with other local chemokines such as CXCL12 in retaining immune cells by preventing their tissue exit. In contrast, high levels of CXCL14 could function as chemoattractant for CXCL14 receptor expressing target cells, including monocytes and TR14 cells. Discovery of the cognate receptor of CXCL14 will, without a doubt, facilitate further advancement in our understanding of CXCL14, the least understood chemokine known today.

Chapter 7: References

Abel, A. M. *et al.* (2018) 'Natural killer cells: Development, maturation, and clinical utilization', *Frontiers in Immunology*. Frontiers Media S.A. doi: 10.3389/fimmu.2018.01869.

Abtin, A. *et al.* (2014) 'Perivascular macrophages mediate neutrophil recruitment during bacterial skin infection', *Nature Immunology*, 15(1), pp. 45–53. doi: 10.1038/ni.2769.

Agace, W. W. *et al.* (2000) 'Constitutive expression of stromal derived factor-1 by mucosal epithelia and its role in HIV transmission and propagation', *Current Biology*, 10(6), pp. 325–328. doi: 10.1016/S0960-9822(00)00380-8.

Ahuja, V., Miller, S. E. and Howell, D. N. (1995) 'Identification of two subpopulations of rat monocytes expressing disparate molecular forms and quantities of CD43', *Cellular Immunology*, 163(1), pp. 59–69. doi: 10.1006/cimm.1995.1099.

Allen, S. J., Crown, S. E. and Handel, T. M. (2007) 'Chemokine:Receptor Structure, Interactions, and Antagonism', *Annual Review of Immunology*. Annual Reviews, 25(1), pp. 787–820. doi: 10.1146/annurev.immunol.24.021605.090529.

Angel, C. E. *et al.* (2006) ' Cutting Edge: CD1a + Antigen-Presenting Cells in Human Dermis Respond Rapidly to CCR7 Ligands ', *The Journal of Immunology*. The American Association of Immunologists, 176(10), pp. 5730–5734. doi: 10.4049/jimmunol.176.10.5730.

Ara, T. *et al.* (2003) 'Long-term hematopoietic stem cells require stromal cell-derived factor-1 for colonizing bone marrow during ontogeny', *Immunity*, 19(2), pp. 257–267. doi: 10.1016/S1074-7613(03)00201-2.

Arenzana-Seisdedos, F. and Parmentier, M. (2006) 'Genetics of resistance to HIV infection: Role of co-receptors and co-receptor ligands', *Seminars in Immunology*. (Human {Genetics} of {Infectious} {Diseases}: {Immunological} {Implications}), 18(6), pp. 387–403. doi: 10.1016/j.smim.2006.07.007.

Auffray, C., Sieweke, M. H. and Geissmann, F. (2009) 'Blood Monocytes: Development, Heterogeneity, and Relationship with Dendritic Cells', *Annual Review of Immunology*. Annual Reviews, 27(1), pp. 669–692. doi: 10.1146/annurev.immunol.021908.132557.

Augsten, M. *et al.* (2009) 'CXCL14 is an autocrine growth factor for fibroblasts and acts as a multi-modal stimulator of prostate tumor growth', *Proceedings of the National Academy of Sciences of the United States of America*, 106(9), pp. 3414–3419. doi: 10.1073/pnas.0813144106.

Autengruber, A. *et al.* (2012) 'Impact of enzymatic tissue disintegration on the level of surface molecule expression and immune cell function', *European Journal of Microbiology and Immunology*. Akademiai Kiado Zrt., 2(2), pp. 112–120. doi: 10.1556/eujmi.2.2012.2.3.

Bachelerie, F. *et al.* (2013) 'International Union of Pharmacology. LXXXIX. Update on the Extended Family of Chemokine Receptors and Introducing a New Nomenclature for Atypical Chemokine Receptors', *Pharmacological Reviews*, 66(1), pp. 1–79. doi:

10.1124/pr.113.007724.

Baggiolini, M. (1995) 'Interleukin 8 and related chemotactic cytokines (chemokines)', *Atemwegs- und Lungenkrankheiten*, 21(7), pp. 336–338. doi: 10.1378/chest.105.3_supplement.95s.

Baggiolini, M., Dewald, B. and Moser, B. (1997) 'Human Chemokines: An Update', *Annual Review of Immunology*, 15(1), pp. 675–705. doi: 10.1146/annurev.immunol.15.1.675.

Baggiolini, M., Walz, A. and Kunkel, S. L. (1989) 'Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils.', *Journal of Clinical Investigation*, 84(4), pp. 1045–1049.

Bain, C. C. *et al.* (2013) 'Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6C hi monocyte precursors', *Mucosal Immunology*, 6(3), pp. 498–510. doi: 10.1038/mi.2012.89.

Bain, C. C. and Schridde, A. (2018) 'Origin, differentiation, and function of intestinal macrophages', *Frontiers in Immunology*. Frontiers Media S.A., 9(NOV). doi: 10.3389/fimmu.2018.02733.

Banchereau, J. and Steinman, R. M. (1998) 'Dendritic cells and the control of immunity.', *Nature*, 392(6673), pp. 245–252.

Bardi, G., Niggli, V. and Loetscher, P. (2003) 'Rho kinase is required for CCR7-mediated polarization and chemotaxis of T lymphocytes', *FEBS Letters*, 542(1–3), pp. 79–83. doi: 10.1016/S0014-5793(03)00351-X.

Bassler, K. *et al.* (2019) 'The Myeloid Cell Compartment—Cell by Cell', *Annual Review of Immunology*. Annual Reviews, 37(1), pp. 269–293. doi: 10.1146/annurev-immunol-042718-041728.

Basu, S. and Broxmeyer, H. E. (2009) ' CCR5 Ligands Modulate CXCL12-Induced Chemotaxis, Adhesion, and Akt Phosphorylation of Human Cord Blood CD34 + Cells ', *The Journal of Immunology*, 183(11), pp. 7478–7488. doi: 10.4049/jimmunol.0900542.

Bazan, J. F. *et al.* (1997) 'A new class of membrane-bound chemokine with a CX3C motif', *Nature*, 385(6617), pp. 640–642. doi: 10.1038/385640a0.

Belge, K.-U. *et al.* (2002) ' The Proinflammatory CD14 + CD16 + DR ++ Monocytes Are a Major Source of TNF ', *The Journal of Immunology*, 168(7), pp. 3536–3542. doi: 10.4049/jimmunol.168.7.3536.

Bennett, C. L. *et al.* (2005) 'Inducible ablation of mouse Langerhans cells diminishes but fails to abrogate contact hypersensitivity', *Journal of Cell Biology*, 169(4), pp. 569–576. doi: 10.1083/jcb.200501071.

Bieber, T. (2007) 'The pro- and anti-inflammatory properties of human antigen-presenting cells expressing the high affinity receptor for IgE (FcεRI)', *Immunobiology*, 212(6), pp. 499–503. doi: 10.1016/j.imbio.2007.03.001.

Bigley, V. *et al.* (2011) 'The human syndrome of dendritic cell, monocyte, B and NK lymphoid deficiency', *Journal of Experimental Medicine*, 208(2), pp. 227–234. doi:

10.1084/jem.20101459.

Bilgin, Y. M. and De Greef, G. E. (2016) 'Plerixafor for stem cell mobilization: The current status', *Current Opinion in Hematology*, 23(1), pp. 67–71. doi: 10.1097/MOH.0000000000000200.

Blanpain, C. and Fuchs, E. (2009) 'Epidermal homeostasis: A balancing act of stem cells in the skin', *Nature Reviews Molecular Cell Biology*, pp. 207–217. doi: 10.1038/nrm2636.

Bleul, C. C. *et al.* (1996) 'A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1)', *Journal of Experimental Medicine*, 184(3), pp. 1101–1109. doi: 10.1084/jem.184.3.1101.

Blumenstein, M. *et al.* (1996) 'Cytokine production precedes the expansion of CD14+CD16+ monocytes in human sepsis a case report of a patient with self-induced septicemia', *Sensor Review*, 16(1), pp. 73–75. doi: 10.1097/00024382-199707000-00012.

Bonneville, M., O'Brien, R. L. and Born, W. K. (2010) 'γ δ T cell effector functions: A blend of innate programming and acquired plasticity', *Nature reviews. Immunology*, 10(7), pp. 467–478. doi: 10.1038/nri2781.

Boring, L. *et al.* (1997) 'Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice', *Journal of Clinical Investigation*, 100(10), pp. 2552–2561. doi: 10.1172/JCI119798.

Borkowski, T. A. *et al.* (1996) 'A role for endogenous transforming growth factor β1 in Langerhans cell biology: The skin of transforming growth factor β1 null mice is devoid of epidermal Langerhans cells', *Journal of Experimental Medicine*, 184(6), pp. 2417–2422. doi: 10.1084/jem.184.6.2417.

Borroni, E. M. *et al.* (2018) 'Chemokines sound the alarmin: The role of atypical chemokine in inflammation and cancer', *Seminars in Immunology*. Academic Press, pp. 63–71. doi: 10.1016/j.smim.2018.10.005.

Borst, J. *et al.* (1991) 'Tissue distribution and repertoire selection of human ???? T cells: Comparison with the murine system', in *Current Topics in Microbiology and Immunology*, pp. 41–46. doi: 10.1007/978-3-642-76492-9_7.

Brandes, M., Willimann, K. and Moser, B. (2005) 'Immunology: Professional antigen-presentation function by human γδ cells', *Science*, 309(5732), pp. 264–268. doi: 10.1126/science.1110267.

Breitfeld, D. *et al.* (2000) 'Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production', *Journal of Experimental Medicine*, 192(11), pp. 1545–1551. doi: 10.1084/jem.192.11.1545.

Brinkmann, V. *et al.* (2004) 'Neutrophil Extracellular Traps Kill Bacteria', *Science*, 303(5663), pp. 1532–1535. doi: 10.1126/science.1092385.

Bukowski, R. M. *et al.* (1994) 'Phase I trial of subcutaneous recombinant macrophage colony-stimulating factor: Clinical and immunomodulatory effects', *Journal of Clinical Oncology*, 12(1), pp. 97–106. doi: 10.1200/JCO.1994.12.1.97.

Burd, P. R. *et al.* (1987) 'Cloning and characterization of a novel T cell activation gene', *Journal of Immunology*, 139(9), pp. 3126–3131. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3499466 (Accessed: 27 October 2019).

Burg, J. S. *et al.* (2015) 'Structural basis for chemokine recognition and activation of a viral G protein-coupled receptor', *Science*. American Association for the Advancement of Science, 347(6226), pp. 1113–1117. doi: 10.1126/science.aaa5026.

Byrd, A. L., Belkaid, Y. and Segre, J. A. (2018) 'The human skin microbiome', *Nature Reviews Microbiology*, pp. 143–155. doi: 10.1038/nrmicro.2017.157.

Cao, Q. *et al.* (2013) 'Genetic deletion of Cxcl14 in mice alters uterine NK cells', *Biochemical and Biophysical Research Communications*, 435(4), pp. 664–670. doi: 10.1016/j.bbrc.2013.04.106.

Cao, X. *et al.* (2000) 'Molecular Cloning and Characterization of a Novel CXC Chemokine Macrophage Inflammatory Protein-2γ Chemoattractant for Human Neutrophils and Dendritic Cells', *The Journal of Immunology*, 165(5), pp. 2588–2595. doi: 10.4049/jimmunol.165.5.2588.

Carlin, L. M. *et al.* (2013) 'Nr4a1-dependent Ly6Clow monocytes monitor endothelial cells and orchestrate their disposal', *Cell*, 153(2), pp. 362–375. doi: 10.1016/j.cell.2013.03.010.

Cecchinato, V. *et al.* (2016) 'Chemokine interaction with synergy-inducing molecules: fine tuning modulation of cell trafficking', *Journal of Leukocyte Biology*, 99(6), pp. 851–855. doi: 10.1189/jlb.1mr1015-457r.

Cecchini, M. G. *et al.* (1994) 'Role of colony stimulating factor-1 in the establishment and regulation of tissue macrophages during postnatal development of the mouse', *Development*, 120(6), pp. 1357–1372.

Cella, M. *et al.* (1999) 'Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon', *Nature Medicine*, 5(8), pp. 919–923. doi: 10.1038/11360.

Cereijo, R. *et al.* (2018) 'CXCL14, a Brown Adipokine that Mediates Brown-Fat-to-Macrophage Communication in Thermogenic Adaptation', *Cell Metabolism*, 28(5), pp. 750-763.e6. doi: 10.1016/j.cmet.2018.07.015.

Chen, L. *et al.* (2010) 'Overexpression of CXC Chemokine Ligand 14 Exacerbates Collagen-Induced Arthritis', *The Journal of Immunology*, 184(8), pp. 4455–4459. doi: 10.4049/jimmunol.0900525.

Chorro, L. *et al.* (2009) 'Langerhans cell (LC) proliferation mediates neonatal development, homeostasis, and inflammation-associated expansion of the epidermal LC network', *Journal of Experimental Medicine*, 206(13), pp. 3089–3100. doi: 10.1084/jem.20091586.

Christopoulos, A. (2014) 'Advances in G protein-coupled receptor allostery: From function to structure', *Molecular Pharmacology*, 86(5), pp. 463–478. doi: 10.1124/mol.114.094342.

Chu, C. C. *et al.* (2012) 'Resident CD141 (BDCA3) + dendritic cells in human skin produce

IL-10 and induce regulatory T cells that suppress skin inflammation', *Journal of Experimental Medicine*, 209(5), pp. 935–945. doi: 10.1084/jem.20112583.

Clark-Lewis, Moser, B., *et al.* (1991) 'Chemical synthesis, purification, and characterization of two inflammatory proteins, neutrophil activating peptide 1 (interleukin-8) and neutrophil activating peptide', *Biochemistry*, 30(12), pp. 3128–3135. doi: 10.1021/bi00226a021.

Clark-Lewis, Schumacher, C., *et al.* (1991) 'Structure-activity relationships of interleukin-8 determined using chemically synthesized analogs: Critical role of NH2-terminal residues and evidence for uncoupling of neutrophil chemotaxis, exocytosis, and receptor binding activities', *Journal of Biological Chemistry*, 266(34), pp. 23128–23134.

Clark-Lewis, I. *et al.* (1994) 'Structural requirements for interleukin-8 function identified by design of analogs and CXC chemokine hybrids', *Journal of Biological Chemistry*, 269(23), pp. 16075–16081.

Clark-Lewis, I. *et al.* (1995) 'Structure-activity relationships of chemokines', *Journal of Leukocyte Biology*, 57(5), pp. 703–711. doi: 10.1002/jlb.57.5.703.

Clark, R. A. *et al.* (2006) 'The vast majority of CLA+ T cells are resident in normal skin.', *Journal of immunology (Baltimore, Md. : 1950)*, 176(7), pp. 4431–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16547281.

Cole, K. E. *et al.* (1998) 'Interferon-inducible T cell alpha chemoattractant (I-TAC): A novel non- ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3', *Journal of Experimental Medicine*, 187(12), pp. 2009–2021. doi: 10.1084/jem.187.12.2009.

Collins, P. J. (2016) *Characterisation of cxcl14 function and target cells in blood and tissues*. Available at: https://search.proquest.com/docview/1857851315?accountid=12543%0Ahttp://opurl.bib.umontreal.ca:9003/sfx_local?url_ver=Z39.88-2004&rft_val_fmt=info:ofi/fmt:kev:mtx:dissertation&genre=dissertations+%26+theses&sid=ProQ:ProQuest+Dissertations+%26+Theses+Globa.

Collins, P. J. *et al.* (2017) 'Epithelial chemokine CXCL14 synergizes with CXCL12 via allosteric modulation of CXCR4', *FASEB Journal*, 31(7), pp. 3084–3097. doi: 10.1096/fj.201700013R.

Contento, R. L. *et al.* (2008) 'CXCR4-CCR5: A couple modulating T cell functions', *Proceedings of the National Academy of Sciences of the United States of America*, 105(29), pp. 10101–10106. doi: 10.1073/pnas.0804286105.

Cooke, R. M. *et al.* (2015) 'Structures of G protein-coupled receptors reveal new opportunities for drug discovery', *Drug Discovery Today*, pp. 1355–1364. doi: 10.1016/j.drudis.2015.08.003.

Cooper, D. L. *et al.* (2012) 'FcγRIIIa expression on monocytes in rheumatoid arthritis: Role in immune-complex stimulated TNF production and non-response to methotrexate therapy', *PLoS ONE*, 7(1). doi: 10.1371/journal.pone.0028918.

Cros, J. *et al.* (2010) 'Human CD14dim Monocytes Patrol and Sense Nucleic Acids and

Viruses via TLR7 and TLR8 Receptors', *Immunity*, 33(3), pp. 375–386. doi: 10.1016/j.immuni.2010.08.012.

Crowley, T., Buckley, C. D. and Clark, A. R. (2018) 'Stroma: the forgotten cells of innate immune memory', *Clinical and Experimental Immunology*. Blackwell Publishing Ltd, pp. 24–36. doi: 10.1111/cei.13149.

Crump, M. P. (1997) 'Solution structure and basis for functional activity of stromal cell-derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1', *The EMBO Journal*, 16(23), pp. 6996–7007. doi: 10.1093/emboj/16.23.6996.

Cyster, J. C. (1999) 'Chemokines and cell migration in secondary lymphoid organs', *Science*, 286(5447), pp. 2098–2102. doi: 10.1126/science.286.5447.2098.

Dai, C. *et al.* (2015) ' CXCL14 Displays Antimicrobial Activity against Respiratory Tract Bacteria and Contributes to Clearance of Streptococcus pneumoniae Pulmonary Infection ', *The Journal of Immunology*. American Association of Immunologists, 194(12), pp. 5980–5989. doi: 10.4049/jimmunol.1402634.

Dai, X. M. *et al.* (2002) 'Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects', *Blood*, 99(1), pp. 111–120. doi: 10.1182/blood.V99.1.111.

Van Damme, J. *et al.* (1988) 'A novel, NH2-terminal sequence-characterized human monokine possessing neutrophil chemotactic, skin-reactive, and granulocytosis-promoting activity', *Journal of Experimental Medicine*, 167(4), pp. 1364–1376. doi: 10.1084/jem.167.4.1364.

Davies, L. C., Rosas, M., *et al.* (2013) 'Distinct bone marrow-derived and tissue-resident macrophage lineages proliferate at key stages during inflammation', *Nature Communications*, 4. doi: 10.1038/ncomms2877.

Davies, L. C., Jenkins, S. J., *et al.* (2013) 'Tissue-resident macrophages', *Nature Immunology*, pp. 986–995. doi: 10.1038/ni.2705.

Debard, N., Sierro, F. and Kraehenbuhl, J. P. (1999) 'Development of Peyer's patches, follicle-associated epithelium and M cell: Lessons from immunodeficient and knockout mice', *Seminars in Immunology*, 11(3), pp. 183–191. doi: 10.1006/smim.1999.0174.

Doebel, T., Voisin, B. and Nagao, K. (2017) 'Langerhans Cells – The Macrophage in Dendritic Cell Clothing', *Trends in Immunology*. Elsevier Ltd, pp. 817–828. doi: 10.1016/j.it.2017.06.008.

Dyer, D. P. *et al.* (2019) 'Chemokine Receptor Redundancy and Specificity Are Context Dependent', *Immunity*. Cell Press, 50(2), pp. 378-389.e5. doi: 10.1016/j.immuni.2019.01.009.

El-Asmar, L. *et al.* (2004) 'Evidence for Negative Binding Cooperativity within CCR5-CCR2b Heterodimers', *Molecular Pharmacology*, 67(2), pp. 460–469. doi: 10.1124/mol.104.003624.

Epelman, S. *et al.* (2014) 'Embryonic and adult-derived resident cardiac macrophages are maintained through distinct mechanisms at steady state and during inflammation',

Immunity, 40(1), pp. 91–104. doi: 10.1016/j.immuni.2013.11.019.

Ernst, O. P. *et al.* (2007) 'Monomeric G protein-coupled receptor rhodopsin in solution activates its G protein transducin at the diffusion limit', *Proceedings of the National Academy of Sciences of the United States of America*, 104(26), pp. 10859–10864. doi: 10.1073/pnas.0701967104.

Eyerich, S. *et al.* (2018) 'Cutaneous Barriers and Skin Immunity: Differentiating A Connected Network', *Trends in Immunology*, pp. 315–327. doi: 10.1016/j.it.2018.02.004.

Facchetti, F. *et al.* (1988) 'Plasmacytoid T cells. Immunohistochemical evidence for their monocyte/macrophage origin', *American Journal of Pathology*, 133(1), pp. 15–21.

Ferrer, I. R. *et al.* (2019) 'A single wave of monocytes is sufficient to replenish the long-term Langerhans cell network after immune injury', *bioRxiv*, 4(38), p. 617514. doi: 10.1101/617514.

Flier, J. S., Underhill, L. H. and Weller, P. F. (1991) 'The immunobiology of eosinophils', *New England Journal of Medicine*, pp. 1110–1118. doi: 10.1056/NEJM199104183241607.

Förster, R., Braun, A. and Worbs, T. (2012) 'Lymph node homing of T cells and dendritic cells via afferent lymphatics', *Trends in Immunology*, pp. 271–280. doi: 10.1016/j.it.2012.02.007.

Franco, C. B. *et al.* (2010) 'Distinguishing Mast Cell and Granulocyte Differentiation at the Single-Cell Level', *Cell Stem Cell*, 6(4), pp. 361–368. doi: 10.1016/j.stem.2010.02.013.

Frederick, M. J. *et al.* (2000) 'In vivo expression of the novel CXC chemokine BRAK in normal and cancerous human tissue', *American Journal of Pathology*. American Society for Investigative Pathology Inc., 156(6), pp. 1937–1950. doi: 10.1016/S0002-9440(10)65067-5.

Frick, I.-M. *et al.* (2011) ' Constitutive and Inflammation-Dependent Antimicrobial Peptides Produced by Epithelium Are Differentially Processed and Inactivated by the Commensal Finegoldia magna and the Pathogen Streptococcus pyogenes ', *The Journal of Immunology*, 187(8), pp. 4300–4309. doi: 10.4049/jimmunol.1004179.

Fuchs, E. (2007) 'Scratching the surface of skin development', *Nature*, pp. 834–842. doi: 10.1038/nature05659.

Fulkerson, P. C. *et al.* (2004) 'Negative regulation of eosinophil recruitment to the lung by the chemokine monokine induced by IFN-γ (Mig, CXCL9)', *Proceedings of the National Academy of Sciences of the United States of America*, 101(7), pp. 1987–1992. doi: 10.1073/pnas.0308544100.

Van Furth, R. *et al.* (1985) 'The characterization, origin, and kinetics of skin macrophages during inflammation', *Journal of Investigative Dermatology*, 85(5), pp. 398–402. doi: 10.1111/1523-1747.ep12277056.

van Furth, R. and Cohn, Z. A. (1968) *The origin and kinetics of mononuclear phagocytes.*, *The Journal of experimental medicine*. doi: 10.1084/jem.128.3.415.

Van Furth, R. and Sluiter, W. (1986) 'Distribution of blood monocytes between a marginating and a circulatng pool', *Journal of Experimental Medicine*, 163(2), pp. 474–479. doi: 10.1084/jem.163.2.474.

García-Andreś, C. and Torres, M. (2010) 'Comparative expression pattern analysis of the highly conserved chemokines {SDF}1 and {CXCL}14 during amniote embryonic development', *Developmental Dynamics*, 239(10), pp. 2769–2777. doi: 10.1002/dvdy.22413.

Gebhardt, T. *et al.* (2009) 'Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus', *Nature Immunology*, 10(5), pp. 524–530. doi: 10.1038/ni.1718.

Geissmann, F. *et al.* (1998) 'Transforming growth factor β1, in the presence of granulocyte/macrophage colony-stimulating factor and interleukin 4, induces differentiation of human peripheral blood monocytes into dendritic langerhans cells', *Journal of Experimental Medicine*, 187(6), pp. 961–966. doi: 10.1084/jem.187.6.961.

Geissmann, F. *et al.* (2008) 'Blood monocytes: Distinct subsets, how they relate to dendritic cells, and their possible roles in the regulation of T-cell responses', *Immunology and Cell Biology*, pp. 398–408. doi: 10.1038/icb.2008.19.

Geissmann, F. *et al.* (2010) 'Development of monocytes, macrophages, and dendritic cells', *Science*. American Association for the Advancement of Science, pp. 656–661. doi: 10.1126/science.1178331.

Geissmann, F., Jung, S. and Littman, D. R. (2003) 'Blood monocytes consist of two principal subsets with distinct migratory properties', *Immunity*. Cell Press, 19(1), pp. 71–82. doi: 10.1016/S1074-7613(03)00174-2.

Ginhoux, F. *et al.* (2006) 'Langerhans cells arise from monocytes in vivo', *Nature Immunology*, 7(3), pp. 265–273. doi: 10.1038/ni1307.

Ginhoux, F. and Guilliams, M. (2016) 'Tissue-Resident Macrophage Ontogeny and Homeostasis', *Immunity*. Cell Press, pp. 439–449. doi: 10.1016/j.immuni.2016.02.024.

Ginhoux, F. and Jung, S. (2014) 'Monocytes and macrophages: Developmental pathways and tissue homeostasis', *Nature Reviews Immunology*. Nature Publishing Group, pp. 392–404. doi: 10.1038/nri3671.

Gordon, C. T. *et al.* (2011) 'CXCL14 expression during chick embryonic development', *The International Journal of Developmental Biology*, 55(3), pp. 335–340. doi: 10.1387/ijdb.103258cg.

Gordon, S. and Taylor, P. R. (2005) 'Monocyte and macrophage heterogeneity', *Nature Reviews Immunology*, pp. 953–964. doi: 10.1038/nri1733.

Goujon, M. *et al.* (2010) 'A new bioinformatics analysis tools framework at EMBL-EBI', *Nucleic Acids Research*, 38(SUPPL. 2). doi: 10.1093/nar/gkq313.

Gouwy, M. *et al.* (2004) 'Synergy between proinflammatory ligands of G protein-coupled receptors in neutrophil activation and migration', *Journal of Leukocyte Biology*, 76(1), pp. 185–194. doi: 10.1189/jlb.1003479.

Gouwy, M. *et al.* (2008) 'Synergy between coproduced CC and CXC chemokines in monocyte chemotaxis through receptor-mediated events', *Molecular Pharmacology*, 74(2), pp. 485–495. doi: 10.1124/mol.108.045146.

Gouwy, M. *et al.* (2011) 'CXCR4 and CCR5 ligands cooperate in monocyte and lymphocyte migration and in inhibition of dual-tropic (R5/X4) HIV-1 infection', *European Journal of Immunology*, 41(4), pp. 963–973. doi: 10.1002/eji.201041178.

Gouwy, M. *et al.* (2012) 'Possible mechanisms involved in chemokine synergy fine tuning the inflammatory response', *Immunology Letters*, 145(1–2), pp. 10–14. doi: 10.1016/j.imlet.2012.04.005.

Govaerts, C. *et al.* (2003) 'Activation of CCR5 by chemokines involves an aromatic cluster between transmembrane helices 2 and 3', *Journal of Biological Chemistry*, 278(3), pp. 1892–1903. doi: 10.1074/jbc.M205685200.

Graham, G. J. *et al.* (2012) 'The biochemistry and biology of the atypical chemokine receptors', *Immunology Letters*, pp. 30–38. doi: 10.1016/j.imlet.2012.04.004.

Gregorio, J. *et al.* (2010) 'Plasmacytoid dendritic cells sense skin injury and promote wound healing through type i interferons', *Journal of Experimental Medicine*, 207(13), pp. 2921–2930. doi: 10.1084/jem.20101102.

Gregory, H. *et al.* (1988) 'Structure determination of a human lymphocyte derived neutrophil activating peptide (LYNAP)', *Biochemical and Biophysical Research Communications*, 151(2), pp. 883–890. doi: 10.1016/S0006-291X(88)80364-4.

Griffith, J. W., Sokol, C. L. and Luster, A. D. (2014) 'Chemokines and Chemokine Receptors: Positioning Cells for Host Defense and Immunity', *Annual Review of Immunology*, 32(1), pp. 659–702. doi: 10.1146/annurev-immunol-032713-120145.

Groom, J. R. and Luster, A. D. (2011) 'CXCR3 in T cell function', *Experimental Cell Research*. (Special {Issue}: {Chemokines}), 317(5), pp. 620–631. doi: 10.1016/j.yexcr.2010.12.017.

Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) 'A new generation of Ca^{2+} indicators with greatly improved fluorescence properties', *Journal of Biological Chemistry*, 260(6), pp. 3440–3450.

Guilliams, M. *et al.* (2014) 'Dendritic cells, monocytes and macrophages: A unified nomenclature based on ontogeny', *Nature Reviews Immunology*. Nature Publishing Group, pp. 571–578. doi: 10.1038/nri3712.

Gunn, M. D. *et al.* (1999) 'Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization', *Journal of Experimental Medicine*, 189(3), pp. 451–460. doi: 10.1084/jem.189.3.451.

Gustavsson, M. *et al.* (2017) 'Structural basis of ligand interaction with atypical chemokine receptor 3', *Nature Communications*, 8. doi: 10.1038/ncomms14135.

Hamel, D. J. *et al.* (2009) 'Chapter 4 Interactions of Chemokines with Glycosaminoglycans', in *Methods in Enzymology*. Academic Press (Chemokines, {Part} {B}), pp. 71–102. doi: 10.1016/S0076-6879(09)05404-4.

Handel, T. M. *et al.* (2005) 'Regulation of Protein Function By Glycosaminoglycans—As Exemplified By Chemokines', *Annual Review of Biochemistry*, 74(1), pp. 385–410. doi: 10.1146/annurev.biochem.72.121801.161747.

Haniffa, Ginhoux, F., *et al.* (2009) 'Differential rates of replacement of human dermal dendritic cells and macrophages during hematopoietic stem cell transplantation', *Journal of Experimental Medicine*, 206(2), pp. 371–385. doi: 10.1084/jem.20081633.

Haniffa, Collin, M. P., *et al.* (2009) 'Mesenchymal stem cells: The fibroblasts' new clothes?', *Haematologica*, pp. 258–263. doi: 10.3324/haematol.13699.

Haniffa, M. *et al.* (2012) 'Human Tissues Contain CD141 hi Cross-Presenting Dendritic Cells with Functional Homology to Mouse CD103 + Nonlymphoid Dendritic Cells', *Immunity*, 37(1), pp. 60–73. doi: 10.1016/j.immuni.2012.04.012.

Haniffa, M., Bigley, V. and Collin, M. (2015) 'Human mononuclear phagocyte system reunited', *Seminars in Cell and Developmental Biology*. Academic Press, pp. 59–69. doi: 10.1016/j.semcdb.2015.05.004.

Haniffa, M., Collin, M. and Ginhoux, F. (2013) 'Ontogeny and Functional Specialization of Dendritic Cells in Human and Mouse', in *Advances in Immunology*, pp. 1–49. doi: 10.1016/B978-0-12-417028-5.00001-6.

Haniffa, M., Gunawan, M. and Jardine, L. (2015) 'Human skin dendritic cells in health and disease', *Journal of Dermatological Science*, pp. 85–92. doi: 10.1016/j.jdermsci.2014.08.012.

Hanna, R. N. *et al.* (2015) 'Patrolling monocytes control tumor metastasis to the lung', *Science*. American Association for the Advancement of Science, 350(6263), pp. 985–990. doi: 10.1126/science.aac9407.

Hara, T. and Tanegashima, K. (2012) 'Pleiotropic functions of the CXC-type chemokine CXCL14 in mammals', *Journal of Biochemistry*, pp. 469–476. doi: 10.1093/jb/mvs030.

Hartwig, T. *et al.* (2015) 'Dermal IL-17-producing γδ T cells establish long-lived memory in the skin', *European Journal of Immunology*, 45(11), pp. 3022–3033. doi: 10.1002/eji.201545883.

Hauser, M. A. *et al.* (2016) 'Inflammation-Induced CCR7 Oligomers Form Scaffolds to Integrate Distinct Signaling Pathways for Efficient Cell Migration', *Immunity*, 44(1), pp. 59–72. doi: 10.1016/j.immuni.2015.12.010.

Hayday, A. C. (2000) 'γδ Cells: A Right Time and a Right Place for a Conserved Third Way of Protection', *Annual Review of Immunology*. Annual Reviews, 18(1), pp. 975–1026. doi: 10.1146/annurev.immunol.18.1.975.

Hazeldine, J. *et al.* (2014) 'Impaired neutrophil extracellular trap formation: A novel defect in the innate immune system of aged individuals', *Aging Cell*, 13(4), pp. 690–698. doi: 10.1111/acel.12222.

Hemmi, H. *et al.* (2001) 'Skin antigens in the steady state are trafficked to regional lymph nodes by transforming growth factor-β1-dependent cells', *International Immunology*, 13(5), pp. 695–704. doi: 10.1093/intimm/13.5.695.

Hettinger, J. *et al.* (2013) 'Origin of monocytes and macrophages in a committed progenitor', *Nature Immunology*, 14(8), pp. 821–830. doi: 10.1038/ni.2638.

Hevezi, P. *et al.* (2009) 'Genome-wide analysis of gene expression in primate taste buds reveals links to diverse processes', *PLoS ONE*, 4(7). doi: 10.1371/journal.pone.0006395.

Hjelmström, P. *et al.* (2000) 'Lymphoid tissue homing chemokines are expressed in chronic inflammation', *American Journal of Pathology*, 156(4), pp. 1133–1138. doi: 10.1016/S0002-9440(10)64981-4.

Holtmeier, W. and Kabelitz, D. (2005) 'γδ T Cells Link Innate and Adaptive Immune Responses', in *Mechanisms of Epithelial Defense*, pp. 151–183. doi: 10.1159/000086659.

Hromas, R. *et al.* (1999) 'Cloning of BRAK, a novel divergent CXC chemokine preferentially expressed in normal versus malignant cells', *Biochemical and Biophysical Research Communications*. Academic Press Inc., 255(3), pp. 703–706. doi: 10.1006/bbrc.1999.0257.

Hughes, C. E. and Nibbs, R. J. B. (2018) 'A guide to chemokines and their receptors', *FEBS Journal*, 285(16), pp. 2944–2971. doi: 10.1111/febs.14466.

Hume, D. A. (2006) 'The mononuclear phagocyte system', *Current Opinion in Immunology*, pp. 49–53. doi: 10.1016/j.coi.2005.11.008.

Iijima, N. *et al.* (2007) 'Vaginal epithelial dendritic cells renew from bone marrow precursors', *Proceedings of the National Academy of Sciences of the United States of America*, 104(48), pp. 19061–19066. doi: 10.1073/pnas.0707179104.

Imai, T. *et al.* (1997) 'Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion', *Cell*. Cell Press, 91(4), pp. 521–530. doi: 10.1016/S0092-8674(00)80438-9.

Ingersoll, M. A. *et al.* (2014) 'Comparison of gene expression profiles between human and mouse monocyte subsets Comparison of gene expression profiles between human and mouse monocyte subsets'. doi: 10.1182/blood-2009-07-235028.

Ishida, Y., Gao, J.-L. and Murphy, P. M. (2008) 'Chemokine Receptor CX3CR1 Mediates Skin Wound Healing by Promoting Macrophage and Fibroblast Accumulation and Function', *The Journal of Immunology*, 180(1), pp. 569–579. doi: 10.4049/jimmunol.180.1.569.

Isik, N., Hereld, D. and Jin, T. (2008) 'Fluorescence resonance energy transfer imaging reveals that chemokine-binding modulates heterodimers of CXCR4 and CCR5 receptors', *PLoS ONE*, 3(10), p. e3424. doi: 10.1371/journal.pone.0003424.

Islam, S. A. *et al.* (2011) 'Mouse CCL8, a CCR8 agonist, promotes atopic dermatitis by recruiting IL-5+TH2 cells', *Nature Immunology*, 12(2), pp. 167–177. doi: 10.1038/ni.1984.

Issafras, H. *et al.* (2002) 'Constitutive agonist-independent CCR5 oligomerization and antibody-mediated clustering occurring at physiological levels of receptors', *Journal of Biological Chemistry*, 277(38), pp. 34666–34673. doi: 10.1074/jbc.M202386200.

Iwata, M. *et al.* (2004) 'Retinoic acid imprints gut-homing specificity on T cells', *Immunity*, 21(4), pp. 527–538. doi: 10.1016/j.immuni.2004.08.011.

Jaeger, K. *et al.* (2019) 'Structural Basis for Allosteric Ligand Recognition in the Human CC Chemokine Receptor 7', *Cell*. Elsevier BV, 178(5), pp. 1222-1230.e10. doi: 10.1016/j.cell.2019.07.028.

Jakubzick, C. *et al.* (2013) 'Minimal differentiation of classical monocytes as they survey steady-state tissues and transport antigen to lymph nodes', *Immunity*, 39(3), pp. 599–610. doi: 10.1016/j.immuni.2013.08.007.

Janssens, R. *et al.* (2017) 'Truncation of CXCL12 by CD26 reduces its CXC chemokine receptor 4- and atypical chemokine receptor 3-dependent activity on endothelial cells and lymphocytes', *Biochemical Pharmacology*, 132, pp. 92–101. doi: 10.1016/j.bcp.2017.03.009.

Joffre, O. P. *et al.* (2012) 'Cross-presentation by dendritic cells', *Nature Reviews Immunology*, pp. 557–569. doi: 10.1038/nri3254.

Jung, S. *et al.* (2000) 'Analysis of Fractalkine Receptor CX3CR1 Function by Targeted Deletion and Green Fluorescent Protein Reporter Gene Insertion', *Molecular and Cellular Biology*. American Society for Microbiology, 20(11), pp. 4106–4114. doi: 10.1128/mcb.20.11.4106-4114.2000.

Jung, S. *et al.* (2002) 'In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens', *Immunity*, 17(2), pp. 211–220. doi: 10.1016/S1074-7613(02)00365-5.

Kabashima, K. *et al.* (2019) 'The immunological anatomy of the skin', *Nature Reviews Immunology*. Nature Publishing Group, pp. 19–30. doi: 10.1038/s41577-018-0084-5.

Kaliński, P. *et al.* (1999) 'T-cell priming by type-1 and type-2 polarized dendritic cells: The concept of a third signal', *Immunology Today*, pp. 561–567. doi: 10.1016/S0167-5699(99)01547-9.

Kang, H. *et al.* (2010) 'Hair follicles from alopecia areata patients exhibit alterations in immune privilege-associated gene expression in advance of hair loss', *Journal of Investigative Dermatology*, pp. 2677–2680. doi: 10.1038/jid.2010.180.

Kanitakis, J. *et al.* (2011) 'Self-renewal capacity of human epidermal Langerhans cells: Observations made on a composite tissue allograft', *Experimental Dermatology*, pp. 145–146. doi: 10.1111/j.1600-0625.2010.01146.x.

Kanitakis, J., Petruzzo, P. and Dubernard, J. M. (2004) 'Turnover of epidermal langerhans' cells [7]', *New England Journal of Medicine*, pp. 2661–2662. doi: 10.1056/NEJM200412163512523.

Karin, N., Wildbaum, G. and Thelen, M. (2016) ' Biased signaling pathways via CXCR3 control the development and function of CD4 + T cell subsets ', *Journal of Leukocyte Biology*, 99(6), pp. 857–862. doi: 10.1189/jlb.2mr0915-441r.

Kashem, S. W., Haniffa, M. and Kaplan, D. H. (2017) 'Antigen-Presenting Cells in the Skin', *Annual Review of Immunology*. Annual Reviews, 35(1), pp. 469–499. doi: 10.1146/annurev-immunol-051116-052215.

Kehrl, J. H. (1998) 'Heterotrimeric G protein signaling: Roles in immune function and fine- tuning by RGS proteins', *Immunity*, 8(1), pp. 1–10. doi: 10.1016/S1074-7613(00)80453-7.

Kenakin, T. and Christopoulos, A. (2013) 'Signalling bias in new drug discovery: Detection, quantification and therapeutic impact', *Nature Reviews Drug Discovery*, 12(3), pp. 205–216. doi: 10.1038/nrd3954.

Kim, W.-K. *et al.* (2010) 'Monocyte heterogeneity underlying phenotypic changes in monocytes according to SIV disease stage', *Journal of Leukocyte Biology*, 87(4), pp. 557–567. doi: 10.1189/jlb.0209082.

Kissenpfennig, A. *et al.* (2005) 'Dynamics and function of langerhans cells in vivo: Dermal dendritic cells colonize lymph node areasdistinct from slower migrating langerhans cells', *Immunity*, 22(5), pp. 643–654. doi: 10.1016/j.immuni.2005.04.004.

Kleist, A. B. *et al.* (2016) 'New paradigms in chemokine receptor signal transduction: Moving beyond the two-site model', *Biochemical Pharmacology*. ({GPCR} {Function} in {Immunity}), 114, pp. 53–68. doi: 10.1016/j.bcp.2016.04.007.

Kleiveland, C. and Kleiveland, C. (2015) 'Peripheral blood mononuclear cells', in *The Impact of Food Bioactives on Health: In Vitro and Ex Vivo Models*. Springer International Publishing, pp. 161–167. doi: 10.1007/978-3-319-16104-4_15.

Koenen, R. R. *et al.* (2009) 'Disrupting functional interactions between platelet chemokines inhibits atherosclerosis in hyperlipidemic mice', *Nature Medicine*, 15(1), pp. 97–103. doi: 10.1038/nm.1898.

Kofuku, Y. *et al.* (2009) 'Structural basis of the interaction between chemokine stromal cell-derived factor-1/CXCL12 and its G-protein-coupled receptor CXCR4', *Journal of Biological Chemistry*, 284(50), pp. 35240–35250. doi: 10.1074/jbc.M109.024851.

Kohout, T. A. *et al.* (2004) 'Differential desensitization, receptor phosphorylation, β-arrestin recruitment, and ERK1/2 activation by the two endogenous ligands for the CC chemokine receptor 7', *Journal of Biological Chemistry*, 279(22), pp. 23214–23222. doi: 10.1074/jbc.M402125200.

Krug, A. *et al.* (2002) 'Cutting Edge: IFN-Producing Cells Respond to CXCR3 Ligands in the Presence of CXCL12 and Secrete Inflammatory Chemokines upon Activation', *The Journal of Immunology*, 169(11), pp. 6079–6083. doi: 10.4049/jimmunol.169.11.6079.

Kuang, H., Chen, Q., Fan, X., *et al.* (2009) *CXCL14 inhibits trophoblast outgrowth via a paracrine/autocrine manner during early pregnancy in mice*, *Journal of Cellular Physiology*. doi: 10.1002/jcp.21877.

Kuang, H., Chen, Q., Zhang, Y., *et al.* (2009) 'The cytokine gene CXCL14 restricts human trophoblast cell invasion by suppressing gelatinase activity', *Endocrinology*, 150(12), pp. 5596–5605. doi: 10.1210/en.2009-0570.

Kubo, A. *et al.* (2009) 'External antigen uptake by Langerhans cells with reorganization of epidermal tight junction barriers', *Journal of Experimental Medicine*, 206(13), pp. 2937–2946. doi: 10.1084/jem.20091527.

Kuehnle, I. and Goodell, M. A. (2002) 'The therapeutic potential of stem cells from

adults', *British Medical Journal*, 325(7360), pp. 372–376. doi: 10.1136/bmj.325.7360.372.

Kufareva, I. *et al.* (2017) 'What Do Structures Tell Us About Chemokine Receptor Function and Antagonism?', *Annual Review of Biophysics*, 46(1), pp. 175–198. doi: 10.1146/annurev-biophys-051013-022942.

Kumamoto, T. *et al.* (2003) 'Hair follicles serve as local reservoirs of skin mast cell precursors', *Blood*, 102(5), pp. 1654–1660. doi: 10.1182/blood-2003-02-0449.

Kurosaki, T., Shinohara, H. and Baba, Y. (2010) 'B Cell Signaling and Fate Decision', *Annual Review of Immunology*. Annual Reviews, 28(1), pp. 21–55. doi: 10.1146/annurev.immunol.021908.132541.

Kurth, I. *et al.* (2001) 'Monocyte selectivity and tissue localization suggests a role for breast and kidney-expressed chemokine (BRAK) in macrophage development', *Journal of Experimental Medicine*, 194(6), pp. 855–861. doi: 10.1084/jem.194.6.855.

Kuscher, K. *et al.* (2009) 'Synergy-inducing chemokines enhance CCR2 ligand activities on monocytes', *European Journal of Immunology*, 39(4), pp. 1118–1128. doi: 10.1002/eji.200838906.

Kuszak, A. J. *et al.* (2009) 'Purification and functional reconstitution of monomeric μ-opioid receptors. Allosteric modulation of agonists binding by Gi2', *Journal of Biological Chemistry*, 284(39), pp. 26732–26741. doi: 10.1074/jbc.M109.026922.

Larsen, C. P. *et al.* (1990) 'Migration and maturation of langerhans cells in skin transplants and explants', *Journal of Experimental Medicine*, 172(5), pp. 1483–1493. doi: 10.1084/jem.172.5.1483.

Lavoie, P. M. and Levy, O. (2017) 'Mononuclear Phagocyte System', in *Fetal and Neonatal Physiology*. Elsevier, pp. 1208-1216.e3. doi: 10.1016/b978-0-323-35214-7.00125-6.

Lawrence, S. M., Corriden, R. and Nizet, V. (2018) 'The Ontogeny of a Neutrophil: Mechanisms of Granulopoiesis and Homeostasis', *Microbiology and Molecular Biology Reviews*. American Society for Microbiology, 82(1). doi: 10.1128/mmbr.00057-17.

Lebien, T. W. and Tedder, T. F. (2008) 'B lymphocytes: How they develop and function', *Blood*, 112(5), pp. 1570–1580. doi: 10.1182/blood-2008-02-078071.

Legler, D. F. *et al.* (1998) 'B cell-attracting chemokine 1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CXCR5', *Journal of Experimental Medicine*, 187(4), pp. 655–660. doi: 10.1084/jem.187.4.655.

Legler, D. F. *et al.* (2017) 'Modulation of Chemokine Receptor Function by Cholesterol: New Prospects for Pharmacological Intervention', *Molecular pharmacology*, 91(4), pp. 331–338. doi: 10.1124/mol.116.107151.

Lindberg, J. *et al.* (2006) 'Effect of infliximab on mRNA expression profiles in synovial tissue of rheumatoid arthritis patients', *Arthritis Research and Therapy*, 8. doi: 10.1186/ar2090.

Liu, F. T., Goodarzi, H. and Chen, H. Y. (2011) 'IgE, mast cells, and eosinophils in atopic

dermatitis', *Clinical Reviews in Allergy and Immunology*, 41(3), pp. 298–310. doi: 10.1007/s12016-011-8252-4.

Liu, K. and Nussenzweig, M. C. (2010) 'Origin and development of dendritic cells', *Immunological Reviews*, pp. 45–54. doi: 10.1111/j.0105-2896.2009.00879.x.

Loetscher, M. *et al.* (1994) *Cloning of a human seven-transmembrane domain receptor, LESTR, that is highly expressed in leukocytes*, *Journal of Biological Chemistry*.

Loetscher, M. *et al.* (1996) 'Chemokine receptor specific for IP10 and Mig: Structure, function, and expression in activate T-Lymphocytes', *Journal of Experimental Medicine*, 184(3), pp. 963–969. doi: 10.1084/jem.184.3.963.

Loetscher, P. *et al.* (1998) 'CCR5 is characteristic of Th1 lymphocytes [6]', *Nature*. Nature Publishing Group, 391(6665), pp. 344–345. doi: 10.1038/34814.

Loetscher, Pius *et al.* (1998) 'N-terminal peptides of stromal cell-derived factor-1 with CXC chemokine receptor 4 agonist and antagonist activities', *Journal of Biological Chemistry*, 273(35), pp. 22279–22283. doi: 10.1074/jbc.273.35.22279.

Loos, T., Mortier, A. and Proost, P. (2009) 'Chapter 1 Isolation, Identification, and Production of Posttranslationally Modified Chemokines', in *Methods in Enzymology*, pp. 3–29. doi: 10.1016/S0076-6879(09)05401-9.

López-Cotarelo, P. *et al.* (2017) 'Beyond Chemoattraction: Multifunctionality of Chemokine Receptors in Leukocytes', *Trends in Immunology*, 38(12), pp. 927–941. doi: 10.1016/j.it.2017.08.004.

Lu, E. and Cyster, J. G. (2019) 'G-protein coupled receptors and ligands that organize humoral immune responses', *Immunological Reviews*, pp. 158–172. doi: 10.1111/imr.12743.

Luo, Q. *et al.* (2016) 'Role of Stromal-Derived Factor-1 in Mesenchymal Stem Cell Paracrinemediated Tissue Repair', *Current Stem Cell Research & Therapy*. Bentham Science Publishers Ltd., 11(7), pp. 585–592. doi: 10.2174/1574888x11666160614102629.

Lusso, P. (2006) 'New EMBO Member's Review - HIV and the chemokine system: 10 years later', *EMBO Journal*, 25(3), pp. 447–456. doi: 10.1038/sj.emboj.7600947.

Luster, A. D., Unkeless, J. C. and Ravetch, J. V. (1985) 'γ-Interferon transcriptionally regulates an early-response gene containing homology to platelet proteins', *Nature*, 315(6021), pp. 672–676. doi: 10.1038/315672a0.

Luttrell, L. M. and Lefkowitz, R. J. (2002) 'The role of β-arrestins in the termination and transduction of G-protein-coupled receptor signals', *Journal of Cell Science*, 115(3), pp. 455–465. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11861753.

MacDonald, K. P. A. *et al.* (2002) 'Characterization of human blood dendritic cell subsets', *Blood*. doi: 10.1182/blood-2001-11-0097.

Maerki, C. *et al.* (2009) 'Potent and Broad-Spectrum Antimicrobial Activity of CXCL14 Suggests an Immediate Role in Skin Infections', *The Journal of Immunology*, 182(1), pp. 507–514. doi: 10.4049/jimmunol.182.1.507.

Malissen, B., Tamoutounour, S. and Henri, S. (2014) 'The origins and functions of dendritic cells and macrophages in the skin', *Nature Reviews Immunology*. Nature Publishing Group, pp. 417–428. doi: 10.1038/nri3683.

Malosse, C. and Henri, S. (2016) 'Isolation of mouse dendritic cell subsets and macrophages from the skin', *Methods in Molecular Biology*, 1423, pp. 129–137. doi: 10.1007/978-1-4939-3606-9_9.

Mantovani, A., Sica, A. and Locati, M. (2005) 'Macrophage polarization comes of age', *Immunity*, pp. 344–346. doi: 10.1016/j.immuni.2005.10.001.

Manzo, A. *et al.* (2005) 'Systematic microanatomical analysis of CXCL13 and CCL21 in situ production and progressive lymphoid organization in rheumatoid synovitis', *European Journal of Immunology*, 35(5), pp. 1347–1359. doi: 10.1002/eji.200425830.

Marsal, J. and Agace, W. W. (2012) 'Targeting T-cell migration in inflammatory bowel disease', *Journal of Internal Medicine*, pp. 411–429. doi: 10.1111/j.1365-2796.2012.02588.x.

Martín-Fontecha, A. *et al.* (2003) 'Regulation of dendritic cell migration to the draining lymph node: Impact on T lymphocyte traffic and priming', *Journal of Experimental Medicine*, 198(4), pp. 615–621. doi: 10.1084/jem.20030448.

Martínez Muñoz, L. *et al.* (2009) 'Dynamic Regulation of CXCR1 and CXCR2 Homo- and Heterodimers', *The Journal of Immunology*, 183(11), pp. 7337–7346. doi: 10.4049/jimmunol.0901802.

Masopust, D. and Schenkel, J. M. (2013) 'The integration of T cell migration, differentiation and function', *Nature Reviews Immunology*, 13(5), pp. 309–320. doi: 10.1038/nri3442.

Mass, E. *et al.* (2016) 'Specification of tissue-resident macrophages during organogenesis', *Science*. American Association for the Advancement of Science, 353(6304). doi: 10.1126/science.aaf4238.

Mazzucchelli, L. *et al.* (1999) 'BCA-1 is highly expressed in Helicobacter pylori-induced mucosa- associated lymphoid tissue and gastric lymphoma', *Journal of Clinical Investigation*, 104(10), pp. R49--R54. doi: 10.1172/JCI7830.

McCully, M. L. *et al.* (2012) 'Epidermis instructs skin homing receptor expression in human T cells', *Blood*, 120(23), pp. 4591–4598. doi: 10.1182/blood-2012-05-433037.

McCully, M. L. *et al.* (2015) 'Skin Metabolites Define a New Paradigm in the Localization of Skin Tropic Memory T Cells', *The Journal of Immunology*, 195(1), pp. 96–104. doi: 10.4049/jimmunol.1402961.

McCully, M. L. *et al.* (2018) 'CCR8 Expression Defines Tissue-Resident Memory T Cells in Human Skin', *The Journal of Immunology*, p. ji1701377. doi: 10.4049/jimmunol.1701377.

McCully, M. L., Kouzeli, A. and Moser, B. (2018) 'Peripheral Tissue Chemokines: Homeostatic Control of Immune Surveillance T Cells', *Trends in Immunology*, 39(9), pp. 734–747. doi: 10.1016/j.it.2018.06.003.

McCully, M. L. and Moser, B. (2011) 'The human cutaneous chemokine system', *Frontiers in Immunology*, 2(AUG). doi: 10.3389/fimmu.2011.00033.

McGovern, N. *et al.* (2014) 'Human dermal CD14+ cells are a transient population of monocyte-derived macrophages', *Immunity*, 41(3), pp. 465–477. doi: 10.1016/j.immuni.2014.08.006.

McWilliam, H. *et al.* (2013) 'Analysis Tool Web Services from the EMBL-EBI.', *Nucleic acids research*, 41(Web Server issue). doi: 10.1093/nar/gkt376.

Mellado, M., Rodríguez-Frade, J. M., Mañes, S., *et al.* (2001) ' C HEMOKINE S IGNALING AND F UNCTIONAL R ESPONSES : The Role of Receptor Dimerization and TK Pathway Activation ', *Annual Review of Immunology*, 19(1), pp. 397–421. doi: 10.1146/annurev.immunol.19.1.397.

Mellado, M., Rodríguez-Frade, J. M., Vila-Coro, A. J., *et al.* (2001) 'Chemokine receptor homo- or heterodimerization activates distinct signaling pathways', *EMBO Journal*, 20(10), pp. 2497–2507. doi: 10.1093/emboj/20.10.2497.

Meller, S. *et al.* (2007) 'Chemokine responses distinguish chemical-induced allergic from irritant skin inflammation: Memory T cells make the difference', *Journal of Allergy and Clinical Immunology*, 119(6), pp. 1470–1480. doi: 10.1016/j.jaci.2006.12.654.

Merad, M. *et al.* (2002) 'Langerhans cells renew in the skin throughout life under steady-state conditions', *Nature Immunology*, 3(12), pp. 1135–1141. doi: 10.1038/ni852.

Merad, M. *et al.* (2013) 'The Dendritic Cell Lineage: Ontogeny and Function of Dendritic Cells and Their Subsets in the Steady State and the Inflamed Setting', *Annual Review of Immunology*. Annual Reviews, 31(1), pp. 563–604. doi: 10.1146/annurev-immunol-020711-074950.

Merad, M., Ginhoux, F. and Collin, M. (2008) 'Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells', *Nature Reviews Immunology*, pp. 935–947. doi: 10.1038/nri2455.

Mercier, F. E., Ragu, C. and Scadden, D. T. (2012) 'The bone marrow at the crossroads of blood and immunity', *Nature Reviews Immunology*, 12(1), pp. 49–60. doi: 10.1038/nri3132.

Metzemaekers, M. *et al.* (2016) 'Regulation of chemokine activity - A focus on the role of dipeptidyl peptidase IV/CD26', *Frontiers in Immunology*, 7(NOV). doi: 10.3389/fimmu.2016.00483.

Meuter, S. *et al.* (2007) 'Murine CXCL14 Is Dispensable for Dendritic Cell Function and Localization within Peripheral Tissues', *Molecular and Cellular Biology*, 27(3), pp. 983–992. doi: 10.1128/mcb.01648-06.

Meuter, S. and Moser, B. (2008) 'Constitutive expression of CXCL14 in healthy human and murine epithelial tissues', *Cytokine*, 44(2), pp. 248–255. doi: 10.1016/j.cyto.2008.08.009.

Meyer, M. *et al.* (2001) 'Cross reactivity of three T cell attracting murine chemokines stimulating the CXC chemokine receptor CXCR3 and their induction in cultured cells and during allograft rejection', *European Journal of Immunology*, 31(8), pp. 2521–2527. doi:

10.1002/1521-4141(200108)31:8<2521::AID-IMMU2521>3.0.CO;2-Q.

Miller, M. D. *et al.* (1990) 'Sequence and chromosomal location of the I-309 gene. Relationship to genes encoding a family of inflammatory cytokines.', *Journal of immunology (Baltimore, Md. : 1950)*, 145(8), pp. 2737–44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2212659 (Accessed: 27 October 2019).

Mizoguchi, A. *et al.* (2002) 'Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation', *Immunity*, 16(2), pp. 219–230. doi: 10.1016/S1074-7613(02)00274-1.

Moelants, E. A. V *et al.* (2013) 'In vivo regulation of chemokine activity by post-translational modification', *Immunology and Cell Biology*, pp. 402–407. doi: 10.1038/icb.2013.16.

Mokhtar, N. M. *et al.* (2009) 'Progestin regulates chemokine (C-X-C motif) ligand 14 transcript level in human endometrium', *Molecular Human Reproduction*, 16(3), pp. 170–177. doi: 10.1093/molehr/gap100.

Moniuszko, M. *et al.* (2009) 'Enhanced frequencies of CD14++CD16+, but not CD14+CD16+, peripheral blood monocytes in severe asthmatic patients', *Clinical Immunology*, 130(3), pp. 338–346. doi: 10.1016/j.clim.2008.09.011.

Monneau, Y., Arenzana-Seisdedos, F. and Lortat-Jacob, H. (2016) 'The sweet spot: how GAGs help chemokines guide migrating cells', *Journal of Leukocyte Biology*, 99(6), pp. 935–953. doi: 10.1189/jlb.3mr0915-440r.

Monteclaro, F. S. and Charo, I. F. (1996) 'The Amino-terminal Extracellular Domain of the MCP-1 Receptor, but Not the RANTES/MIP-1a Receptor, Confers Chemokine Selectivity EVIDENCE FOR A TWO-STEP MECHANISM FOR MCP-1 RECEPTOR ACTIVATION', *Journal of Biological Chemistry*. United States, 271(32), pp. 19084–19092. doi: 10.1074/jbc.271.32.19084.

Monteclaro, F. S. and Charo, I. F. (1997) 'The amino-terminal domain of CCR2 is both necessary and sufficient for high affinity binding of monocyte chemoattractant protein 1: Receptor activation by a pseudo-tethered ligand', *Journal of Biological Chemistry*, 272(37), pp. 23186–23190. doi: 10.1074/jbc.272.37.23186.

Moretta, L. and Moretta, A. (2004) 'Killer immunoglobulin-like receptors', *Current Opinion in Immunology*, pp. 626–633. doi: 10.1016/j.coi.2004.07.010.

Mortier, A., Van Damme, J. and Proost, P. (2008) 'Regulation of chemokine activity by posttranslational modification', *Pharmacology and Therapeutics*, 120(2), pp. 197–217. doi: 10.1016/j.pharmthera.2008.08.006.

Moser, B. *et al.* (2004) 'Chemokines: Multiple levels of leukocyte migration control', *Trends in Immunology*, pp. 75–84. doi: 10.1016/j.it.2003.12.005.

Mosser, D. M. and Edwards, J. P. (2008) 'Exploring the full spectrum of macrophage activation', *Nature Reviews Immunology*, pp. 958–969. doi: 10.1038/nri2448.

Mueller, S. N., Zaid, A. and Carbone, F. R. (2014) 'Tissue-resident T cells: Dynamic players in skin immunity', *Frontiers in Immunology*. doi: 10.3389/fimmu.2014.00332.

Murphy, P. M. *et al.* (2000) 'International union of pharmacology. XXII. Nomenclature for chemokine receptors', *Pharmacological Reviews*, 52(1), pp. 145–176.

Murphy, P. M. and Heusinkveld, L. (2018) 'Multisystem multitasking by CXCL12 and its receptors CXCR4 and ACKR3', *Cytokine*, 109, pp. 2–10. doi: 10.1016/j.cyto.2017.12.022.

Nagasawa, T. *et al.* (1996) 'Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1', *Nature*, 382(6592), pp. 635–638. doi: 10.1038/382635a0.

Nagasawa, T. (2014) 'CXC chemokine ligand 12 (CXCL12) and its receptor CXCR4', *Journal of Molecular Medicine*, 92(5), pp. 433–439. doi: 10.1007/s00109-014-1123-8.

Nakayamada, S. *et al.* (2012) 'Helper T cell diversity and plasticity', *Current Opinion in Immunology*, 24(3), pp. 297–302. doi: 10.1016/j.coi.2012.01.014.

Nara, N. *et al.* (2007) 'Disruption of CXC motif chemokine ligand-14 in mice ameliorates obesity-induced insulin resistance', *Journal of Biological Chemistry*, 282(42), pp. 30794–30803. doi: 10.1074/jbc.M700412200.

Nassari, S. *et al.* (2017) 'The chemokines CXCL12 and CXCL14 differentially regulate connective tissue markers during limb development', *Scientific Reports*. Nature Publishing Group, 7(1). doi: 10.1038/s41598-017-17490-z.

Nathan, C. (2006) 'Neutrophils and immunity: Challenges and opportunities', *Nature Reviews Immunology*, pp. 173–182. doi: 10.1038/nri1785.

Neptune, E. R. and Bourne, H. R. (1997) 'Receptors induce chemotaxis by releasing the βγ subunit of G(i), not by activating G(q) or G(s)', *Proceedings of the National Academy of Sciences of the United States of America*, 94(26), pp. 14489–14494. doi: 10.1073/pnas.94.26.14489.

Nesmelova, I. V. *et al.* (2005) 'Platelet factor 4 and interleukin-8 CXC chemokine heterodimer formation modulates function at the quaternary structural level', *Journal of Biological Chemistry*, 280(6), pp. 4948–4958. doi: 10.1074/jbc.M405364200.

Nestle, F. O. *et al.* (2009) 'Skin immune sentinels in health and disease', *Nature Reviews Immunology*, pp. 679–691. doi: 10.1038/nri2622.

Nibbs, R. J. B. and Graham, G. J. (2013) 'Immune regulation by atypical chemokine receptors', *Nature Reviews Immunology*, 13(11), pp. 815–829. doi: 10.1038/nri3544.

Nomiyama, H. *et al.* (2008) 'Extensive expansion and diversification of the chemokine gene family in zebrafish: Identification of a novel chemokine subfamily CX', *BMC Genomics*, 9(1), p. 222. doi: 10.1186/1471-2164-9-222.

Nowarski, R., Jackson, R. and Flavell, R. A. (2017) 'The Stromal Intervention: Regulation of Immunity and Inflammation at the Epithelial-Mesenchymal Barrier', *Cell*. Cell Press, pp. 362–375. doi: 10.1016/j.cell.2016.11.040.

Nygaard, R. *et al.* (2009) 'Ligand binding and micro-switches in 7TM receptor structures', *Trends in Pharmacological Sciences*, 30(5), pp. 249–259. doi: 10.1016/j.tips.2009.02.006.

O'Hayre, M. *et al.* (2008) 'Chemokines and cancer: Migration, intracellular signalling and

intercellular communication in the microenvironment', *Biochemical Journal*, 409(3), pp. 635–649. doi: 10.1042/BJ20071493.

Obaru, K. *et al.* (1986) 'A cDNA clone used to study mRNA inducible in human tonsillar lymphocytes by a tumor promoter', *Journal of Biochemistry*, 99(3), pp. 885–894. doi: 10.1093/oxfordjournals.jbchem.a135549.

Oberlin, E. *et al.* (1996) 'The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1', *Nature*, 382(6594), pp. 833–835. doi: 10.1038/382833a0.

Oestreich, K. J. and Weinmann, A. S. (2012) 'Master regulators or lineage-specifying? Changing views on CD4+ T cell transcription factors', *Nature Reviews Immunology*, pp. 799–804. doi: 10.1038/nri3321.

Ogilvie, P. *et al.* (2001) 'Eotaxin is a natural antagonist for CCR2 and an agonist for CCR5', *Blood*, 97(7), pp. 1920–1924. doi: 10.1182/blood.V97.7.1920.

Ohl, L. *et al.* (2004) 'CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions', *Immunity*, 21(2), pp. 279–288. doi: 10.1016/j.immuni.2004.06.014.

Ojeda, A. F., Munjaal, R. P. and Lwigale, P. Y. (2013) 'Expression of CXCL12 and CXCL14 during eye development in chick and mouse', *Gene Expression Patterns*, 13(8), pp. 303–310. doi: 10.1016/j.gep.2013.05.006.

Ojeda, A. F., Munjaal, R. P. and Lwigale, P. Y. (2017) 'Knockdown of CXCL14 disrupts neurovascular patterning during ocular development', *Developmental Biology*. Academic Press, 423(1), pp. 77–91. doi: 10.1016/j.ydbio.2017.01.008.

Otero, C. *et al.* (2008) 'Distinct motifs in the chemokine receptor CCR7 regulate signal transduction, receptor trafficking and chemotaxis', *Journal of Cell Science*, 121(16), pp. 2759–2767. doi: 10.1242/jcs.029074.

Otero, C., Groettrup, M. and Legler, D. F. (2006) 'Opposite Fate of Endocytosed CCR7 and Its Ligands: Recycling versus Degradation', *The Journal of Immunology*, 177(4), pp. 2314–2323. doi: 10.4049/jimmunol.177.4.2314.

Otte, M. *et al.* (2014) 'CXCL14 is no direct modulator of CXCR4', *FEBS Letters*, 588(24), pp. 4769–4775. doi: 10.1016/j.febslet.2014.11.009.

Ouyang, W. *et al.* (2000) 'Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment', *Immunity*, 12(1), pp. 27–37. doi: 10.1016/S1074-7613(00)80156-9.

Ozawa, S. *et al.* (2006) 'BRAK/CXCL14 expression suppresses tumor growth in vivo in human oral carcinoma cells', *Biochemical and Biophysical Research Communications*, 348(2), pp. 406–412. doi: 10.1016/j.bbrc.2006.07.070.

Pablos, J. L. *et al.* (1999) 'Stromal-cell derived factor is expressed by dendritic cells and endothelium in human skin', *American Journal of Pathology*, 155(5), pp. 1577–1586. doi: 10.1016/S0002-9440(10)65474-0.

Palframan, R. T. *et al.* (2001) 'Inflammatory chemokine transport and presentation in

HEV: A remote control mechanism for monocyte recruitment to lymph nodes in inflamed tissues', *Journal of Experimental Medicine*, 194(9), pp. 1361–1373. doi: 10.1084/jem.194.9.1361.

Pang, L. *et al.* (2013) 'Pseudogout-Associated Inflammatory Calcium Pyrophosphate Dihydrate Microcrystals Induce Formation of Neutrophil Extracellular Traps', *The Journal of Immunology*, 190(12), pp. 6488–6500. doi: 10.4049/jimmunol.1203215.

Paoletti, S. *et al.* (2005) 'A rich chemokine environment strongly enhances leukocyte migration and activities', *Blood*, 105(9), pp. 3405–3412. doi: 10.1182/blood-2004-04-1648.

Pasparakis, M., Haase, I. and Nestle, F. O. (2014) 'Mechanisms regulating skin immunity and inflammation', *Nature Reviews Immunology*, pp. 289–301. doi: 10.1038/nri3646.

Passlick, B., Flieger, D. and Loms Ziegler-Heitbrock, H. W. (1989) 'Identification and characterization of a novel monocyte subpopulation in human peripheral blood', *Blood*. American Society of Hematology, 74(7), pp. 2527–2534. doi: 10.1182/BLOOD.V74.7.2527.BLOODJOURNAL7472527.

Patel, J., Channon, K. M. and McNeill, E. (2013) 'The downstream regulation of chemokine receptor signalling: Implications for atherosclerosis', *Mediators of Inflammation*, 2013, p. 459520. doi: 10.1155/2013/459520.

Paus, R. *et al.* (2003) 'The hair follicle and immune privilege', in *Journal of Investigative Dermatology Symposium Proceedings*, pp. 188–194. doi: 10.1046/j.1087-0024.2003.00807.x.

Pease, J. E. *et al.* (1998) 'The N-terminal extracellular segments of the chemokine receptors CCR1 and CCR3 are determinants for MIP-1α and eotaxin binding, respectively, but a second domain is essential for efficient receptor activation', *Journal of Biological Chemistry*, 273(32), pp. 19972–19976. doi: 10.1074/jbc.273.32.19972.

Pelicano, H. *et al.* (2009) 'Mitochondrial dysfunction and reactive oxygen species imbalance promote breast cancer cell motility through a CXCL14-mediated mechanism', *Cancer Research*, 69(6), pp. 2375–2383. doi: 10.1158/0008-5472.CAN-08-3359.

Penk, A. *et al.* (2019) 'NMR and molecular modeling reveal specificity of the interactions between CXCL14 and glycosaminoglycans', *Glycobiology*, 29(10), pp. 715–725. doi: 10.1093/glycob/cwz047.

Percherancier, Y. *et al.* (2005) 'Bioluminescence resonance energy transfer reveals ligand-induced conformational changes in CXCR4 homo- and heterodimers', *Journal of Biological Chemistry*, 280(11), pp. 9895–9903. doi: 10.1074/jbc.M411151200.

Perdiguero, E. G. and Geissmann, F. (2016) 'The development and maintenance of resident macrophages', *Nature Immunology*, pp. 2–8. doi: 10.1038/ni.3341.

Perussia, B., Fanning, V. and Trinchieri, G. (1985) 'A leukocyte subset bearing HLA-DR antigens is responsible for in vitro alpha interferon production in response to viruses', *Natural Immunity and Cell Growth Regulation*, 4(3), pp. 120–137.

Peterson, F. C. *et al.* (2006) 'Structural Determinants Involved in the Regulation of CXCL14/BRAK Expression by the 26 S Proteasome', *Journal of Molecular Biology*, 363(4),

pp. 813–822. doi: 10.1016/j.jmb.2006.08.057.

Petkovic, V. *et al.* (2004a) 'Eotaxin-3/CCL26 is a natural antagonist for CC chemokine receptors 1 and 5: A human chemokine with a regulatory role', *Journal of Biological Chemistry*, 279(22), pp. 23357–23363. doi: 10.1074/jbc.M309283200.

Petkovic, V. *et al.* (2004b) 'I-TAC/CXCL11 is a natural antagonist for CCR5', *Journal of Leukocyte Biology*, 76(3), pp. 701–708. doi: 10.1189/jlb.1103570.

Pitzalis, C. *et al.* (2014) 'Ectopic lymphoid-like structures in infection, cancer and autoimmunity', *Nature Reviews Immunology*, 14(7), pp. 447–462. doi: 10.1038/nri3700.

Proost, P. *et al.* (1993) 'Human and Bovine Granulocyte Chemotactic Protein-2: Complete Amino Acid Sequence and Functional Characterization as Chemokines', *Biochemistry*, 32(38), pp. 10170–10177. doi: 10.1021/bi00089a037.

Proudfoot, A. E. I. *et al.* (2003) 'Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines', *Proceedings of the National Academy of Sciences of the United States of America*, 100(4), pp. 1885–1890. doi: 10.1073/pnas.0334864100.

Proudfoot, A. E. I. and Uguccioni, M. (2016) 'Modulation of chemokine responses: Synergy and cooperativity', *Frontiers in Immunology*. Frontiers, 7(MAY), p. 183. doi: 10.3389/fimmu.2016.00183.

Purvanov, V. *et al.* (2018) 'Fluorescently tagged CCL19 and CCL21 to monitor CCR7 and ACKR4 functions', *International Journal of Molecular Sciences*, 19(12), p. 3876. doi: 10.3390/ijms19123876.

Qin, L. *et al.* (2015) 'Crystal structure of the chemokine receptor CXCR4 in complex with a viral chemokine', *Science*. American Association for the Advancement of Science, 347(6226), pp. 1117–1122. doi: 10.1126/science.1261064.

Qin, S. *et al.* (1998) 'The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions', *Journal of Clinical Investigation*, 101(4), pp. 746–754. doi: 10.1172/JCI1422.

Rajagopal, S. *et al.* (2013) 'Biased agonism as a mechanism for differential signaling by chemokine receptors', *Journal of Biological Chemistry*, 288(49), pp. 35039–35048. doi: 10.1074/jbc.M113.479113.

Rajarathnam, K. *et al.* (1994) 'Neutrophil activation by monomeric interleukin-8', *Science*, 264(5155), pp. 90–92. doi: 10.1126/science.8140420.

Ramírez-Valle, F., Gray, E. E. and Cyster, J. G. (2015) 'Inflammation induces dermal Vγ4+ γδT17 memory-like cells that travel to distant skin and accelerate secondary IL-17-driven responses', *Proceedings of the National Academy of Sciences of the United States of America*, 112(26), pp. 8046–8051. doi: 10.1073/pnas.1508990112.

Randolph, G. J. *et al.* (2002) 'The CD16+ (FcγRIII+) subset of human monocytes preferentially becomes migratory dendritic cells in a model tissue setting', *Journal of Experimental Medicine*. Rockefeller University Press, 196(4), pp. 517–527. doi: 10.1084/jem.20011608.

Randolph, G. J., Ochando, J. and Partida-Sánchez, S. (2008) 'Migration of Dendritic Cell Subsets and their Precursors', *Annual Review of Immunology*, 26(1), pp. 293–316. doi: 10.1146/annurev.immunol.26.021607.090254.

Raphael, I. *et al.* (2015) 'T cell subsets and their signature cytokines in autoimmune and inflammatory diseases', *Cytokine*. Academic Press, pp. 5–17. doi: 10.1016/j.cyto.2014.09.011.

Ritter, S. L. and Hall, R. A. (2009) 'Fine-tuning of GPCR activity by receptor-interacting proteins', *Nature Reviews Molecular Cell Biology*, 10(12), pp. 819–830. doi: 10.1038/nrm2803.

Robbins, S. H. *et al.* (2008) 'Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling', *Genome Biology*, 9(1). doi: 10.1186/gb-2008-9-1-r17.

Rodríguez-Frade, J. M. *et al.* (2016) 'Chemokine Detection Using Receptors Immobilized on an SPR Sensor Surface', *Methods in Enzymology*. Academic Press Inc., 570, pp. 1–18. doi: 10.1016/bs.mie.2015.09.013.

Roozendaal, R. and Mebius, R. E. (2011) 'Stromal Cell–Immune Cell Interactions', *Annual Review of Immunology*. Annual Reviews, 29(1), pp. 23–43. doi: 10.1146/annurev-immunol-031210-101357.

Rosenkilde, M. M. *et al.* (2004) 'Molecular mechanism of AMD3100 antagonism in the CXCR4 receptor: Transfer of binding site to the CXCR3 receptor', *Journal of Biological Chemistry*, 279(4), pp. 3033–3041. doi: 10.1074/jbc.M309546200.

Sadler, J. E. (2008) 'Von Willebrand factor, ADAMTS13, and thrombotic thrombocytopenic purpura Clinical features of idiopathic TTP', *Blood*, 112, pp. 11–18. doi: 10.1182/blood-2008.

Sallusto, F. *et al.* (2014) 'Two subsets of memory T lymphocytes with distinct homing potentials and effector functions', *Journal of Immunology*, 192(3), pp. 840–844. doi: 10.1126/science.1058867.

Sallusto, F. and Lanzavecchi, A. (1994) 'Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus iuterleukin 4 and downregulated by tumor necrosis factor α', *Journal of Experimental Medicine*, 179(4), pp. 1109–1118. doi: 10.1084/jem.179.4.1109.

Salogni, L. *et al.* (2009) 'Activin A induces dendritic cell migration through the polarized release of CXC chemokine ligands 12 and 14', *Blood*, 113(23), pp. 5848–5856. doi: 10.1182/blood-2008-12-194597.

Sánchez-Torres, C. *et al.* (2001) 'CD16+ and CD16- human blood monocyte subsets differentiate in vitro to dendritic cells with different abilities to stimulate CD4+ T cells', *International Immunology*, 13(12), pp. 1571–1581. doi: 10.1093/intimm/13.12.1571.

Di Santo, J. P. (2006) 'NATURAL KILLER CELL DEVELOPMENTAL PATHWAYS: A Question of Balance', *Annual Review of Immunology*, 24(1), pp. 257–286. doi: 10.1146/annurev.immunol.24.021605.090700.

Sato, N. *et al.* (2000) 'CC chemokine receptor (CCR)2 is required for langerhans cell

migration and localization of T helper cell type 1 (Th1)-inducing dendritic cells: Absence of CCR2 shifts the Leishmania major - Resistant phenotype to a susceptible state dominated by Th2 cytok', *Journal of Experimental Medicine*, 192(2), pp. 205–218. doi: 10.1084/jem.192.2.205.

Sauty, A. *et al.* (2001) 'CXCR3 Internalization Following T Cell-Endothelial Cell Contact: Preferential Role of IFN-Inducible T Cell α Chemoattractant (CXCL11)', *The Journal of Immunology*, 167(12), pp. 7084–7093. doi: 10.4049/jimmunol.167.12.7084.

Schaerli, P. *et al.* (2000) 'CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function', *Journal of Experimental Medicine*, 192(11), pp. 1553–1562. doi: 10.1084/jem.192.11.1553.

Schaerli, P. *et al.* (2004) 'A Skin-selective Homing Mechanism for Human Immune Surveillance T Cells', *Journal of Experimental Medicine*, 199(9), pp. 1265–1275. doi: 10.1084/jem.20032177.

Schaerli, P. *et al.* (2005) 'Cutaneous {CXCL}14 targets blood precursors to epidermal niches for langerhans cell differentiation', *Immunity*. Cell Press, 23(3), pp. 331–342. doi: 10.1016/j.immuni.2005.08.012.

Schenkel, J. M. and Masopust, D. (2014) 'Tissue-resident memory T cells', *Immunity*, pp. 886–897. doi: 10.1016/j.immuni.2014.12.007.

Schiraldi, M. *et al.* (2012) 'HMGB1 promotes recruitment of inflammatory cells to damaged tissues by forming a complex with CXCL12 and signaling via CXCR4', *Journal of Experimental Medicine*, 209(3), pp. 551–563. doi: 10.1084/jem.20111739.

Schmitt, N. and Ueno, H. (2015) 'Regulation of human helper T cell subset differentiation by cytokines', *Current Opinion in Immunology*, 34, pp. 130–136. doi: 10.1016/j.coi.2015.03.007.

Scholten, D. J. *et al.* (2012) 'Pharmacological modulation of chemokine receptor function', *British Journal of Pharmacology*, pp. 1617–1643. doi: 10.1111/j.1476-5381.2011.01551.x.

Schuler, G. and Steinman, R. M. (1985) 'Murine epidermal langerhans cells mature into potent immunostimulatory dendritic cells in vitro', *Journal of Experimental Medicine*, 161(3), pp. 526–546. doi: 10.1084/jem.161.3.526.

Schulz, C. *et al.* (2012) 'A lineage of myeloid cells independent of myb and hematopoietic stem cells', *Science*. American Association for the Advancement of Science, 335(6077), pp. 86–90. doi: 10.1126/science.1219179.

Schwarze, S. R. *et al.* (2005) 'Modulation of CXCL14 (BRAK) expression in prostate cancer', *Prostate*, 64(1), pp. 67–74. doi: 10.1002/pros.20215.

Scott, C. L., Henri, S. and Guilliams, M. (2014) 'Mononuclear phagocytes of the intestine, the skin, and the lung', *Immunological Reviews*. Blackwell Publishing Ltd, 262(1), pp. 9–24. doi: 10.1111/imr.12220.

Sebastiani, S. *et al.* (2005) 'CCL22-induced responses are powerfully enhanced by synergy inducing chemokines via CCR4: Evidence for the involvement of first β-strand of chemokine', *European Journal of Immunology*, 35(3), pp. 746–756. doi:

10.1002/eji.200525800.

Segura, E. and Villadangos, J. A. (2009) 'Antigen presentation by dendritic cells in vivo', *Current Opinion in Immunology*, pp. 105–110. doi: 10.1016/j.coi.2009.03.011.

Seki, N. *et al.* (2004) 'IL-4-Induced GATA-3 Expression Is a Time-Restricted Instruction Switch for Th2 Cell Differentiation', *The Journal of Immunology*, 172(10), pp. 6158–6166. doi: 10.4049/jimmunol.172.10.6158.

Shellenberger, T. D. *et al.* (2004) 'BRAK/CXCL14 is a potent inhibitor of angiogenesis and a chemotactic factor for immature dendritic cells', *Cancer Research*, 64(22), pp. 8262–8270. doi: 10.1158/0008-5472.CAN-04-2056.

Shi, C. and Pamer, E. G. (2011) 'Monocyte recruitment during infection and inflammation', *Nature Reviews Immunology*, 11(11), pp. 762–774. doi: 10.1038/nri3070.

Shurin, G. V. *et al.* (2006) 'Loss of new chemokine CXCL14 in tumor tissue is associated with low infiltration by dendritic cells (DC), while restoration of human CXCL14 expression in tumor cells causes attraction of DC both in vitro and in vivo.', *The Journal of Immunology*, 176(6), pp. 3840.2-3840. doi: 10.4049/jimmunol.176.6.3840-a.

Sidney, L. E. *et al.* (2014) 'Concise review: Evidence for CD34 as a common marker for diverse progenitors', *Stem Cells*. AlphaMed Press, pp. 1380–1389. doi: 10.1002/stem.1661.

Sievers, F. *et al.* (2011) 'Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega', *Molecular Systems Biology*, 7. doi: 10.1038/msb.2011.75.

Sieweke, M. H. and Allen, J. E. (2013) 'Beyond stem cells: Self-renewal of differentiated macrophages', *Science*. doi: 10.1126/science.1242974.

Sin, Y. M. *et al.* (1986) 'Mast cells in newly formed lining tissue during acute inflammation: A six day air pouch model in the mouse', *Annals of the Rheumatic Diseases*, 45(10), pp. 873–877. doi: 10.1136/ard.45.10.873.

Singer, I. I. *et al.* (2001) 'CCR5, CXCR4, and CD4 Are Clustered and Closely Apposed on Microvilli of Human Macrophages and T Cells', *Journal of Virology*, 75(8), pp. 3779–3790. doi: 10.1128/jvi.75.8.3779-3790.2001.

Skrzeczyńska-Moncznik, J. *et al.* (2008) 'Peripheral blood CD14high CD16+ monocytes are main producers of IL-10', *Scandinavian Journal of Immunology*, 67(2), pp. 152–159. doi: 10.1111/j.1365-3083.2007.02051.x.

Sleeman, M. A. *et al.* (2000) 'B cell- and monocyte-activating chemokine (BMAC), a novel non-ELR α-chemokine', *International Immunology*, 12(5), pp. 677–689. doi: 10.1093/intimm/12.5.677.

Smith, J. R. *et al.* (2003) 'Expression of B-cell-attracting chemokine 1 (CXCL13) by malignant lymphocytes and vascular endothelium in primary central nervous system lymphoma', *Blood*, 101(3), pp. 815–821. doi: 10.1182/blood-2002-05-1576.

Snook, J. P., Kim, C. and Williams, M. A. (2018) 'TCR signal strength controls the differentiation of CD4+ effector and memory T cells', *Science Immunology*. American

Association for the Advancement of Science, 3(25). doi: 10.1126/sciimmunol.aas9103.

Sokol, C. L. and Medzhitov, R. (2010) 'Emerging functions of basophils in protective and allergic immune responses', *Mucosal Immunology*, pp. 129–137. doi: 10.1038/mi.2009.137.

Song, E. Y. *et al.* (2010) 'Epigenetic {Mechanisms} of {Promigratory} {Chemokine} {CXCL}14 {Regulation} in {Human} {Prostate} {Cancer} {Cells}', *Cancer Research*, 70(11), pp. 4394 LP--4401. Available at: http://cancerres.aacrjournals.org/content/70/11/4394.abstract.

Southern, C. *et al.* (2013) 'Screening β-arrestin recruitment for the identification of natural ligands for orphan G-protein-coupled receptors', *Journal of Biomolecular Screening*, 18(5), pp. 599–609. doi: 10.1177/1087057113475480.

Spits, H. *et al.* (2013) 'Innate lymphoid cells-a proposal for uniform nomenclature', *Nature Reviews Immunology*, 13(2), pp. 145–149. doi: 10.1038/nri3365.

Stallaert, W., Christopoulos, A. and Bouvier, M. (2011) 'Ligand functional selectivity and quantitative pharmacology at G protein-coupled receptors', *Expert Opinion on Drug Discovery*, 6(8), pp. 811–825. doi: 10.1517/17460441.2011.586691.

Stanford, M. M. and Issekutz, T. B. (2003) 'The relative activity of CXCR3 and CCR5 ligands in T lymphocyte migration: concordant and disparate activities in vitro and in vivo', *Journal of Leukocyte Biology*, 74(5), pp. 791–799. doi: 10.1189/jlb.1102547.

Starnes, T. *et al.* (2006) 'The chemokine CXCL14 (BRAK) stimulates activated NK cell migration: Implications for the downregulation of CXCL14 in malignancy', *Experimental Hematology*, 34(8), pp. 1101–1105. doi: 10.1016/j.exphem.2006.05.015.

Steinman, R. M., Adams, J. C. and Cohn, Z. A. (1975) 'Identification of a novel cell type in peripheral lymphoid organs of mice. IV. Identification and distribution in mouse spleen', *Journal of Experimental Medicine*, 141(4), pp. 804–820. doi: 10.1084/jem.141.4.804.

Stoitzner, P. *et al.* (2005) 'Migratory langerhans cells in mouse lymph nodes in steady state and inflammation', *Journal of Investigative Dermatology*, 125(1), pp. 116–125. doi: 10.1111/j.0022-202X.2005.23757.x.

Stossel, T. P. (1994) 'The machinery of cell crawling.', *Scientific American*, 271(3), pp. 54–55, 58. doi: 10.1038/scientificamerican0994-54.

Strieter, R. M. *et al.* (1995) 'The functional role of the ELR motif in CXC chemokine-mediated angiogenesis', *Journal of Biological Chemistry*, 270(45), pp. 27348–27357. doi: 10.1074/jbc.270.45.27348.

Strong, A. E. *et al.* (2006) 'Synthetic chemokines directly labeled with a fluorescent dye as tools for studying chemokine and chemokine receptor interactions', *European Cytokine Network*, 17(1), pp. 49–59.

Struyf, S. *et al.* (2001) 'Identification of a blood-derived chemoattractant for neutrophils and lymphocytes as a novel CC chemokine, Regakine-1', *Blood*, 97(8), pp. 2197–2204. doi: 10.1182/blood.V97.8.2197.

Struyf, S. *et al.* (2005) 'Chemokines synergize in the recruitment of circulating

neutrophils into inflamed tissue', *European Journal of Immunology*, 35(5), pp. 1583–1591. doi: 10.1002/eji.200425753.

Struyf, S., Proost, P. and Van Damme, J. (2003) 'Regulation of the Immune Response by the Interaction of Chemokines and Proteases', *Advances in Immunology*, 81, pp. 1–44. doi: 10.1016/S0065-2776(03)81001-5.

Suratt, B. T. *et al.* (2004) 'Role of the CXCR4/SDF-1 chemokine axis in circulating neutrophil homeostasis', *Blood*, 104(2), pp. 565–571. doi: 10.1182/blood-2003-10-3638.

Tacke, F. and Randolph, G. J. (2006) 'Migratory fate and differentiation of blood monocyte subsets', *Immunobiology*. Elsevier GmbH, pp. 609–618. doi: 10.1016/j.imbio.2006.05.025.

Tamoutounour, S. *et al.* (2013) 'Origins and functional specialization of macrophages and of conventional and monocyte-derived dendritic cells in mouse skin', *Immunity*, 39(5), pp. 925–938. doi: 10.1016/j.immuni.2013.10.004.

Tanegashima, K. *et al.* (2010) 'CXCL14 deficiency in mice attenuates obesity and inhibits feeding behavior in a novel environment', *PLoS ONE*, 5(4), p. e10321. doi: 10.1371/journal.pone.0010321.

Tanegashima, K., Suzuki, K., *et al.* (2013) 'CXCL14 is a natural inhibitor of the CXCL12-CXCR4 signaling axis', *FEBS Letters*, 587(12), pp. 1731–1735. doi: 10.1016/j.febslet.2013.04.046.

Tanegashima, K., Tsuji, K., *et al.* (2013) 'Dimeric peptides of the C-terminal region of CXCL14 function as CXCL12 inhibitors', *FEBS Letters*, 587(23), pp. 3770–3775. doi: 10.1016/j.febslet.2013.10.017.

Taniuchi, I. (2018) 'CD4 Helper and CD8 Cytotoxic T Cell Differentiation', *Annual Review of Immunology*. Annual Reviews, 36(1), pp. 579–601. doi: 10.1146/annurev-immunol-042617-053411.

Tasker, P. A. and Sklar, L. (1975) 'Crystal and molecular structure of di(perchlorato)(1,4,8,11-tetraazacyclotetradecane)copper (II). Cu(cyclam) (ClO4)2', *Journal of Crystal and Molecular Structure*. Kluwer Academic Publishers-Plenum Publishers, 5(5), pp. 329–344. doi: 10.1007/BF01270621.

Tessema, M. *et al.* (2010) 'Re-expression of CXCL14, a common target for epigenetic silencing in lung cancer, induces tumor necrosis', *Oncogene*, 29(37), pp. 5159–5170. doi: 10.1038/onc.2010.255.

Thelen, M. *et al.* (1988) 'Mechanism of neutrophil activation by NAF, a novel monocyte-derived peptide agonist', *FASEB Journal*, 2(11), pp. 2702–2706. doi: 10.1096/fasebj.2.11.2840318.

Thelen, M. *et al.* (2010) 'Chemokine receptor oligomerization: functional considerations', *Current Opinion in Pharmacology*, 10(1), pp. 38–43. doi: 10.1016/j.coph.2009.09.004.

Thieu, V. T. *et al.* (2008) 'Signal Transducer and Activator of Transcription 4 Is Required for the Transcription Factor T-bet to Promote T Helper 1 Cell-Fate Determination', *Immunity*, 29(5), pp. 679–690. doi: 10.1016/j.immuni.2008.08.017.

Tsou, C. L. *et al.* (2007) 'Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites', *Journal of Clinical Investigation*, 117(4), pp. 902–909. doi: 10.1172/JCI29919.

Tyler, C. J. *et al.* (2015) 'Human Vγ9/Vδ2 T cells: Innate adaptors of the immune system', *Cellular Immunology*. Academic Press Inc., 296(1), pp. 10–21. doi: 10.1016/j.cellimm.2015.01.008.

Uguccioni, M. *et al.* (1999) 'Increased expression of IP-10, IL-8, MCP-1, and MCP-3 in ulcerative colitis', *American Journal of Pathology*, 155(2), pp. 331–336. doi: 10.1016/S0002-9440(10)65128-0.

Vacchini, A., Locati, M. and Borroni, E. M. (2016) 'Overview and potential unifying themes of the atypical chemokine receptor family', *Journal of Leukocyte Biology*, 99(6), pp. 883–892. doi: 10.1189/jlb.2mr1015-477r.

Valent, P. and Bettelheim, P. (1992) 'Cell Surface Structures on Human Basophils and Mast Cells: Biochemical and Functional Characterization', *Advances in Immunology*, 52(C), pp. 333–423. doi: 10.1016/S0065-2776(08)60879-2.

Valladeau, J. *et al.* (2000) 'Langerin, a novel C-type lectin specific to langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules', *Immunity*, 12(1), pp. 71–81. doi: 10.1016/S1074-7613(00)80160-0.

Valladeau, J. and Saeland, S. (2005) 'Cutaneous dendritic cells', *Seminars in Immunology*, 17(4), pp. 273–283. doi: 10.1016/j.smim.2005.05.009.

Vanbervliet, B. *et al.* (2003) 'The inducible CXCR3 ligands control plasmacytoid dendritic cell responsiveness to the constitutive chemokine stromal cell-derived factor 1 (SDF-1)/CXCL12', *Journal of Experimental Medicine*, 198(5), pp. 823–830. doi: 10.1084/jem.20020437.

Vantourout, P. and Hayday, A. (2013) 'Six-of-the-best: Unique contributions of γδ T cells to immunology', *Nature Reviews Immunology*, 13(2), pp. 88–100. doi: 10.1038/nri3384.

Veldkamp, C. T. *et al.* (2016) 'Production of Recombinant Chemokines and Validation of Refolding', in *Methods in Enzymology*. Academic Press Inc., pp. 539–565. doi: 10.1016/bs.mie.2015.09.031.

Venetz, D. *et al.* (2010) 'Perivascular expression of CXCL9 and CXCL12 in primary central nervous system lymphoma: T-cell infiltration and positioning of malignant B cells', *International Journal of Cancer*, 127(10), pp. 2300–2312. doi: 10.1002/ijc.25236.

Vermijlen, D. *et al.* (2018) 'γδ T cell responses: How many ligands will it take till we know?', *Seminars in Cell and Developmental Biology*, pp. 75–86. doi: 10.1016/j.semcdb.2017.10.009.

Villadangos, J. A. and Schnorrer, P. (2007) 'Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo', *Nature Reviews Immunology*, pp. 543–555. doi: 10.1038/nri2103.

Villanova, F. *et al.* (2014) 'Characterization of innate lymphoid cells in human skin and blood demonstrates increase of NKp44+ ILC3 in psoriasis', *Journal of Investigative Dermatology*, 134(4), pp. 984–991. doi: 10.1038/jid.2013.477.

Vinuesa, C. G. *et al.* (2016) 'Follicular Helper T Cells', *Annual Review of Immunology*. Annual Reviews, 34(1), pp. 335–368. doi: 10.1146/annurev-immunol-041015-055605.

Vishwanath, M. *et al.* (2006) 'Development of intravital intermittent confocal imaging system for studying Langerhans cell turnover', *Journal of Investigative Dermatology*, 126(11), pp. 2452–2457. doi: 10.1038/sj.jid.5700448.

Vivier, E. *et al.* (2008) 'Functions of natural killer cells', *Nature Immunology*, pp. 503–510. doi: 10.1038/ni1582.

Walz, A. *et al.* (1987) 'Purification and amino acid sequencing of NAF, a novel neutrophil-activating factor produced by monocytes', *Biochemical and Biophysical Research Communications*, 149(2), pp. 755–761. doi: 10.1016/0006-291X(87)90432-3.

Wang, H. *et al.* (2012) 'The CXCR7 chemokine receptor promotes B-cell retention in the splenic marginal zone and serves as a sink for CXCL12', *Blood*, 119(2), pp. 465–468. doi: 10.1182/blood-2011-03-343608.

Wente, M. N. *et al.* (2008) 'CXCL14 expression and potential function in pancreatic cancer', *Cancer Letters*, 259(2), pp. 209–217. doi: 10.1016/j.canlet.2007.10.021.

Wiktor-Jedrzejczak, W. and Gordon, S. (1996) 'Cytokine regulation of the macrophage (Mϕ) system studied using the colony stimulating factor-1-deficient op/op mouse', *Physiological Reviews*. American Physiological Society, 76(4), pp. 927–947. doi: 10.1152/physrev.1996.76.4.927.

Willimann, K. *et al.* (1998) 'The chemokine SLC is expressed in T cell areas of lymph nodes and mucosal lymphoid tissues and attracts activated T cells via CCR7', *European Journal of Immunology*, 28(6), pp. 2025–2034. doi: 10.1002/(SICI)1521-4141(199806)28:06<2025::AID-IMMU2025>3.0.CO;2-C.

Wolf, M. and Moser, B. (2012) 'Antimicrobial activities of chemokines: Not just a side-effect?', *Frontiers in Immunology*, 3(JUL). doi: 10.3389/fimmu.2012.00213.

Wollenberg, A. *et al.* (1996) 'Immunomorphological and ultrastructural characterization of Langerhans cells and a novel, inflammatory dendritic epidermal cell (IDEC) population in lesional skin of atopic eczema', *Journal of Investigative Dermatology*, 106(3), pp. 446–453. doi: 10.1111/1523-1747.ep12343596.

Wong, K. L. *et al.* (2011) 'Gene expression profiling reveals the defining features of the classical, intermediate,and nonclassical human monocyte subsets', *Blood*, 118(5). doi: 10.1182/blood-2010-12-326355.

Woollard, S. M. and Kanmogne, G. D. (2015) 'Maraviroc: A review of its use in hivinfection and beyond', *Drug Design, Development and Therapy*, 9, pp. 5447–5468. doi: 10.2147/DDDT.S90580.

Wynn, T. A., Chawla, A. and Pollard, J. W. (2013) 'Macrophage biology in development, homeostasis and disease', *Nature*, pp. 445–455. doi: 10.1038/nature12034.

Xu, L. *et al.* (2015) 'Roles of chemokines and chemokine receptors in obesity-associated insulin resistance and nonalcoholic fatty liver disease', *Biomolecules*, pp. 1563–1579. doi: 10.3390/biom5031563.

Yajima, N., Izukuri, K. and Hata, R.-I. (2010) 'Production of conditional knockout mice for chemokine BMAC/cxcl14 gene by Cre/loxP system', *Inflammation and Regeneration*. Japanese Society of Inflammation and Regeneration, 30(6), pp. 536–541. doi: 10.2492/inflammregen.30.536.

Yamane, T. (2018) 'Mouse yolk sac hematopoiesis', *Frontiers in Cell and Developmental Biology*. Frontiers Media S.A. doi: 10.3389/fcell.2018.00080.

Yona, S. *et al.* (2013) 'Fate Mapping Reveals Origins and Dynamics of Monocytes and Tissue Macrophages under Homeostasis', *Immunity*, 38(1), pp. 79–91. doi: 10.1016/j.immuni.2012.12.001.

Yoshimura, T. *et al.* (1987) 'Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines.', *Proceedings of the National Academy of Sciences of the United States of America*, 84(24), pp. 9233–9237. doi: 10.1073/pnas.84.24.9233.

Yoshimura, T. (2015) 'Discovery of IL-8/CXCL8 (the story from Frederick)', *Frontiers in Immunology*, 6(JUN). doi: 10.3389/fimmu.2015.00278.

Zaba, L. C. *et al.* (2007) 'Normal human dermis contains distinct populations of CD11c +BDCA-1+ dendritic cells and CD163+FXIIIA + macrophages', *Journal of Clinical Investigation*, 117(9), pp. 2517–2525. doi: 10.1172/JCI32282.

Zawada, A. M. *et al.* (2011) 'SuperSAGE evidence for CD14 ++CD16 + monocytes as a third monocyte subset', *Blood*, 118(12). doi: 10.1182/blood-2011-01-326827.

Zhang, Y. *et al.* (2016) 'Migratory and adhesive cues controlling innate-like lymphocyte surveillance of the pathogen-exposed surface of the lymph node', *eLife*, 5(AUGUST). doi: 10.7554/eLife.18156.

Zhou, L. J. and Tedder, T. F. (1996) 'CD14+ blood monocytes can differentiate into functionally mature CD83+ dendritic cells', *Proceedings of the National Academy of Sciences of the United States of America*, 93(6), pp. 2588–2592. doi: 10.1073/pnas.93.6.2588.

Ziarek, J. J. *et al.* (2017) 'Structural basis for chemokine recognition by a G protein-coupled receptor and implications for receptor activation', *Science Signaling*. American Association for the Advancement of Science, 10(471). doi: 10.1126/scisignal.aah5756.

Zidar, D. A. *et al.* (2009) 'Selective engagement of G protein coupled receptor kinases (GRKs) encodes distinct functions of biased ligands', *Proceedings of the National Academy of Sciences of the United States of America*, 106(24), pp. 9649–9654. doi: 10.1073/pnas.0904361106.

Ziegler-Heitbrock, H. W. L. (1996) 'Heterogeneity of human blood monocytes: The CD14+CD16+ subpopulation', *Immunology Today*. Elsevier Ltd, pp. 424–428. doi: 10.1016/0167-5699(96)10029-3.

Ziegler-Heitbrock, H. W. L. (2000) 'Definition of human blood monocytes', *Journal of Leukocyte Biology*, 67(5), pp. 603–606. doi: 10.1002/jlb.67.5.603.

Ziegler-Heitbrock, L. *et al.* (2010) 'Nomenclature of monocytes and dendritic cells in blood', *Blood*, 116(16). doi: 10.1182/blood-2010-02-258558.

Ziegler-Heitbrock, L. (2014) ‘Reprint of: Monocyte subsets in man and other species’, *Cellular Immunology*. Academic Press Inc., 291(1–2), pp. 11–15. doi: 10.1016/j.cellimm.2014.06.008.

Zlotnik, A. (2006) ‘Chemokines and cancer’, *International Journal of Cancer*, 119(9), pp. 2026–2029. doi: 10.1002/ijc.22024.

Zou, Y. R. *et al.* (1998) ‘Function of the chemokine receptor CXCR4 in heaematopolesis and in cerebellar development’, *Nature*, 393(6685), pp. 595–599. doi: 10.1038/31269.

Zwijnenburg, P. J. G. *et al.* (2003) ‘CXC-chemokines KC and macrophage inflammatory protein-2 (MIP-2) synergistically induce leukocyte recruitment to the central nervous system in rats’, *Immunology Letters*, 85(1), pp. 1–4. doi: 10.1016/S0165-2478(02)00200-6.

Chapter 8: Appendix

8.1 Supplementary Data

8.1.1 CXCL14 Target Cells

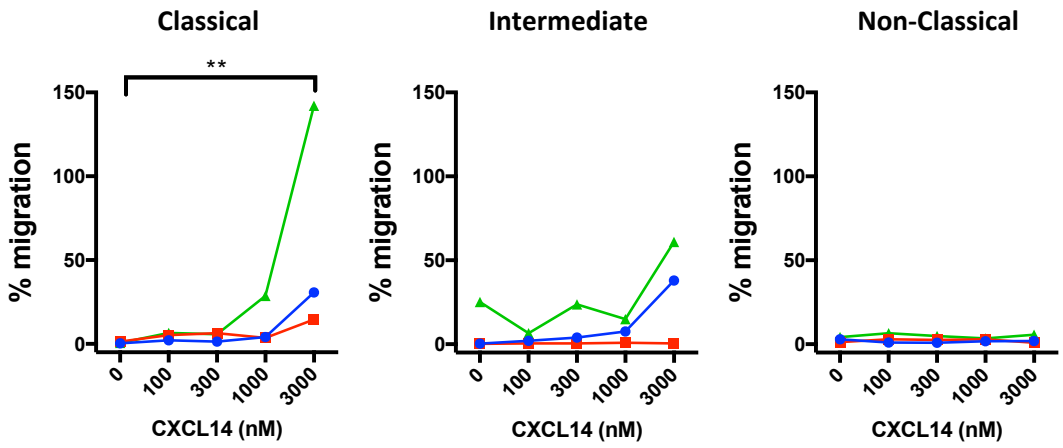


Figure 8.1. Migratory responses of human peripheral blood monocytes towards CXCL14.

Migration of classical, intermediate and non-classical monocytes in response to CXCL14. Data are mean + SEM of 3 donors from 3 independent experiments. Responses from each donor are shown in different colours. **p<0.01, Friendman test followed by Dunn's multiple comparisons test.

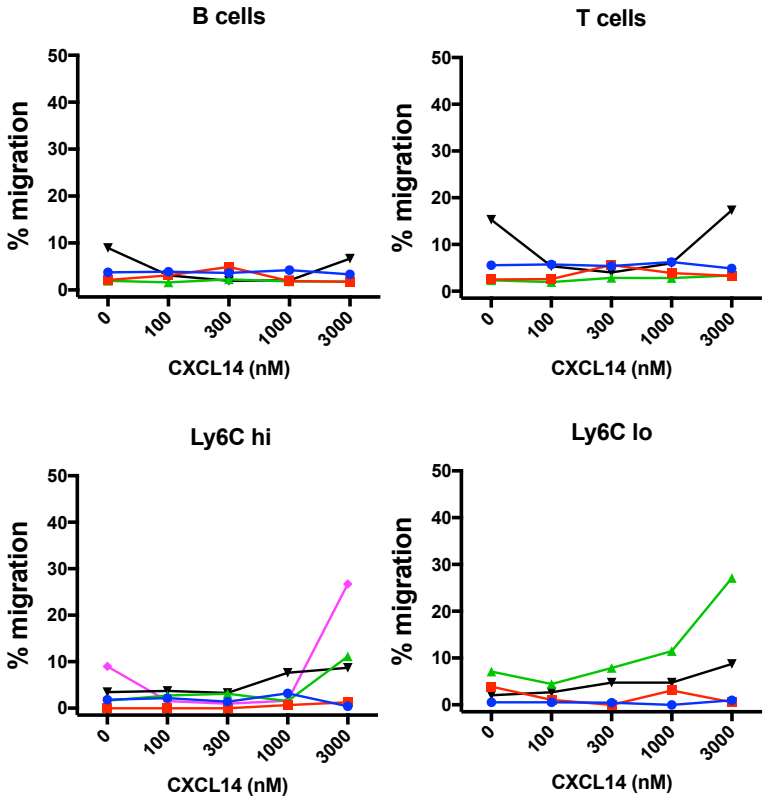


Figure 8.2. Migratory responses of murine splenocytes towards CXCL14.
Migratory responses were tested by transwell migration. Migration of B cells (identified by B220+ staining), T cells (identified by CD3+ staining) and monocytes (identified by Ly6C staining) towards CXCL14 are shown. Data are mean + SD of 4-5 independent experiments. Each experiment is shown in different colours.

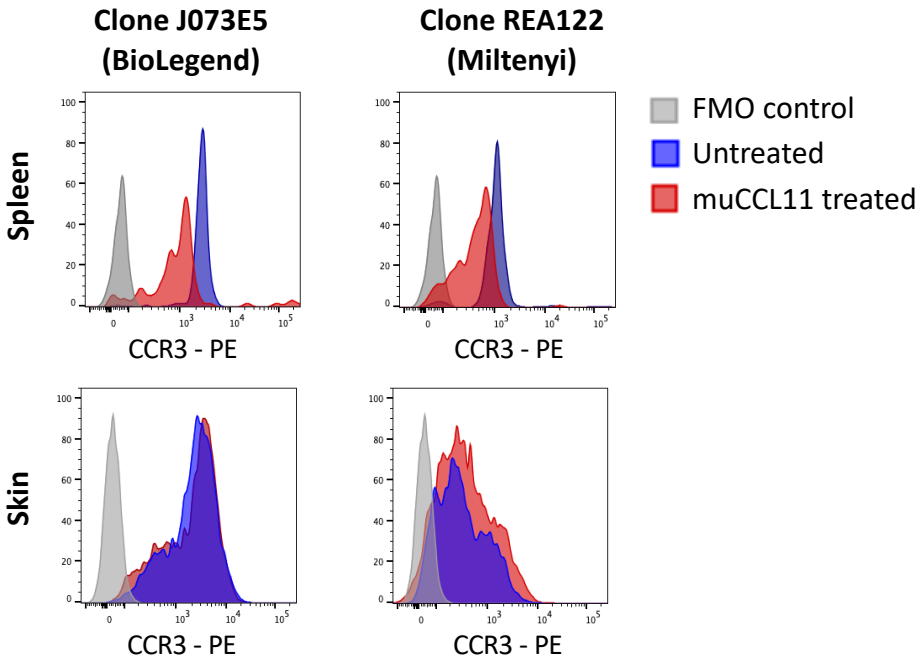


Figure 8.3 Staining of skin TR14 cells and spleen cells with CCR3-PE antibodies.
Skin TR14 cells or CD45+SSChi spleen cells were stained with PE-conjugated CCR3 antibodies from different suppliers (Biolegend and Miltenyi) and of different clones. muCCL11 treatment was used to induce internalisation of the receptor and therefore a shift in fluorescence. This experiment was performed once.

8.2 Publications During this PhD

Vermijlen, D., Gatti, D., **Kouzeli, A.**, Rus, T., Eberl, M. (2018) 'γδ T cell responses: How many ligands will it take till we know?', Seminars in Cell and Developmental Biology, pp. 75–86. doi: 10.1016/j.semcdb.2017.10.009.

McCully, M. L., **Kouzeli, A.** and Moser, B. (2018) 'Peripheral Tissue Chemokines: Homeostatic Control of Immune Surveillance T Cells', Trends in Immunology, 39(9), pp. 734–747. doi: 10.1016/j.it.2018.06.003.

8.3 Presentations During my PhD Studies

8.3.1 Poster Presentations

- Cardiff University Division of Infection and Immunity Annual Meeting, Cardiff, 2016. Title "Human γδT-APCs: Processing of Tumour Antigens and Induction of Anti-Tumour Immunity"
- Gamma delta conference, London, 2016. Title "Human γδT-APCs: Processing of Tumour Antigens and Induction of Anti-Tumour Immunity"
- British Society of Immunology Annual Congress, Liverpool, 2017. Title "Morphology and function analysis of a novel subset of CXCL14-responsive cells present in healthy tissues"
- European Chemokine and Cell Migration Conference, Cardiff, 2017– Title "Morphology and function analysis of a novel subset of CXCL14-responsive cells present in healthy tissues"
- Cardiff University Division of Infection and Immunity Annual Meeting, Cardiff, 2017. Title "Morphology and function analysis of a novel subset of CXCL14-responsive cells present in healthy tissues"
- 31st Cardiff University Annual School of Medicine and Dentistry Postgraduate Research Day, Cardiff, 2017. Title "Morphology and function analysis of a novel subset of CXCL14-responsive cells present in healthy tissues"
- 32nd Cardiff University Annual School of Medicine and Dentistry Postgraduate Research Day, Cardiff, 2018. Title "Morphology and function analysis of a novel subset of CXCL14-responsive cells present in healthy tissues"
- Cardiff University Division of Infection and Immunity Annual Meeting, Cardiff, 2018. Title "Identification of CXCL14-responsive cells in healthy peripheral tissues"
- Gordon Research Seminar on Chemotactic Cytokines, Newry, ME United States, 2018. Title "Identification of CXCL14-responsive cells in healthy peripheral tissues"

- Gordon Research Conference on Chemotactic Cytokines, Newry, ME United States, 2018. Title “Identification of CXCL14-responsive cells in healthy peripheral tissues”
- European Chemokine and Cell Migration Conference, Madrid, 2019. Title “Synergistic interaction of CXCL14 with other chemokines”

8.3.2 Oral Presentations

- Cardiff University Division of Infection and Immunity Annual Meeting, Cardiff, 2018. Title “Identification of CXCL14-responsive cells in healthy peripheral tissues”
- Cardiff University Division of Infection and Immunity Seminar series, Cardiff, 2018. Title “Identification of CXCL14-responsive cells in healthy peripheral tissues”
- Gordon Research Seminar on Chemotactic Cytokines, Newry, ME United States, 2018. Title “Identification of CXCL14-responsive cells in healthy peripheral tissues”
- Gordon Research Conference on Chemotactic Cytokines, Newry, ME United States, 2018. Title “Identification of CXCL14-responsive cells in healthy peripheral tissues”
- European Chemokine and Cell Migration Conference, Madrid, 2019. Title “Synergistic interaction of CXCL14 with other chemokines”