



Koumbas Foley, Lily (2026) *Defining inflammatory chemokine dynamics in embryonic and adult tissues*. PhD thesis.

<https://theses.gla.ac.uk/85910/>

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

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

This work cannot be reproduced or quoted extensively from without first obtaining permission from the author

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

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

Enlighten: Theses

<https://theses.gla.ac.uk/>
research-enlighten@glasgow.ac.uk



Defining Inflammatory Chemokine Dynamics in Embryonic and Adult Tissues

Lily Koumbas Foley

*A thesis submitted in fulfilment of the requirements of the
University of Glasgow for the degree of Doctor of
Philosophy*

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

September 2025

Abstract

Chemokines orchestrate immune cell migration via interaction with their specific cognate receptors during both homeostasis and inflammation. The inflammatory CC-chemokine receptors CCR1, CCR2, CCR3 and CCR5 (iCCRs) are predominantly expressed by myeloid cells. These receptors exhibit high promiscuity, binding to multiple CC-chemokines, which themselves can engage various receptors, creating a complex and dynamic signalling network. iCCRs share ligands with ACKR2, an atypical, non-signalling chemokine receptor. ACKR2 functions as a scavenger of inflammatory chemokines that signal through chemokine receptors CCR1-5, modulating chemokine availability and contributing to the resolution of inflammation. ACKR2 is highly expressed by trophoblasts and lymphatic endothelial cells. ACKR2 expressed by trophoblasts prevents maternal inflammatory chemokines from entering the embryo circulation. In ACKR2-deficient mice, transfer of maternal chemokines disrupts leukocyte distribution in embryonic tissues. In particular a novel F4/80^{int} macrophage subset is reduced in embryonic skin, highlighting emerging roles for inflammatory chemokine signalling in embryogenesis.

This thesis extends these findings by mapping chemokine expression in embryonic tissues and characterising inflammatory chemokine receptor profiles on embryonic myeloid subsets. Homeostatic CXC-chemokines were robustly detected across tissues analysed. Inflammatory chemokines and receptors were more highly detected in the lung and skin than the liver and heart, with particularly high CCL11 expression detected in the lung. This suggests a broader role for inflammatory chemokines in embryonic development. Furthermore, this thesis determines whether macrophage distribution is altered in naïve adult ACKR2-deficient mice. We found that specific subsets of macrophages across multiple adult tissues were reduced in numbers in ACKR2-deficient mice. This could not be attributed to altered cell recruitment caused by dysregulated chemokine levels and also had functional consequences in bacterial infection. Transcriptomic data of lung macrophages demonstrated that ACKR2 has limited effects on gene expression profiles of macrophage populations. Together, these findings indicate that inflammatory chemokine expression in the embryo is tightly regulated and patterned, suggesting important and specific roles, and highlight that ACKR2 expression impacts macrophage numbers beyond the embryo.

Table of Contents

Abstract.....	2
List of Tables.....	6
List of Figures	7
Acknowledgements	10
Publications.....	11
Author's Declaration.....	12
Abbreviations	13
1 Introduction	16
1.1 The Immune System and Inflammation.....	16
1.2 Leukocyte Development.....	16
1.3 The Innate Immune Response	19
1.4 A Closer Look at Neutrophils, Monocytes, Macrophages and Eosinophils.....	20
1.4.1 Neutrophils.....	20
1.4.2 Monocytes.....	21
1.4.3 Macrophages.....	21
1.4.4 Eosinophils	23
1.5 The Chemokine System	23
1.5.1 Overview	23
1.5.2 Chemotactic Cytokines.....	24
1.5.3 Chemokine Receptors	25
1.6 Chemokine Signalling Function	28
1.6.1 The Leukocyte Adhesion Cascade	28
1.6.2 Arrest in Shear Flow	28
1.6.3 The Role of Glycosaminoglycan Binding.....	28
1.6.4 Tight Adhesion.....	29
1.6.5 Trans-Endothelial Migration.....	29
1.7 Chemokine and Receptor Evolution	30
1.8 Inflammatory Chemokine Receptors: CCR1, CCR2, CCR3 and CCR5.....	31
1.8.1 Overview	31
1.8.2 CCR1	32
1.8.3 CCR2	33
1.8.4 CCR3	35
1.8.5 CCR5	36
1.9 The Complexity of Chemokine Signalling.....	37
1.10 Atypical Chemokine Receptors	41
1.10.1 Overview.....	41
1.10.2 ACKR1	42
1.10.3 ACKR2	44
1.10.4 The Role of ACKR2 in the Placenta	46
1.10.5 ACKR2 and Macrophage Distribution and Function	50
1.10.6 ACKR3	51
1.10.7 ACKR4	52
1.10.8 ACKR5	53
1.11 Aims of the Project.....	53
2 Materials and Methods	55

2.1	Mouse Models	55
2.1.1	Wild Type Mice.....	55
2.1.2	ACKR2 Knock Out Mice.....	55
2.1.3	iCCR-Reporter Mice	55
2.2	Embryo Age and Sex Determination	56
2.2.1	Phenotyping Age	56
2.2.2	Sex Genotyping.....	56
2.3	Transcriptional Analysis.....	58
2.3.1	RNA Isolation and Purification.....	58
2.3.2	RNA Quantification.....	58
2.4	Proteomic Analysis.....	59
2.4.1	Protein Isolation	59
2.4.2	Protein quantification	60
2.5	Tissue Processing for Flow Cytometry	61
2.5.1	Foetal Tissues	61
2.5.2	Adult Tissues.....	62
2.6	Flow Cytometry.....	64
2.6.1	Cell Staining.....	64
2.6.2	Cell Counts.....	67
2.7	Fluorescence Activated Cell Sorting	67
2.8	Imaging.....	67
2.8.1	Wholemount Imaging of the Peritoneal Wall.....	67
2.8.2	RNAScope	68
2.8.3	Co-staining with Antibodies	69
2.9	Peritoneal Infection of Mice with <i>E. coli</i>	69
2.10	Bioinformatics.....	69
2.10.1	Bulk-RNA sequencing.....	69
2.11	Statistical Analysis.....	70
3	<i>Chemokine Ligand and Receptor Expression in the Developing Embryo</i>	71
3.1	Introduction.....	71
3.2	Chemokine Ligand Expression in Embryonic Tissues at E15, E17.5 and E18.5	73
3.2.1	Chemokine Expression in Embryonic Heart	75
3.2.2	Chemokine Expression in the Embryonic Lung	83
3.2.3	Chemokine Expression in the Embryonic Skin.....	94
3.2.4	Chemokine Expression in the Embryonic Liver	103
3.3	Chemokine Receptor Expression.....	114
3.3.1	Chemokine Receptor Expression in E17.5 Lung Myeloid Populations	114
3.3.2	Chemokine Receptor Expression in E17.5 Skin Myeloid Populations	120
3.3.3	Chemokine Receptor Expression in E17.5 Liver Myeloid Populations	123
3.3.4	Chemokine Receptor Expression in E17.5 Spleen Myeloid Populations	126
3.4	Discussion.....	129
4	<i>ACKR2 Deficiency is Associated with Altered Peritoneal Macrophages</i>	134
4.1	Introduction.....	134
4.2	Results.....	137
4.2.1	SPM Numbers are Reduced in the Peritoneal Cavity and Peritoneal Wall of Naïve ACKR2-Deficient Mice.....	137
4.2.2	Monocytes are Reduced in Numbers in the Peritoneal Wall of Naïve ACKR2-Deficient Mice	145
4.2.3	ACKR2 RNA is Expressed across Lymphatic Vessels in the Peritoneal Wall.....	147

4.2.4	Inflammatory Chemokine Receptors (iCCRs) are Expressed in the Peritoneal Wall	149
4.2.5	ACKR2 Does Not Scavenge Chemokines in the Peritoneal Cavity or the Peritoneal Wall at Rest 152	
4.2.6	Bacterial Clearance is Defective in ACKR2-Deficient Mice	157
4.2.7	Examination of Protein Levels of Other Potential Mediators.	163
4.2.8	ACKR2 is Associated with Reduced Numbers in a Specific Lung Macrophage Population.....	166
4.3	Discussion.....	169
5	<i>Transcriptomic Analysis of Lung Macrophage Populations</i>	173
5.1	Introduction.....	173
5.2	Results.....	175
5.2.1	The Impact of ACKR2 on the Number of Alveolar Macrophages and Monocytes in the Naïve Lung	175
5.2.2	iCCR Expression by MHCII ⁻ and MHCII ⁺ Lung Macrophages	178
5.2.3	Transcriptomic Analysis of MHCII ⁻ and MHCII ⁺ Macrophage Populations	181
5.3	Discussion.....	198
6	<i>Final Discussion.....</i>	201
6.1	Conclusion	209
	<i>List of References.....</i>	211

List of Tables

Table 1-1 Chemokine Receptors and Cognate Ligands.....	39
Table 2-1. Antibodies Used for Flow Cytometry of Myeloid Cells Obtained from Embryonic Tissues	65
Table 2-2 Antibodies Used for Flow Cytometry of Myeloid Cells Obtained from Adult Tissues.....	66
Table 3-1 qPCR and Luminex Targets with Rationale for Target Exclusion	74
Table 3-2 Differential expression of CX3CL1 in the Embryonic Heart Across Developmental Stages	81
Table 3-3 Differential Expression of CC-Chemokine Genes in the Embryonic Lung Across Developmental Stages	86
Table 3-4 Differential Expression of CXC/CX3C-Chemokine Genes in the Embryonic Lung Across Developmental Stages	92
Table 3-5 Differential Expression of CC-Chemokine Genes in the Embryonic Skin Across Developmental Stages.....	96
Table 3-6 Differential Expression of CXC-Chemokine Genes in the Embryonic Skin Across Developmental Stages.....	101
Table 3-7 Differential Expression of CC-Chemokine Genes in the Embryonic Liver Across Developmental Stages.....	105
Table 3-8 Differential Expression of CXC-Chemokine Genes in the Embryonic Liver Across Developmental Stages.....	109
Table 5-1 Most Downregulated (Log ₂ Fold) Genes in Naïve Wild Type MHCII ⁻ Versus MHCII ⁺ Interstitial Lung Macrophages	193
Table 5-2 Most Upregulated (Log ₂ Fold) Genes in Naïve Wild Type MHCII ⁻ Versus MHCII ⁺ Interstitial Lung Macrophages	194

List of Figures

Figure 1-1 Primitive and Definitive Haematopoiesis	18
Figure 1-2 Classical and Atypical Chemokine Receptor Activation Pathways.....	27
Figure 1-3 Chemokine Receptor Interactions	45
Figure 1-4 Chemokine Compartmentalisation at the Placenta-Embryo Interface	49
Figure 2-1 Representative Gel: Sex Genotyping E15 Mouse Embryos by Simplex PCR...	57
Figure 3-1 CC-Chemokine Transcription in Embryonic Heart.....	76
Figure 3-2 CC-Chemokine Protein Expression in Embryonic Heart.....	78
Figure 3-3 CXC/CX3C/XC-Chemokine Transcription in Embryonic Heart.....	80
Figure 3-4 CXC-Chemokine Protein Expression in Embryonic Heart.....	82
Figure 3-5 CC-Chemokine Transcription in Embryonic Lung	85
Figure 3-6 CC-Chemokine Protein Expression in Embryonic Lung	88
Figure 3-7 CXC/CX3C-Chemokine Transcription in Embryonic Lung.....	91
Figure 3-8 CXC-Chemokine Protein Expression in Embryonic Lung	93
Figure 3-9 CC-Chemokine Transcription in Embryonic Skin	95
Figure 3-10 CC-Chemokine Protein Expression in Embryonic Skin	98
Figure 3-11 CXC/CX3C/XC-Chemokine Transcription in Embryonic Skin	100
Figure 3-12 CXC-Chemokine Protein Expression in Embryonic Skin	102
Figure 3-13 CC-Chemokine Transcription in Embryonic Liver.....	104
Figure 3-14 CC-Chemokine Protein Expression in Embryonic Liver.....	106
Figure 3-15 CXC/CX3C-Chemokine Transcription in Embryonic Liver	108
Figure 3-16 CXC-Chemokine Protein Expression in Embryonic Liver	111
Figure 3-17 Summary of Chemokine Expression Pattern in Embryonic Heart, Lung, Liver and Skin Across E15, E17.5 and E18.5	113
Figure 3-18 Representative Gating Strategy of Myeloid Cells in Embryonic Lung at E17.5	115
Figure 3-19 iCCR Expression Across Myeloid Cells in Total Embryonic Lung at E17.5	117
Figure 3-20 qRT-PCR Analysis of iCCR Gene Expression in Total Embryo Lung at E17.5	119
Figure 3-21 Representative Gating Strategy of Myeloid Cells in Embryonic Skin at E17.5	121
Figure 3-22 iCCR Expression Across Myeloid Cells in Embryonic Skin at E17.5.....	122
Figure 3-23 Representative Gating Strategy of Myeloid Cells in Embryonic Liver at E17.5	124

Figure 3-24 iCCR Expression Across Myeloid Cells in Embryonic Liver at E17.5	125
Figure 3-25 Representative Gating Strategy of Myeloid Cells in Embryonic Spleen at E17.5	127
Figure 3-26 iCCR Expression Across Myeloid Cells in Embryonic Spleen at E17.5	128
Figure 4-1 Schematic Diagram Summarising LPM and SPM Origins and Characteristics	136
Figure 4-2 Gating Strategy to Define Cell Populations in the Peritoneal Cavity	138
Figure 4-3 The Number of Small Peritoneal Macrophages in the Peritoneal Cavity of Wild Type and ACKR2-Deficient Mice at Rest	139
Figure 4-4 Gating Strategy to Define Cell Populations in the Peritoneal Wall	141
Figure 4-5 The Numbers of Specific Macrophages in the Peritoneal Wall, Omentum, Spleen and Mesenteric Lymph Node of Wild Type and ACKR2-Deficient Mice at Rest	143
Figure 4-6 Monocyte Numbers in the Peritoneal Cavity, Peritoneal Wall, Blood and Bone Marrow of Wild Type and ACKR2-Deficient Mice at Rest	146
Figure 4-7 ACKR2 RNA Expression in the Peritoneal Wall at Rest.....	148
Figure 4-8 Inflammatory Chemokine Receptor Expression in the Peritoneal Wall at Rest	150
Figure 4-9 ACKR2 Ligand Protein Expression in the Peritoneal Cavity at Rest	153
Figure 4-10 ACKR2 Ligand Expression in the Peritoneal Wall at Rest.....	155
Figure 4-11 Non-ACKR2- Ligand Chemokine Protein Expression in the Peritoneal Wall at Rest.....	156
Figure 4-12 CFU counts in Wild Type and ACKR2-Deficient Tissues Following <i>E. coli</i> Infection	159
Figure 4-13 Leukocyte Numbers in the Peritoneal Cavity and Peritoneal Wall of Wild Type and ACKR2-Deficient Mice Following Intraperitoneal <i>E. Coli</i> Infection	160
Figure 4-14 Protein Concentration of Other Potential Mediators in the Peritoneal Wall at Rest.....	165
Figure 4-15 Numbers of Macrophages Across Multiple Tissues in Wild Type and ACKR2-Deficient Mice at Rest.....	167
Figure 5-1 Gating Strategy Used to Define Cell Types in the Lung.....	176
Figure 5-2 The Number of Alveolar Macrophages and Monocytes in the Lungs of Wild Type and ACKR2-Deficient Mice at Rest	177
Figure 5-3 iCCR Reporter Gating Strategy in the Lung	179
Figure 5-4 iCCR Expression Across Macrophage Subpopulations in the Lung at Rest....	180
Figure 5-5 Overall Distribution of Gene Expression Across Samples Used for Transcriptomic Analysis	182

Figure 5-6 Principal Component Analysis of Gene Expression Across Samples Used for Transcriptomic Analysis	184
Figure 5-7 The Number of Differentially Expressed Genes Between Wild Type and ACKR2-Deficient MHCII ⁻ and MHCII ⁺ Lung Macrophages at Rest.....	186
Figure 5-8 Transcriptional Differences Between Wild Type and ACKR2-Deficient MHCII ⁻ Lung Macrophages at Rest.....	187
Figure 5-9 Transcriptional Differences Between Wild Type and ACKR2-Deficient MHCII ⁺ Lung Macrophages at Rest	189
Figure 5-10 Mean Expression and Log2Fold Change of Genes in Naïve Wild Type MHCII ⁻ Versus MHCII ⁺ Lung Macrophages	191
Figure 5-11 Pathway Analysis of Genes Upregulated in MHCII ⁻ Lung Macrophages versus MHCII ⁺ Lung Macrophages at Rest	196
Figure 5-12 Pathway Analysis of Genes Upregulated in MHCII ⁺ Lung Macrophages versus MHCII ⁻ Lung Macrophages at Rest	197
Figure 6-1 Tile Scan of the E17.5 Embryo at 20X Magnification.....	204

Acknowledgements

I would first like to acknowledge Gerry Graham and Gill Wilson. Gerry, thank you for all your support (even when my work was ‘torture’!) and for offering so much of your time and insight. I’ve learnt a huge amount these past four years. Gill, you taught me everything I know in the lab with endless kindness and fun. We’ve had so many good times, giggling and gossiping on big experiment days, working to Beyonce and dancing at conferences. I will miss you so much!

Thank you to the CRG, you are all absolute legends! Elise, thank you for reading my thesis and offering great advice, you’re still in the wrong profession, don’t forget us when you’re a big shot comedy writer! Extra special thanks to Ayumi for spending hours helping with embryo dissections and for the CURSED bone marrow RNAscope! You always brightened my day, I hope you won’t miss my drama too much. Thank you, Fabian, for our 2-minute side project and for all your help with the Taqman arrays, I’ll miss your bigfoot antics! Heather, thank you for helping on the sorter in the evenings, even when you had to run mid-sort to move your car. Robin, thanks for everything, the memory of you shouting ‘GO HOME’ was honestly a lifeline. Thank you to all the Level 3 babygirls and to our lab managers; Catherine, Jen and Maddy. Thank you to all the staff at the CRF and WRF, especially Connie! I would also like to acknowledge the staff at the Glasgow Imaging Facility and Flow Core Facility, with particular thanks to Susan, Diane, Alana and Daniel.

Thank you to the extra hot nerds Zuzanna and Kirstyn for the giggles, tears and rants. We are bonded together forever now. I couldn’t be happier about it! Love you both!

To Christie, Holly, Kate and Eloise, your love really carried me through, always will!

Thank you so much to Laurie, we have slogged through together and I couldn’t have finished this without you. Thank you for nourishing me with delicious food all year and for all of our adventures. I love you!

Big up my family!!! Adrian, Alisha, Arun, Amira, Hannah and the Koumbas gals, you all make home the best place in the world! Thank you to Dad, to Mia and the biggest thank you to Mum. Words obviously can’t describe how grateful I am for all your love and encouragement.

Finally, I would like to acknowledge the animals that contributed to this study.

Publications

Wilson GJ, **Koumbas Foley L**, Pocalun Z, Pitmon E, Fukuoka A, Lee KM, Fernandez L, Mathie H, Bartolini R, Cole JJ, Graham GJ. 2024. Eosinophils promote monocyte to macrophage differentiation and anti-bacterial immunity. bioRxiv. doi: 10.1101/2024.11.25.625164 (*preprint*)

Fukuoka A, Wilson GJ, Pitmon E, **Koumbas Foley L**, Johnsson H, Pingen M, Graham GJ. 2024. Disruption of placental ACKR3 impairs growth and hematopoietic development of offspring. *Development*. 15;151(4). doi: 10.1242/dev.202333

Strizova Z, Benesova I, Bartolini R, Novysedlak R, Cecrdlova E, **Koumbas Foley L**, Striz I. 2023. M1/M2 macrophages and their overlaps – myth or reality? *Clin Sci (Lond)*. 137(15):1067–1093. doi: 10.1042/CS20220531

Author's Declaration

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

Printed Name: Lily Koumbas Foley

Signature:

Abbreviations

ACKR	Atypical Chemokine Receptor
APC	Antigen Presenting Cell
BAC	Bacterial Artificial Chromosome
BMDM	Bone Marrow Derived Macrophages
CCL	CC-type Chemokine Ligand
CCR	CC-type Chemokine Receptor
Chil-1	Chitinase 3-like-1
CKR	Chemokine Receptor
CSF	Cerebrospinal Fluid
Ct	Cycle Threshold
CXCL	CXC-type Chemokine Ligand
CXCR	CXC-type Chemokine Receptor
DAMP	Damage Associated Molecular Pattern
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle Medium
E15	Embryonic Day 15
E17.5	Embryonic Day 17.5
E18.5	Embryonic Day 18.5
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EMP	Erythro-Myeloid Progenitor
FACS	Fluorescence-Activated Cell Sorting
FBS	Foetal Bovine Serum
FC	Fold Change
FMO	Fluorescence Minus One
GAG	Glycosaminoglycans
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GPCR	G Protein-Coupled Receptor
HIV	Human Immunodeficiency Virus
HSC	Hematopoietic Stem Cell
iCCR	Inflammatory Chemokine Receptor CCR1, CCR2, CCR3 and/or CCR5

IFN Interferon

Ig Immunoglobulin

IL Interleukin

REP Inflammatory CC-Chemokine Receptor Reporter

KO Knock Out

LEC Lymphatic Endothelial Cell

LPM Large Peritoneal Macrophage

LPS Lipopolysaccharide

MCSF Macrophage Colony-Stimulating Factor

MFI Mean Fluorescence Intensity

MHC Major Histocompatibility Complex

MMP Matrix Metalloproteinase

MS Multiple Sclerosis

NK Natural Killer Cell

P0 Postnatal Day 0

PAMP Pathogen Associated Molecular Pattern

PBS Phosphate-Buffered Saline

PCR Polymerase Chain Reaction

PFA Paraformaldehyde

PRR Pattern Recognition Receptor

qPCR Quantitative Polymerase Chain Reaction

RA Rheumatoid Arthritis

RBC Red Blood Cell

RNA Ribonucleic Acid

RPM Rotations Per Minute

RT Room Temperature

RT-qPCR- Reverse Transcriptase Quantitative Polymerase Chain Reaction

SEM Standard Error of the Mean

SD Standard Deviation

SPM Small Peritoneal Macrophage

TCR T Cell Receptor

TGF Transforming Growth Factor

Th2 T Helper Cell Type 2

TLR Toll-Like Receptor

TNF Tumour Necrosis Factor

Treg Regulatory T Cell

vCKBP Viral Chemokine Binding Protein

VEGF Vascular Endothelial Growth Factor

WT Wild Type

XCL XC-Type Chemokine Ligand

1 Introduction

1.1 The Immune System and Inflammation

The immune system is composed of a complex network of cells, tissues, and molecules which protect the host from damage by pathogens, allergens or injury. Simultaneously, the immune system plays a critical role in distinguishing self from non-self and ensuring tolerance to non-harmful external stimuli, such as commensal bacteria. The immune response can be broadly categorised as either innate or adaptive. Both consist of diverse immune cell (leukocyte) populations with specialised functions. The innate immune system is characterised by a rapid and non-specific response to infection or damage (Pradeu et al., 2024). In contrast, the adaptive immune response is highly specific to the invading pathogen and requires time to develop. Importantly, adaptive immunity also develops immunological memory, which allows the immune system to recognise specific antigens upon re-encounter. This memory facilitates a faster and more robust response, often preventing the development of symptoms during secondary infection and forms the basis of vaccination (Lam et al., 2024).

Following pathogen detection or tissue injury, inflammation is triggered. Inflammation is characterised by the accumulation of leukocytes and cytokines at the site of infection or injury and serves to eliminate pathogens, remove damaged cells, and initiate repair mechanisms such as wound healing. Whilst acute inflammation is essential for host defence and tissue repair, chronic inflammation, in which inflammatory signalling and leukocyte accumulation is not resolved, can lead to significant pathology i.e. autoimmune disease and cancer (Feehan and Gilroy, 2019). Thus, inflammation is delicately orchestrated by layers of regulatory mechanisms which act to balance a robust protective response with the risk of tissue damage.

1.2 Leukocyte Development

Leukocytes are first seeded in developing tissues during primitive haematopoiesis, a process that occurs during early embryonic development. The earliest leukocytes to emerge

are primitive macrophages, which arise from erythroid myeloid progenitors in the yolk sac (Gomez Perdiguero et al., 2015). These primitive macrophages then migrate to developing tissues, where they persist throughout life as resident macrophages and perform critical tissue-specific roles. For example, microglia, the resident macrophages of the brain, are the earliest resident macrophages (Neo et al., 2021). This wave of differentiation also produces erythrocytes and megakaryocytes. The next wave of haematopoiesis is known as definitive haematopoiesis and is initiated in the aorta-gonad-mesonephros (AGM) region of the embryo. This stage gives rise to multipotent hematopoietic progenitors that establish the hematopoietic stem cell (HSC) pool (Neo et al., 2021). These HSCs colonise the foetal liver, which serves as the primary hematopoietic organ during the second trimester (embryonic day 12-16 in mice) of embryo development (Hossain et al., 2022). HSCs then migrate to and colonise the bone marrow, which supports lifelong blood and immune cell production. Throughout postnatal life, definitive haematopoiesis is confined primarily to the bone marrow (Dzierzak and Bigas, 2018) (Figure 1-1). HSCs differentiate into two major lineages, the myeloid and lymphoid lineages, which give rise to cells such as macrophages, dendritic cells, T cells, and B cells (Hadland and Yoshimoto, 2018) (Figure 1-1). These differentiated leukocyte populations can be broadly categorised as members of the innate or adaptive immune response.

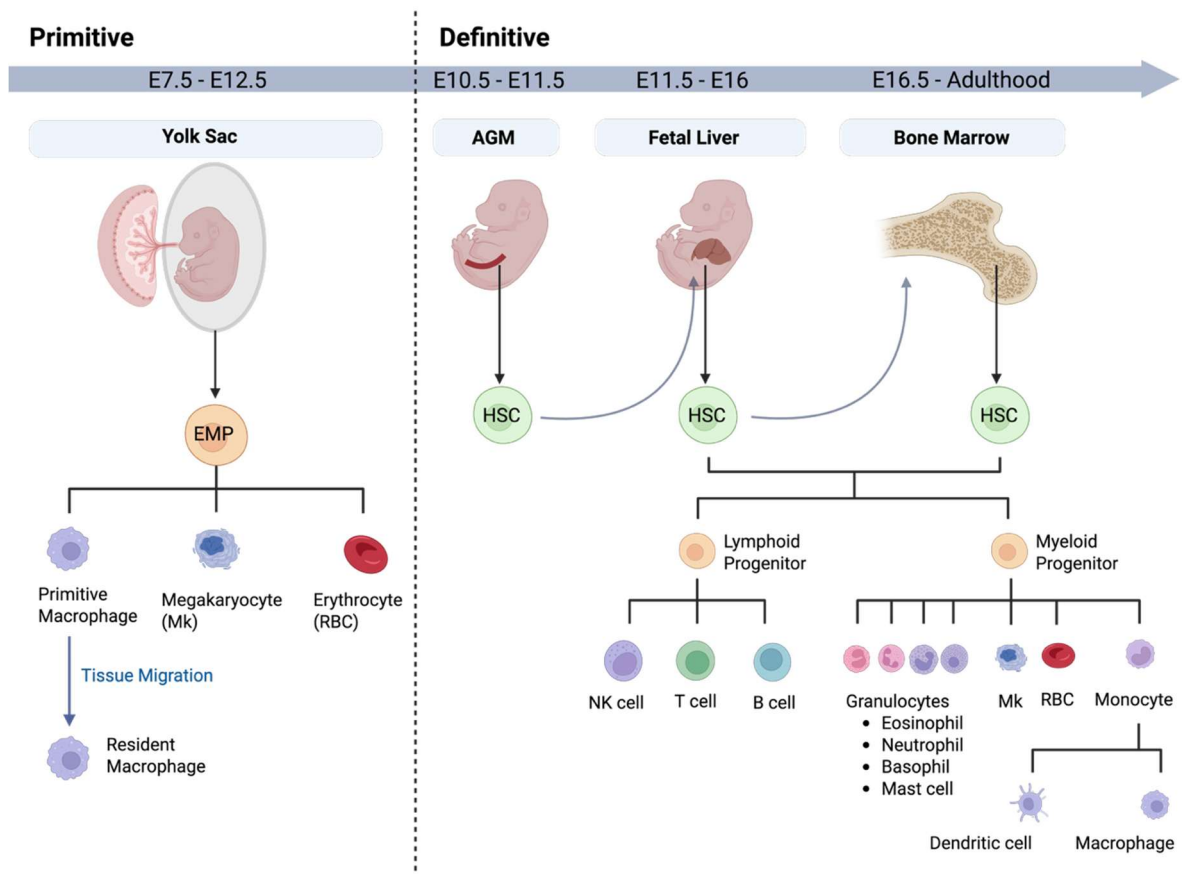


Figure 1-1 Primitive and Definitive Haematopoiesis

Primitive haematopoiesis occurs between E7.5 and E12.5 in the embryonic yolk sac and generates erythromyeloid progenitor cells (EMPs; orange), which give rise to primitive macrophages, megakaryocytes (Mk) and erythrocytes (RBC). Primitive macrophages migrate to developing tissues, where they persist as resident macrophages. Definitive haematopoiesis is initiated in the aorta-gonad-mesonephros (AGM) region at E10.5 and produces multipotent hematopoietic cells (HSCs; green). From E11.5 HSCs colonise the foetal liver, the primary hematopoietic organ during late embryo development and later (E16.5) the bone marrow, the primary hematopoietic organ throughout adulthood. During definitive haematopoiesis HSCs differentiate into lymphoid and myeloid progenitor cells. Lymphoid progenitor cells become NK cells, T cells and B cells. Myeloid progenitor cells differentiate into granulocytes (eosinophils, neutrophils, basophils and mast cells), megakaryocytes (Mk), erythrocytes (RBC) and monocytes. Monocytes further differentiate into macrophages and dendritic cells. Created using BioRender.com

1.3 The Innate Immune Response

Cells of the innate immune response include neutrophils, monocytes, macrophages, dendritic cells, natural killer (NK) cells, eosinophils, basophils and mast cells and are derived primarily from the myeloid lineage (excluding NK cells). Innate immune cells are characterised by their rapid and non-specific response to infection or damage (Marshall et al., 2018). These cells directly target pathogens in response to recognition of conserved features of pathogens (pathogen associated molecular markers (PAMPs) as well as danger-associated molecular patterns (DAMPs) released from damaged cells. An example of a PAMP is lipopolysaccharide (LPS), a component of the bacterial cell wall (Iwasaki and Medzhitov, 2015). This recognition occurs through pattern-recognition receptors (PRRs), primarily toll-like receptors (TLRs), which activate downstream signalling pathways, such as the NF- κ B pathway (Iwasaki and Medzhitov, 2015). Activation of NF- κ B leads to the production of pro-inflammatory cytokines and other immune mediators, which are critical for the rapid recruitment of subsequent additional immune cells to the site of infection and orchestration of local inflammation. Cytokines are small, secreted proteins and include interferons (IFNs), interleukins (ILs), transforming growth factors (TGFs) tumour necrosis factors (TNFs) and chemokines (Kany et al., 2019). Cytokines can be categorised as proinflammatory (e.g., IL-1, IL-6, TNF- α and type 1 interferons (IFNs)) or anti-inflammatory (e.g., IL-4 and TGF- β). Proinflammatory cytokines also promote further cytokine release, and this feedback loop can lead to cytokine storms associated with severe inflammation and pathological consequences, for example in the context of COVID-19 infection (Manik and Singh, 2022). Anti-inflammatory cytokines are often associated with processes such as wound healing (Barrientos et al., 2008).

In addition to cytokine production, various innate immune cells, particularly dendritic cells, macrophages, and neutrophils can phagocytose, which is the process of engulfing pathogens and cellular debris. These cells also function as antigen-presenting cells (APCs), processing the ingested material and presenting it to T cells. For example, dendritic cells (DCs) internalise antigen and present these molecules on major histocompatibility complex (MHC) class I and MHC class II molecules. After antigen uptake, DCs migrate via lymphatic vessels to the lymph nodes, where they localise to the T cell zones. Within these zones, DCs engage with naïve T cells, and antigen presentation via MHC molecules is essential for T cell activation (Ingulli et al., 1997). The process of antigen presentation by innate APCs is crucial for the activation of the adaptive immune response and therefore serves as a bridge between the two systems (Marshall et al., 2018).

1.4 A Closer Look at Neutrophils, Monocytes, Macrophages and Eosinophils

Throughout this thesis, there will be particular focus on the number, localisation and functional capacity of specific cells of the innate immune system, with particular interest in monocytes and macrophages and specific reference to neutrophils and eosinophils.

1.4.1 Neutrophils

Neutrophils are the largest population of circulating granulocytes and are released from the bone marrow as mature cells. Neutrophils contribute to acute inflammation and are mobilised rapidly following activation (Nemeth et al., 2020). Neutrophils play an essential role in immune protection and patients with severe congenital neutropenia are immunodeficient, and highly susceptible to recurrent and potentially life-threatening infection during early life (Skokowa et al., 2017, Dotta et al., 2011, Zeidler et al., 2009). Neutrophils destroy pathogens through several mechanisms including phagocytosis, generation of reactive oxygen species (ROS), release of cytotoxic granules, and the expulsion of DNA to form neutrophil extracellular traps (NETs) that physically capture and kill bacteria (Nemeth et al., 2020, Brinkmann et al., 2004). The cytotoxic granules secreted by neutrophils are classified as primary (azurophilic), secondary (specific) or tertiary (gelatinase), each containing distinct antimicrobial proteins and peptides such as defensins and proteolytic enzymes including matrix metalloproteinases (Pham, 2006, Nemeth et al., 2020). These molecules both directly kill pathogens and stimulate cytokine and chemokine production generating feedback loops that enhance neutrophil function and promote the recruitment and activation of other leukocytes (Nemeth et al., 2020). Furthermore, neutrophils secrete various mediators via extracellular vesicles, including neutrophil-derived Leukotriene B4 (LTB4), which is implicated in autoimmune disease (Liang et al., 2020). LTB4, in combination with chemokines and integrins, is essential for neutrophil swarming. Following the initial arrival of a small number of neutrophils at a site of infection or injury, a larger number of activated neutrophils are recruited and migrate in a coordinated manner, forming clusters around pathogens or at the site of injury (Lammermann et al., 2013, Chtanova et al., 2008).

1.4.2 Monocytes

Monocytes are phagocytic cells derived from myeloid precursors in the foetal liver during embryogenesis and in the bone marrow throughout adult haematopoiesis (Ginhoux and Jung, 2014). Monocytes require colony-stimulating factor 1 (CSF1) signalling via the CSFR1 receptor for survival and maintenance and both CSF1 and CSFR1 deficiency result in profound monocytopenia (Dai et al., 2002). Monocytes are primarily found in the circulation and make up 4 % and 10 % of non-erythrocyte cells in the blood of mice and humans respectively (Ginhoux and Jung, 2014). Ly6C^{high} monocytes are relatively short-lived in the circulation (approx. 20 h) (Patel et al., 2017, Yona et al., 2013). Following inflammation, monocytes extravasate to tissues where they differentiate into either macrophages or DCs depending on cytokine cues within the environment (Jakubzick et al., 2017). There are also monocyte reservoirs within tissues such as the spleen for rapid mobilisation following infection or damage (Swirski et al., 2009, Wu et al., 2016). Furthermore, monocytes can enter non-inflamed tissues and remain as monocytes, as opposed to differentiating into downstream effector cell types (Jakubzick et al., 2013). At rest, these monocytes sample the environment for antigen, which they then present to adaptive immune cells in the draining lymph nodes (Jakubzick et al., 2013).

Monocytes have been broadly divided into two main subsets based on the expression of a widely used mouse monocyte marker Ly6C. Ly6C^{high} expressing monocytes in mice are considered inflammatory and referred to as classical monocytes (CD14⁺ CD16⁺ in humans) (Ingersoll et al., 2010). In comparison, Ly6C^{low} monocytes in mice are referred to as alternative monocytes and are associated with wound healing and repair (CD14^{low} CD16⁺ in humans) (Ziegler-Heitbrock et al., 2010). In addition, Ly6C^{low} monocytes have a well-established role in patrolling the vascular endothelium, where they recruit neutrophils for endothelial repair in instances of damage (Auffray et al., 2007, Carlin et al., 2013). Monocytes can also replace tissue resident macrophages to maintain the populations following cell death or throughout ageing (Bain et al., 2014, Scott et al., 2016).

1.4.3 Macrophages

Macrophages are innate phagocytic APCs, which engulf PAMPs, cellular debris, apoptotic cells, and other particles for both direct clearance and presentation to adaptive immune cells. In addition, macrophages play crucial roles in homeostasis by shaping tissue

architecture and mediating tissue remodelling. This occurs through the production of matrix metalloproteinases (MMPs) which remodel the extracellular matrix; growth factors i.e. vascular endothelial growth factor (VEGF) and thrombospondin, which promote angiogenesis (i.e. during wound healing); and other signalling molecules (Mosser et al., 2021).

Macrophages have two broad lineages; macrophages are either embryonically derived from early progenitor cells within the yolk sac during primitive haematopoiesis, and the foetal liver during embryogenesis, or monocytes derived from the bone marrow. Yolk sac erythro-myeloid progenitors in mice arise at embryonic day 8.5 and become pre-macrophages (Gomez Perdiguero et al., 2015). These macrophages are seeded within specific tissues in mice from embryonic day 9.5 (E9.5), where they become highly differentiated to adapt to that niche throughout organogenesis (Mass et al., 2016). Resident macrophages include microglia in the brain, Kupffer cells in the liver (Schulz et al., 2012), Langerhans cells in the skin (Hoeffel et al., 2012), alveolar macrophages in the lung (Guilliams et al., 2013), large peritoneal macrophages in the peritoneal cavity, red pulp macrophages in the spleen (Yona et al., 2013) and Hofbauer cells in the placenta (Takahashi et al., 1991). Defining features of resident macrophages include self-renewal and long-term maintenance within their respective tissues (Hashimoto et al., 2013). Tissue resident cells are maintained by growth factors released by the macrophage niche, which is composed of surrounding stromal cells (Guilliams et al., 2020). Macrophages, excluding alveolar macrophages, require CSF1/IL-34-CSFR1 signalling for survival and maintenance. Alveolar macrophages require the granulocyte-macrophage colony stimulating factor (GM-CSF) (Guilliams et al., 2013, Guilliams et al., 2020).

In contrast, monocyte-derived macrophages differentiate within tissues and can be short or long lived. These macrophages are replenished by the bone marrow and are associated with inflammation. Many monocyte-derived macrophages can continue down a differentiation pathway and eventually adopt a foetal-derived-like phenotype, contributing to the maintenance of the resident population throughout ageing (Scott et al., 2016, Bain et al., 2014, Bain et al., 2016).

It should be noted that macrophage populations are extremely heterogenous, and this has led to extensive efforts to define and categorise subpopulations across tissues. In some literature, macrophages are broadly classified as either M1 (classical, pro-inflammatory), or M2 (alternatively activated). However, this dichotomy is based on *in vitro* macrophage

differentiation, after stimulation with LPS or IL-4/IL10 respectively. The applicability of this classification to *in vivo* contexts is widely debated as many macrophage subsets fall outside the constraints of this classification system (Strizova et al., 2023).

1.4.4 Eosinophils

Eosinophils are granulocytic innate immune cells which are derived from the bone marrow and released as terminally differentiated cells. Eosinophils make up a small proportion of circulating granulocytes and are therefore a minority population of total innate immune cells (Schetters and Schuijs, 2021). Eosinophils differentiate from myeloid precursors predominantly in response to IL-5 (Yoshida et al., 1996), but also in response to IL-3, IL-33 and GM-CSF (Johnston et al., 2016, Jacobsen et al., 2021). At rest, eosinophils populate mucosal tissues, i.e. the respiratory tract and gastrointestinal tract. Eosinophils play key roles in allergic disease including asthma and atopic dermatitis and have implications in gut pathology (Chu et al., 2014). Eosinophils contribute to inflammation through production and release of the cytokines; IL-4, IL-6, IL-10, IL-13, IL-12, TNF- α , and IFN- γ , which they constitutively express (Chu et al., 2011, Wu et al., 2011, Huang et al., 2014, Blanchard and Rothenberg, 2009).

Furthermore, eosinophils directly attack pathogens through release of cytotoxic proteins stored within their cytoplasm. These proteins include eosinophil major basic protein (MBP) eosinophil peroxidase (EPO), eosinophil derived neurotoxin (EDN) (EPO and EDN are both ribonucleases) and eosinophil cationic protein (ECP) (Jacobsen et al., 2021).

Eosinophils can also contribute to activation of the adaptive immune response by presenting antigen via MHCII and the co-stimulatory molecules CD80 and CD86 (Shi et al., 2004). Eosinophils are associated with the type 2 immune response, which involves particular cell types (i.e. T-helper 2 cells) and cytokine profiles (IL-4, IL-5, and IL-13) which contribute to protection from helminth infection (Arnold and Munitz, 2024).

1.5 The Chemokine System

1.5.1 Overview

Inflammation is triggered by the recognition of infectious pathogens or tissue damage and is characterised by the targeted recruitment of leukocytes to the site of infection or injury. The broad and specialised intrinsic functions of leukocytes previously described must be

coupled with specific localisation of these cells to provide immune protection. Leukocyte migration is regulated by chemokine signalling, a complex and overlapping network which acts to ensure the correct spatiotemporal position of leukocytes. Chemokine signalling also regulates homeostatic processes, such as stem cell homing during organogenesis and immune surveillance (Serbina and Pamer, 2006, Tachibana et al., 1998, Nagasawa et al., 1996). Whilst leukocyte accumulation is typically transient, dysregulated chemokine signalling can lead to prolonged immune cell infiltration, contributing to inflammatory diseases and cancer progression (Chen et al., 2018).

1.5.2 Chemotactic Cytokines

Chemokines are small (67-127 amino acids), highly conserved chemotactic cytokines. They modulate the immune response via chemotaxis, a form of migration which is defined by cellular movement in the direction of a chemical stimulus. Chemokines signal through interactions with G protein-coupled seven-transmembrane receptors on the surface of leukocytes and other non-hematopoietic cells. Chemokines are organised into families based on a conserved N-terminal cysteine motif, which, along with other cysteines in the peptide chain, forms disulfide bridges that stabilise and define their structure. This classification underpins chemokine nomenclature, dividing the chemokine family into CCL, CXCL, XCL, and CX3CL sub-families. In this system, 'C' signifies conserved cysteine residues, 'X' denotes varying amino acids, and 'L' represents ligand. Chemokines are then numbered in the sequence of their chronological discovery (Zlotnik and Yoshie, 2000). Chemokines are expressed exclusively by vertebrates (DeVries et al., 2006, Nomiya et al., 2011b, Bajoghli et al., 2009) and are highly conserved. Sequence identity between the molecules ranges between 20 % and 90 % (Miller and Mayo, 2017), indicating diverse origins across the entire family overall. Chemokines are predominantly secreted proteins; however, two members of the family; CXCL16 and CX3CL1, are membrane-bound. Notably, the cytoplasmic domain of CXCL16 can engage in reverse signalling, in which CXCL16 interaction with its cognate receptor CXCR6 activates signalling pathways within the CXCL16 expressing cell as well as the receptor expressing cell (Adamski et al., 2017). Chemokines can also be categorised as either homeostatic or inflammatory. Homeostatic chemokines are constitutively expressed in selected tissues, with particular expression in thymic and lymphoid tissues (CCL14, CCL19, CCL20, CCL21, CCL25, CCL27, CXCL12 and CXCL13) and act to maintain physiological conditions and immune surveillance (Moser et al., 2004, Bachmann et al., 2006). For example, CCL25 is expressed by the thymus and the small bowel epithelium and plays an essential role in the homing of

CCR9⁺ T cells to the gut. CCL25 is the only CCR9 activating ligand (Trivedi and Adams, 2018) and elevated, dysregulated CCL25 levels in the liver and colonic mucosa is associated with inflammatory bowel disease (Trivedi and Adams, 2018, Eksteen et al., 2004). Furthermore, CCL19 and CCL21 coordinate lymphocyte trafficking within the thymus, and CCL21 coordinates the exit of $\alpha\beta$ T cells from the neonatal thymus (James et al., 2021). The essential homeostatic roles for chemokines are most evident in the case of CXCL12, the most primitive and evolutionarily conserved chemokine (Zlotnik et al., 2006), which is essential for proper embryological development. CXCL12 is critical for organogenesis, as it regulates the development of the heart, brain and gonads by guiding precursor cell migration during embryogenesis (Sierro et al., 2007, Sanchez-Alcaniz et al., 2011, Belmadani et al., 2005, Gerrits et al., 2008). For example, analysis in zebrafish revealed that homozygous CXCL12 or CXCR4 (cognate receptor) deletion leads to sterility. Additionally, CXCL12 homes haematopoietic stem cells to the bone marrow and stromal cells to the lymph nodes (Sugiyama et al., 2006).

In contrast, inflammatory chemokines are widespread across tissues and usually able to bind multiple receptors, they are therefore often referred to as promiscuous. These molecules include CXCL8, CCL2, CCL3, CCL4, CCL5 etc. and are associated with the recruitment of innate immune cells during an inflammatory response, i.e. CXCL8 attracts CXCR1/2⁺ neutrophils to sites of infection or injury (Cambier et al., 2023). However, various chemokines, such as CCL21 and CXCL13 have roles in both homeostasis and inflammation (Chen et al., 2018). Thus, the classification and nomenclature of these molecules is ultimately determined by structure.

1.5.3 Chemokine Receptors

Classical chemokine receptors (CKRs) belong to the G protein-coupled receptor (GPCR) family and are organised and named based on the ligand family with which they interact: CCR, CXCR, XCR and CXC3R (Bachelier et al., 2014, Hughes and Nibbs, 2018). These class A seven transmembrane receptors transduce extracellular ligand binding into intracellular signalling cascades through activation of heterotrimeric G $\alpha\beta\gamma$ proteins (Legler and Thelen, 2018). The N-terminus of the protein is significant in receptor activation and upon ligand binding, conformational change in the receptor exposes the defining DRYLAIV motif, located within the transmembrane domain of the receptor. The DRYLAIV motif binds the G $\alpha\beta\gamma$ complex, and this interaction leads to receptor-mediated nucleotide exchange of the G α subunit and consequential separation of G α i from the

complex. The remaining G $\beta\gamma$ structure is then able to bind various signalling effectors which activate downstream kinases such as Akt/PKC. Ultimately this induces actin reorganisation and cell migration, cell survival and proliferation (Figure 1-2A) (Legler and Thelen, 2018, Arimont et al., 2017).

Conventional chemokine receptors can also couple to β -arrestin, and this triggers distinct downstream effects including receptor internalisation. Internalised receptors contained within endosomes are either destined for lysosomal degradation (i.e. CXCR4) or are recycled to the cell surface (i.e. CCR5) (Liebick et al., 2016). Receptor internalisation allows desensitisation of the receptors by transiently preventing further chemokine signalling cascades and the speed with which the receptors are redistributed on the cell surface contributes to the fine tuning of chemokine gradient detection and the directional movement of cells (Liebick et al., 2016).

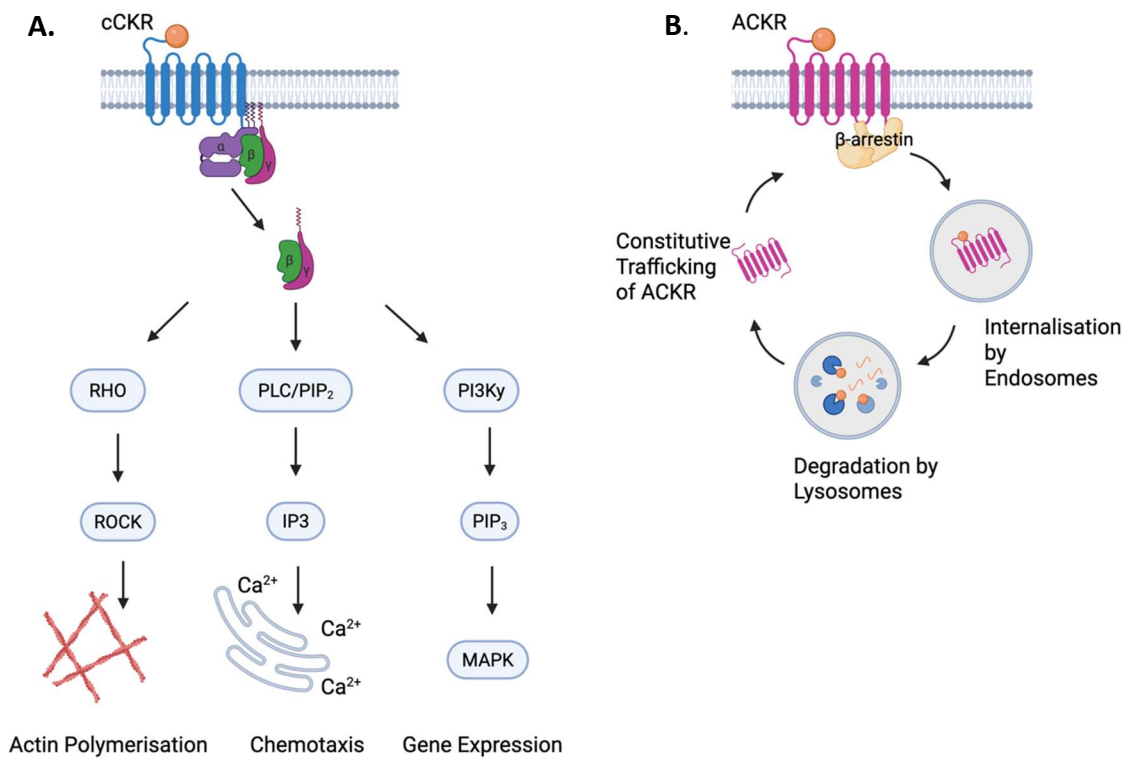


Figure 1-2 Classical and Atypical Chemokine Receptor Activation Pathways

A) Classical chemokine receptors (cCKRs, shown in blue) are activated by their specific ligands (orange), triggering the dissociation of heterotrimeric G-proteins into $G_{\alpha i}$ and $G_{\beta \gamma}$ subunits. These subunits initiate multiple downstream signalling cascades. For example, $G_{\alpha i}$ activates the Akt pathway, whilst $G_{\beta \gamma}$ stimulates phospholipase C (PLC), which cleaves PIP₂ into DAG and IP₃. This leads to intracellular calcium release, actin reorganisation, and ultimately, cell migration. Additionally, activation of the RHO/ROCK pathway promotes actin polymerisation, contributing to cell polarity and directional migration. Other pathways, such as MAPK signalling, drive gene expression changes that support cell survival and proliferation. **B)** In contrast, atypical chemokine receptors (ACKRs, shown in pink) do not signal through G-proteins but instead, couple with β -arrestin, facilitating ligand internalisation into endosomes and subsequent degradation by lysosomes. ACKRs are continuously recycled and trafficked back to the cell surface. Adapted from an image by (Comerford and McColl, 2024), created using BioRender.com

1.6 Chemokine Signalling Function

1.6.1 The Leukocyte Adhesion Cascade

During inflammation, the recruitment of leukocytes to sites of tissue damage requires the transmigration of leukocytes, for example, from circulation through blood vessel walls. This process occurs across three major steps: rolling, activation, and adhesion, known collectively as the leukocyte adhesion cascade (Graham et al., 2019).

1.6.2 Arrest in Shear Flow

Leukocytes such as neutrophils and monocytes first tether to the vascular endothelium under conditions of shear stress. This is mediated by interactions between selectins (P-selectin and E-selectin) on activated endothelial cells and their ligands on leukocytes, such as P-selectin glycoprotein ligand 1 (PSGL1) (McEver and Cummings, 1997) and E selectin ligand (ESL-1) (Ley et al., 2007, Nourshargh and Alon, 2014). A further selectin, L-selectin, is expressed by some leukocytes and mediates secondary leukocyte tethering (Bargatze et al., 1994). These interactions reduce the velocity of leukocytes despite shear flow, allowing them to roll along the vessel wall. Rolling enables leukocytes to scan the endothelium for secreted chemokines bound to glycosaminoglycans (GAGs) that then provide signals for migration (Graham et al., 2019).

1.6.3 The Role of Glycosaminoglycan Binding

In vivo, chemokines are anchored to extracellular matrices by interaction with GAGs, negatively charged polysaccharide chains found at the end of proteoglycans (Handel and Dyer, 2021). GAG binding on the luminal surface of the endothelium retains and localises chemokines, enabling their presentation by endothelial cells to trafficking leukocytes. This facilitates the localisation of chemokines by preventing their dispersion by shear flow in post-capillary venules (Sarris et al., 2012, Weber et al., 2013, Proudfoot et al., 2017). GAG binding is required for specific chemokines, such as CCL2 and CCL5 (Proudfoot et al., 2003b), to recruit cells *in vivo*, and prevention of the human chemokine CXCL8 from forming GAG interactions by the protein TNF-stimulated gene-6 has been shown to reduce CXCL8 driven neutrophil recruitment (Dyer et al., 2016). Various models for chemokine presentation in conditions of shear stress, and particularly within the leukocyte adhesion

cascade have been proposed and discussed, including a model referred to as ‘the cloud model’ (Graham et al., 2019). In this model, it is suggested that chemokines undergo reversible interactions with GAGs within the glycocalyx, the extracellular layer that lines the luminal surface of the vascular endothelium. This creates a dynamic system in which soluble chemokines surround GAG-bound chemokines presented on endothelial cells, which in turn ensures the retention of chemokines at localised sites, whilst also preserving the availability of soluble chemokines for receptor engagement (Graham et al., 2019).

1.6.4 Tight Adhesion

Chemokines within the glycocalyx of the endothelium interact with chemokine receptors on the surface of leukocytes. This interaction then triggers inside-out signalling, which activates leukocyte integrins by inducing a conformational change which results in integrins such as LFA-1 and VLA-4 switching from a low-affinity to a high-affinity state (Nourshargh and Alon, 2014). This change allows integrins to bind strongly to adhesion molecules, for example ICAM-1 binds LFA-1 and VCAM-1 binds VLA-4, and anchors leukocytes strongly to the vessel wall (Berlin et al., 1995). The specific combination and spatial arrangement of integrins expressed by leukocytes determines both the strength of adhesion and the signalling pathways which are activated for transmigration across the endothelium (Mitroulis et al., 2015).

1.6.5 Trans-Endothelial Migration

Following strong adhesion, leukocytes undergo transmigration through vessel walls, the final stage of the leukocyte adhesion cascade. The activation of specific signalling pathways, such as Rho GTPase signalling, leads to rearrangement of the actin cytoskeleton, allowing leukocytes to pass through endothelial cell junctions (Ley et al., 2007). This is facilitated by changes in the structure and function of junctional proteins like PECAM-1 (Platelet Endothelial Cell Adhesion Molecule 1), which plays a crucial role in opening endothelial junctions (Ley et al., 2007, Nourshargh and Alon, 2014). This method of transmigration is referred to as paracellular migration. Alternative methods of transmigration include the direct passage of leukocytes through endothelial cells, known as transcellular migration. Transcellular migration requires the formation of intracellular channels mediated by matrix metalloproteinases (MMPs) and involves the extension of pseudopodia by leukocytes directly into the endothelial cell. Transcellular migration

typically occurs in thinner regions of the endothelium, where there are fewer tight junctions (Ley et al., 2007). Finally, neutrophils can also undergo reverse trans-endothelial migration in which they migrate from the abluminal to luminal side of the endothelium. This process is driven by LTB₄ which induces the proteolytic cleavage of the endothelial junctional protein JAM-C and is associated with the progression of inflammation from local to systemic (Colom et al., 2015).

The leukocyte adhesion cascade is a multistep model that results in the recruitment of specific leukocytes to inflamed sites via differential chemokine ligand and receptor expression patterns and subsequent differential integrin expression and clustering across the cell membrane. The unique integrin engagement with the endothelium in turn mediates specific cell signalling pathways, which facilitate trans-endothelial migration, ensuring leukocytes are transported across the vascular endothelium to the subluminal tissue.

1.7 Chemokine and Receptor Evolution

The chemokine receptor families have expanded and evolved by *en bloc* and tandem duplication events, forming genetic clusters (DeVries et al., 2006, Zlotnik et al., 2006). CCR2 is the oldest of CCRs 1-5 and CCR receptors 1-3 and 5 resulted from the duplication of the CCR2 gene and are clustered within a 170 kb region on human chromosome 3/mouse chromosome 9 (Nomiya et al., 2011a, Dyer et al., 2019a). CCR1 and CCR5 are most homologous to CCR2, with CCR2 and CCR5 sharing approximately 70 % sequence identity (Fantuzzi et al., 2019). These genetic events have diversified the receptor pool, enhancing selectivity and defence against pathogens with receptor antagonists. Most chemokine receptors are located on human chromosome 3 (CCR1, 2, 3, 4, 5, 8, 9, CXCR6, CX3CR1, ACKR2, 4, 5). However, CXCR1, 2, and 4, as well as ACKR3, are found on human chromosome 2, and other receptors are distributed across human chromosomes 10, 6, 11, and X (DeVries et al., 2006). Chemokine ligand diversity also evolved through duplication events (DeVries et al., 2006) and evolutionary pressure to withstand perturbation of chemokine signalling by pathogens.

Pathogens can antagonise the chemokine system by producing chemokine-binding proteins (CKBPs) and peptides that block multiple chemokines (Bhusal et al., 2020, Viejo-Borbolla et al., 2012, van Berkel et al., 2000). For example, poxviruses and herpesviruses encode CKBPs that are non-homologous to host chemokines, enabling immune evasion by

suppressing leukocyte infiltration (Nguyen et al., 2017a). M3, produced by mouse herpesvirus 68, blocks multiple CXCL chemokines and abrogates chemokine signalling (van Berkel et al., 2000). Similarly, gG from human herpes simplex virus-1 binds both CCL and CXCL chemokines but, unlike M3, can both block and enhance chemokine signalling (Viejo-Borbolla et al., 2012).

Furthermore, the bites of ticks, bloodsucking obligatory parasites, typically remain undetected by the host. This is attributed to the presence of chemokine antagonists in tick saliva which effectively inhibit neutrophil and leukocyte infiltration at the site of injury (Bhusal et al., 2020). These antagonistic molecules, known as evasins, are classified into two groups: class A, which targets CC-chemokines, and class B, which targets CXC chemokines. Individual evasins bind unique and specific arrays of chemokines and have shown protective effects in mouse models of inflammatory diseases such as pancreatitis, bleomycin-induced lung fibrosis, and post-infarction myocardial injury, suggesting therapeutic potential (Montecucco et al., 2014, Russo et al., 2011, Braunersreuther et al., 2013). Furthermore, a recently discovered evasin selectively inhibits CCL2, highlighting its potential to target monocyte recruitment in disease (Singh et al., 2017). Additionally, a new subclass of class A evasins, named class A3, has been identified. These molecules exhibit broader chemokine-binding profiles than other class A evasins and can be engineered for selective chemokine inhibition, underscoring their promise as therapeutic agents (Devkota et al., 2023). Finally, it should be noted that chemokine signalling can also be perturbed by pathogens which secrete blockers that antagonise chemokine receptors. For example, the KS-associated herpes virus 8 (KSHV) releases the protein vCCL2, which can bind CCR1, CCR2, CCR3, CCR5, CCR8, CCR10, CXCR4, CX3CR1, and XCR1 (Sozzani et al., 1998) and either acts as an antagonist or agonist depending on the chemokine receptor target.

1.8 Inflammatory Chemokine Receptors: CCR1, CCR2, CCR3 and CCR5

1.8.1 Overview

The inflammatory CC-chemokine receptors: CCR1, CCR2, CCR3, and CCR5 are key mediators of non-neutrophilic myeloid cell recruitment, and these receptors will be of particular focus throughout this study. These inflammatory receptors are often co-

expressed on immune cells, and their combinatorial and discrete contributions to inflammation and immunity are not fully understood. To elucidate these roles, mice lacking CCR1, CCR2, CCR3, and CCR5 either individually or in combination have been generated. These knockout models revealed that the absence of these receptors does not lead to developmental defects, as hematopoietic progenitor cell numbers in embryos and foetal liver monocyte populations are unaffected (Dyer et al., 2019a).

1.8.2 CCR1

CCR1 was the first discovered chemokine receptor and is expressed on the surface of various leukocytes such as monocytes, macrophages, dendritic cells, and specific B cell subsets (Medina-Ruiz et al., 2024, Coates et al., 2004, Armas-Gonzalez et al., 2018). CCR1 is a promiscuous receptor that signals via interaction with multiple ligands, including CCL3, CCL5, CCL6, CCL7, CCL8, CCL9, CCL13, CCL14, CCL15, CCL16, and CCL23 (Bachelier et al., 2014). Notably, CCR1 is constitutively active and has been reported to exert basal signalling independently of ligand interaction (Gilliland et al., 2013). CCR1 deficiency is not lethal and naïve CCR1^{-/-} mice do not display clear phenotypic changes during development (Gao et al., 1997). This finding was reinforced by a study reporting small reductions in monocytes in CCR1-deficient mice but no major disturbances in overall leukocyte distribution (Dyer et al., 2019a). However, following immune challenge, CCR1 deficiency leads to defective immune responses following infection by pathogens such as *Toxoplasma gondii* (Gao et al., 1997, Khan et al., 2001). CCR1 also plays a role in the initiation and progression of inflammatory diseases. For example, in mouse models, CCR1 has been implicated in rheumatoid arthritis (RA), where it drives disease onset in the K/BxN model and exacerbates disease severity in collagen antibody-induced arthritis (Chou et al., 2010, Lewis et al., 2014). Additionally, CCR1 deficiency or pharmacological blockade has been shown to reduce leukocyte infiltration and inflammation in models of peritonitis (Lewis et al., 2014). These studies suggest CCR1 has a role in neutrophil recruitment to sites of inflammation, which aggravates disease. However, it is important to note that neutrophil recruitment is predominantly orchestrated by CXCR2 (Cacalano et al., 1994). A study by Dyer et al., demonstrated that CCR1 is not essential for neutrophil migration at rest or during acute inflammation in mice, as shown by a model of inflammation in which the inflammatory agent carrageenan was injected into a subcutaneous air pouch created on the mouse back (Dyer et al., 2019a). More recently, CCR1 has been implicated in spinal cord injury as well as disc inflammation, with

evidence that CCR1 blockade can ameliorate inflammation in both these instances (Hasan et al., 2025).

Various pharmacological blockers targeting CCR1 have previously been trialled for the treatment of inflammatory diseases such as RA (Tak et al., 2013) and chronic obstructive pulmonary disease (Kerstjens et al., 2010); however, these have not been efficacious. CCR1 has also been associated with various cancers, including liver cancer, multiple myeloma, and non-Hodgkin's lymphoma (Kitamura et al., 2010, Zeissig et al., 2021). In mouse models of Mantle Cell Lymphoma (MCL), an aggressive form of non-Hodgkin's lymphoma, a mechanism has been identified in which tumour-derived CCL3 leads to the chemotactic recruitment of macrophages to the tumour site in a CCR1-dependent manner. At the tumour site, CCR1 signalling promotes tumour survival by driving macrophage polarisation towards a pro-tumour M2 phenotype (Le et al., 2023). Inhibition of CCR1 using the small molecule antagonist BX-471 was shown to reduce both tumour weight and volume in MCL mouse models (Le et al., 2023). Furthermore, investigations in mice have also revealed that dual blockade of CCR1 and CXCR2 may represent a promising therapeutic approach for treatment of colorectal cancer (Masui et al., 2024).

1.8.3 CCR2

The role and significance of CCR2 is highlighted by its requirement for mobilisation of Ly6C^{high} monocytes from the bone marrow to the blood, as CCR2-deficient mice display profound monocytopenia (Serbina and Pamer, 2006). Importantly, knock out of CCR1, 3 and 5, in combination with CCR2, phenocopied this monocytopenia, demonstrating that this mechanism is orchestrated in a non-redundant manner by CCR2 (Dyer et al., 2019a). However, Ly6C^{low} circulating monocytes are not affected by CCR2 deficiency (Dyer et al., 2019a). This may be explained by their predominant expression of CX3CR1 rather than CCR2 (Geissmann et al., 2003). A lack of circulating Ly6C^{high} monocytes in the blood leads to reduced monocyte derived macrophages in tissues. CCR2 demonstrates ligand promiscuity and binds CCL2, CCL7, CCL8 and CCL13 and CCL2 is the most significant CCR2 activating ligand (Shao et al., 2022). Additionally, CCR2 can also scavenge CCL2 from the environment (Shroka et al., 2023). This mechanism occurs independently of classical GPCR signalling and it thought to enable sustained CCR2 responsiveness to chemokine ligands during proinflammatory immune responses (Shroka et al., 2023)

CCR2 is implicated in various inflammatory diseases including RA (Cho et al., 2007, Liou et al., 2020), multiple sclerosis (MS) (Prins et al., 2014) and atherosclerosis (Saederup et al., 2008, Aiello et al., 1999). In RA, CCR2 is upregulated in the synoviocyte cells of patients and its ligand CCL2 has even been proposed to be a suitable indicator of remission/disease state in RA (Liou et al., 2020). In addition, higher levels of CCL2 in serum are found in symptomatic COVID-19 patients compared with asymptomatic patients and severe COVID-19 is associated with high CCL2 in plasma (Bagheri et al., 2024).

Evidence for the role of CCR2 in disease is provided by studies which have shown that the reduced monocyte levels in circulation and tissues, associated with CCR2 deficiency, lead to improved outcomes across various conditions. For example, an investigation measuring the cognitive function of mice following traumatic brain injury has highlighted that CCR2 deficiency is associated with improved recovery (Hsieh et al., 2014). In addition, CCR2-deficiency has been shown to be protective during sepsis as the lack of CCR2 increased survival rate of CLP-induced sepsis in mice (Cebinelli et al., 2021).

Various small-molecule anti-CCR2 drugs have been developed to exploit this signalling pathway for therapeutic purposes. For instance, the small molecule drug MK-0812 has been tested in clinical trials for conditions such as MS, atherosclerosis, and RA. However, it was found to be ineffective in these contexts (Bachelier et al., 2014) (Clinicaltrials.gov, NCT00542022). A modified version of the drug, 15a, demonstrates a significantly longer residency time on human CCR2 compared to the unmodified version (714 minutes vs. 92 minutes, respectively). Preclinical experiments using ApoE^{-/-} mouse models of atherosclerosis showed promising results, as treatment with this small-molecule antagonist reduced atherosclerotic plaque size by 70 % (Bot et al., 2017). However, the role of ApoE in the development of atherosclerotic plaque is far more pronounced in mice than in humans. Nonetheless, this does underline that small modifications in drugs may increase efficacy.

Furthermore, CCR2 has a role in cancer progression, for example, high expression of CCL2 is a prognostic marker for liver cancer and inhibition of the CCR2 signalling pathway by small molecule antagonist has demonstrated reduced tumoral growth in mice through promoting activation of anti-tumorigenic T cells (Li et al., 2017). A significant study using mouse models has highlighted that across various tumour types and models of metastasis, CCR2 inhibition by the antagonist RS504393 improves the efficacy of anti-PD1 checkpoint inhibitor therapy. This appears to be related to an increase in preferential CD8⁺ T cell activation of CD8⁺ T cells and suppression of CD4⁺ Treg activity (Tu et al., 2020). Conversely, CCR2 has also demonstrated protective effects in the context of cancer.

Increased CCR2 expression on KLRG1⁺ NK cells correlates with reduced metastasis in mouse models of spontaneous metastasis (Hansell et al., 2018, Hayes et al., 2024). This is attributed to the enhanced capability of CCR2⁺ NK cells to migrate to tumours, which produce many CCR2 ligands, allowing these cells to carry out effective anti-tumour functions. These findings suggest that upregulation of CCR2 on tumoricidal immune cells could represent a potential therapeutic strategy in protection against metastasis (Hansell et al., 2018, Hayes et al., 2024)

1.8.4 CCR3

CCR3 is predominantly expressed on the surface of eosinophils and is critical for their trafficking under both homeostatic and inflammatory conditions. CCR3 is also expressed on basophils, Th2 cells, and mast cells and the receptor binds chemokines CCL11, CCL13, CCL24, and CCL26 (eotaxins), which are involved in allergic responses (Humbles et al., 2002). During allergic inflammation, CCR3 expression is upregulated, particularly on eosinophils, leading to their accumulation in affected tissues such as the lungs in asthma, the nasal mucosa in allergic rhinitis and the skin in atopic dermatitis, making CCR3 an attractive therapeutic target in various contexts (Yuan et al., 2022, Humbles et al., 2002, Salter et al., 2021, Gaspar et al., 2013).

Additionally, a bone marrow specific CCR3 knock-out mouse model was generated using the Cre-LoxP system (Yuan et al., 2022). In an ovalbumin-induced model of allergic rhinitis, mice with bone marrow-specific CCR3 deficiency displayed fewer lesions, reduced eosinophil infiltration in the nasal mucosa and decreased serum levels of Th2 associated cytokines (IL-4 and IL-5) (Yuan et al., 2022). These protective effects were linked to the PI3K/AKT pathway as silencing the *CCR3* gene led to a reduction in CCR3 protein expression and reduced PI3K/AKT pathway activity in eosinophils. Furthermore, inhibition of the PI3K/AKT pathway using the synthetic blocker Ly294002 reproduced the protective effects observed in the conditional bone marrow CCR3-deficient mice. These findings suggest that the PI3K/AKT pathway acts downstream of CCR3 to promote inflammation in the context of allergic rhinitis (Yuan et al., 2022).

1.8.5 CCR5

CCR5 is expressed by APCs including macrophages and DCs. CCR5 ligands include CCL3, 4, 5, 7, 8, 11, 14 and 16 (Table 1-1). CCR5 is upregulated across macrophage differentiation and maturation, and this occurs concurrently with CCR2 downregulation (Medina-Ruiz et al., 2022). CCR5 deficiency in mice does not lead to developmental defects at rest; however, the receptor has been associated with inflammatory disease. For example, CCR5 expressing neutrophils in the bone marrow have been implicated in the pathogenesis of osteoporosis (Li et al., 2023). These neutrophils are a major source of TGF- β 1 in bone tissue, and elevated levels of TGF- β 1 have been shown to suppress bone formation and promote bone resorption, ultimately leading to reduced bone mass (Li et al., 2023).

CCR5 has been most extensively studied in the context of its role as a coreceptor for HIV entry into macrophages and dendritic cells (Deng et al., 1996, Choe et al., 1996). The naturally occurring polymorphism CCR5 Δ 32, which occurs at high frequency in people of northern European origin, is characterised by a frameshift mutation which results in a 32-base pair deletion in the CCR5 transcript and has provided significant insights into the role of CCR5 in HIV pathology (Liu et al., 1996, Samson et al., 1996, Dean et al., 1996, Huang et al., 1996). Individuals homozygous for the CCR5 Δ 32 mutation are resistant to most strains of HIV-1, as the mutation results in a truncated, nonfunctional receptor, preventing viral entry into target cells. This discovery has underscored the importance of CCR5 as a therapeutic target in HIV treatment. Population data has revealed that individuals with CCR5 Δ 32 are at greater risk of symptomatic West Nile virus (Lim et al., 2010).

Importantly, a small molecule antagonist against CCR5, Maraviroc, is clinically approved for treatment of HIV. Maraviroc is a CCR5-specific inhibitor that prevents HIV from binding to the receptor and entering cells, thereby halting viral replication (Dorr et al., 2005). The therapeutic targeting of CCR5 not only highlights its significance in HIV infection but also represents broader potential for modulating chemokine receptor activity in inflammatory diseases. Maraviroc is one of the few examples of the chemokine system being successfully targeted for approved clinical therapeutic use. However, the presence of another chemokine receptor, CXCR4, which also acts as a coreceptor for HIV on CD4⁺ T cells, can compromise the effectiveness of CCR5 inhibition (Feng et al., 1996), as HIV can exploit this alternative entry route. HIV exhibits coreceptor tropism, in which different viral variants show a preference for different coreceptors. R5-tropic HIV, which prefers

CCR5, is the most commonly transmitted variant and is associated with early infection. In contrast, X4-tropic HIV prefers CXCR4 and is linked to later-stage infection and accelerated, worsened disease (Connell et al., 2020). HIV switches from R5 to X4 tropism post-infection, making CCR5 blockade less effective at perturbing established disease. This switch to X4 tropism has recently been shown to increase in response to immune activation and blocking of CCR5 has also been shown to promote X4 tropism (Connell et al., 2020). Therefore, further understanding of these dynamics is required for future fine tuning of CCR5 targeted HIV therapy.

1.9 The Complexity of Chemokine Signalling

Despite a third of all drugs targeting GPCRs, and the well-established role of chemokines and their receptors in pathogenesis, effective clinical antagonists targeting this system remain relatively few. This scarcity is generally attributed to the inherent complexity of the chemokine network. Multiple chemokines can interact with the same receptor and individual receptors can be activated by numerous chemokines. Furthermore, leukocytes express unique patterns of chemokine receptors, with individual cells often expressing multiple receptors simultaneously (Table 1-1). In addition, different cell types secrete various chemokines, frequently in response to the same stimuli. This creates a highly dynamic and overlapping network, in which the effects of targeting one receptor may be diminished by signalling through alternative pathways. As a result, it has been supposed that chemokines and their receptors may act redundantly to compensate for one another (Dyer, 2020, Baggiolini, 1998). However, recent research challenges this view, as evidence suggests that receptor expression patterns are linked to specific functional roles. For example, CCR2⁺ monocytes display a distinct transcriptional profile compared to CCR1⁺CCR2⁺ monocytes. These cells upregulate neutrophilic genes, indicating differentiation toward specialised functions and recruitment via specific mechanisms in precise inflammatory contexts (Medina-Ruiz et al., 2024).

Additionally, both chemokines and their receptors can exist as multiple splice variants, which can have different functional properties. For example, CXCL12 forms six variants by alternative splicing, each with distinct functions (Yu et al., 2006). CCR2, CCR9, CXCR3 and CXCR4 can form isoforms which confer both different ligand affinity and downstream signalling outcomes (Hughes and Nibbs, 2018, Yu et al., 2000, Gupta and Pillarisetti, 1999, Lasagni et al., 2003, Wong et al., 1997). This receptor heterogeneity

complicates drug development, as targeting one isoform might not achieve the desired therapeutic effect or could lead to off-target effects or unexpected consequences.

Chemokine Receptor	Chemokine Ligand
CCR1	CCL 3, 5, 6 ^M , 7, 8, 9 ^M , 13 ^H , 14 ^H , 15 ^H , 16 ^H , 23 ^H
CCR2	CCL 2, 7, 8, 12 ^M , 13 ^H , 16 ^H
CCR3	CCL 5, 7, 11, 13 ^H , 14 ^H , 15 ^H , 24, 26, 28
CCR4	CCL 17, 22
CCR5	CCL 3, 4, 5, 7, 8, 11, 14 ^H , 16 ^H
CCR6	CCL 20
CCR7	CCL 19, 21
CCR8	CCL 1, 8, 16 ^H
CCR9	CCL25
CCR10	CCL 27, 28
CXCR1	CXCL 6, 7, 8 ^H
CXCR2	CXCL 1, 2 3, 5, 6, 7, 8 ^H
CXCR3	CXCL 4, 9, 10, 11 CXCL4L1
CXCR4	CXCL 12
CXCR5	CXCL 13
CXCR6	CXCL 16
CX3CR1	CX3CL 1, CCL26
XCR1	XCL 1, 2
ACKR1	CCL 2, 5, 7, 11, 13, 14, 17 CXCL 1, 2, 3, 5, 6, 8 ^H , 11
ACKR2	CCL 2, 3, 4, 5, 7, 8, 11, 12 ^M , 13 ^H , 14 ^H , 17, 22, 23 ^H , 24, CXCL 10
ACKR3	CXCL 11, 12
ACKR4	CCL 19, 21, 25, CXCL 13
ACKR5	CXCL 10, 12, 13, others to be confirmed

Table 1-1 Chemokine Receptors and Cognate Ligands

H signifies chemokine is only present in humans, M signifies chemokine is only found in mice, all other chemokines are present in both species (Hughes and Nibbs, 2018, Bachelierie et al., 2014).

Furthermore, chemokine activity is fine-tuned by post-translational modifications such as proteolysis, glycosylation, nitration and citrullination that must also be considered during drug design (Metzemaekers et al., 2017, Nguyen et al., 2017b, Janssens et al., 2017, Stone, 2017). For example, CXCL12 is truncated through enzymatic cleavage by dipeptidyl peptidase IV/CD26, which removes two N-terminal amino acids and results in altered CXCL12 function (Janssens et al., 2017). CD26-truncated CXCL12 does not induce CXCR4-dependent calcium signalling in monocytes and lymphocytes, thus failing to promote downstream chemotaxis of these cells. Similarly, chemotaxis of endothelial cells is diminished following CXCL12 modification by CD26 (Janssens et al., 2017). The truncated form of CXCL12 also loses the ability to recruit β -arrestin-2 to the CXCR4 on the cell membrane but retains the capacity to enhance β -arrestin recruitment to the cell membrane via ACKR3 (Janssens et al., 2017). In addition, CD26 enzymatically cleaves the N-termini of CXCL9, CXCL10, and CXCL11, all of which bind to and activate CXCR3 (Metzemaekers et al., 2017). Notably, recent findings indicate that GAGs can protect these chemokines from proteolytic truncation. It is not yet clear whether the GAGs physically prevent the enzyme from accessing the chemokine N-terminus or whether GAG-mediated oligomerisation of chemokines prevents proteolysis (Metzemaekers et al., 2017, Stone, 2017). Chemokine receptors also undergo post translational modifications which impact their chemokine binding specificity, strengthening arguments against a redundant signalling system (Stone, 2017).

Finally, the variation and specificity in both chemokine GAG binding affinity and the impact of GAG binding on chemokine function introduce further complexity to the chemokine system (Proudfoot et al., 2017, Dyer, 2020). For example, whilst CXCL4 has a particularly high affinity for GAGs, CCL3 and CCL4, the only acidic and thus negatively charged chemokines, have a much lower affinity for GAG binding (Proudfoot et al., 2003a). In addition, the sulfation state of GAGs can influence the binding affinity of certain chemokines (Dyer et al., 2016, Gray et al., 2023). Furthermore, evidence suggests that chemokine-GAG interactions confer specificity of chemokine-driven cell recruitment, for example, immobile, GAG-bound CXCL9 signalling leads to recruitment of CD4⁺ T cells. However, soluble CXCL9 signalling, which occurs in the absence of GAG binding, favours CD8⁺ T cell recruitment (Ridley et al., 2023). This demonstrates that GAG interactions can regulate chemokine function. Understanding of the role of chemokine-GAG binding beyond the presentation of chemokines on the endothelial glycocalyx during the leukocyte adhesion cascade is continuing to develop.

So far, only three drugs that target the chemokine system are approved for clinical use. Plerixafor (AMD3100), is a CXCR4 antagonist and is currently used clinically to mobilise stem cells from the bone marrow, aiding in stem cell collection for transplantation in non-Hodgkin's lymphoma (Devine et al., 2004). As previously mentioned, maraviroc is a small molecule CCR5 inhibitor used in the treatment of HIV infection. Finally, the monoclonal antibody mogamizulibab targets CCR4 and promotes cytotoxic T-cell mediated death during T-cell lymphoma (Ishii et al., 2010, Duvic et al., 2016). It should be noted that none of these drugs treat autoimmune or chronic inflammatory disease. However, there are currently a number of CXCR2 antagonists, often in combination with immune checkpoint inhibitors, undergoing clinical trials for the treatment of solid tumours (Lazennec et al., 2024). For example, AstraZeneca is conducting a Phase I/IIa trial (003346-36) of the CXCR2 inhibitor AZD5069 in combination with the PD-L1 monoclonal antibody durvalumab. This combination aims to target CXCR2-expressing myeloid-derived suppressor cells and neutrophils which promote tumorigenesis in hepatocellular carcinoma (Evans et al., 2023).

1.10 Atypical Chemokine Receptors

1.10.1 Overview

In addition, a further class of non-signalling 7 transmembrane chemokine receptors (ACKR1, ACKR2, ACKR3, ACKR4, and the newly discovered, ACKR5), atypical chemokine receptors (ACKRs) are expressed significantly by stromal cells (Nibbs and Graham, 2013). Whilst conventional chemokine receptors induce downstream signalling via DRYLAIV motif (2nd intracellular loop) interaction with G proteins, atypical receptors lack this motif. Thus, ACKRs do not induce the chemotaxis, ligand-induced receptor phosphorylation or calcium mobilisation associated with conventional chemokine receptor signalling. ACKRs 2-4 have homologous motifs at the N-terminal second intracellular loop (DKYLEIV (ACKR2), DRYLSIT (ACKR3) and DRYVAVT (ACKR4), whilst ACKR1 does not (Crawford and Volkman, 2023). Furthermore, excluding ACKR1, atypical chemokine receptors bind β -arrestin in response to ligand binding to trigger the internalisation of chemokines for degradation. Internalised chemokine is trafficked to endosomes, where it is released at low pH and sent to lysosomes for degradation. Meanwhile, the atypical receptor recycles to the cell surface ready for new rounds of ligand internalisation (Figure 1-2) (Nibbs and Graham, 2013).

Notably, ACKR1 regulates chemokine distribution through inducing transcytosis, a process in which molecules are transported in vesicles across endothelial cells to alternative sites (Bonecchi et al., 2016, Nibbs et al., 2003). Other ACKRs scavenge chemokines to regulate their distribution throughout tissues by forming concentration gradients essential for effective cell homing (ACKR3 and ACKR4) and the localisation and resolution of inflammation (ACKR1, ACKR2) (Bachelierie et al., 2014, Nibbs and Graham, 2013). Aside from resolving inflammation, examples of roles for atypical chemokine receptors in physiology and homeostasis are continuously unfolding and include roles in embryonic development, cardiovascular lymphatic vessel growth and motor function and behaviour (Lee et al., 2019, Schneider et al., 2014, Lee et al., 2014, Wilson et al., 2020, Fukuoka et al., 2024).

1.10.2 ACKR1

ACKR1 is a promiscuous receptor, previously named DARC, which binds CXCL8 in humans and CXCL1 and 2 in humans and mice (neutrophil attracting) as well as other proinflammatory CCL and CXCL chemokines. It is the only ACKR that binds many of both classes of ligands. ARCK1 does not bind homeostatic chemokines (Gardner et al., 2004) and is predominantly expressed in erythroid cells, endothelial cells lining postcapillary venules, and cerebellar Purkinje neurons (Thiriot et al., 2017).

In humans, ACKR1 exists in various isoforms, with the two most common alleles being FyA and FyB. The receptor has gained significant interest due to its function as a binding site for *Plasmodium vivax*, the parasite responsible for a form of malaria, as well as *Plasmodium knowlesi*, both of which target erythrocytes during infection. Notably, resistance to malaria has been observed in individuals carrying the FyES (Fy Erythrocyte Silent) or 'Duffy null' allele, where a single nucleotide polymorphism (SNP) disrupts the GATA1 transcription factor binding site within the ACKR1 gene promoter region. The presence of this SNP leads to the selective silencing of ACKR1 expression in erythrocytes, while preserving its expression and function in endothelial cells (Tournamille et al., 1995). Consequently, this prevents the invasion of *Plasmodium vivax* into red blood cells without compromising the crucial roles ACKR1 plays in endothelial cells (Tournamille et al., 1995). FyES allele individuals are healthy and do not have an increased risk of common disease (Hysong et al., 2025), although genome-wide association studies have shown a

strong association between the Duffy-null polymorphism in *ACKR1* and benign ethnic neutropenia, characterised by lower neutrophil counts in individuals of African descent (Reich et al., 2009). Recent findings have linked this neutropenia to the absence of ACKR1 expression on progenitor nucleated erythroid cells (NECs) in the bone marrow (Duchene et al., 2017). This absence is associated with a reduction in hematopoietic stem cells (HSCs) and significant transcriptional changes in the HSC population, particularly a shift towards myeloid lineage commitment. These changes are thought to result from decreased contact between nucleated erythroid cells and HSCs, leading to modified haematopoiesis that is associated with a neutropenia phenotype observed in individuals with the Duffy null allele (Duchene et al., 2017). This neutropenia is mostly asymptomatic. Additionally, ACKR1 on NECs has been shown to bind chemokines without internalising them, allowing for the retention of these signalling molecules in the environment. Therefore, ACKR1 may play a critical role in the development and maintenance of a niche within the bone marrow that supports HSCs and their differentiation (Duchene et al., 2017).

Endothelial ACKR1 has been suggested to mediate chemokine concentration gradients through active transcytosis of chemokines across barriers, i.e. to the lumen of blood vessels (Nibbs et al., 2003). This allows chemokine depletion at one site, whilst simultaneously directing the molecules to other microanatomical locations, promoting chemokine-driven leukocyte recruitment through redistribution rather than destruction (Nibbs et al., 2003, Bonecchi and Graham, 2016). In an ACKR1-deficient mouse model of endotoxemia, reduced neutrophil recruitment is associated with exacerbated lung and liver damage in response to LPS (Dawson et al., 2000). However, these mice do not show increased susceptibility to peritoneal infection by *Staphylococcus aureus*, even though this may be expected due to decreased neutrophil recruitment. This observation has led some to argue that endothelial ACKR1 is functionally redundant (Luo et al., 2000). Endothelial ACKR1 also plays contradictory roles in cancer, where it demonstrates both pathogenic and protective effects in a context-dependent manner. For example, ACKR1 deficiency in mouse models of prostate cancer leads to reduced tumour density and increased tumour necrosis, correlating with increased levels of tumoral CXCL1 and CXCL2 (Shen et al., 2006). In contrast, ACKR1 is associated with recurrence free survival in human breast cancer (Li et al., 2018, Jenkins et al., 2019).

1.10.3 ACKR2

ACKR2, previously named D6, is highly promiscuous and can recognise and internalise various inflammatory chemokines, which signal via interaction with CCRs 1-5 (Figure 1-3). Thus, ACKR2 acts to reciprocally coordinate the signalling axes of these inflammatory chemokine receptors to localise and resolve inflammation (Graham et al., 2012). ACKR2 was previously thought to exclusively bind CCL chemokines (Fra et al., 2003), but it is now established that the receptor also interacts with CXCL10 (Fra et al., 2003, Chevigné et al., 2021). ACKR2 is expressed most strikingly on trophoblast cells but is also expressed across LECs, pulmonary vascular endothelial cells and some leukocyte subsets, including splenic B cells and peritoneal B1 cells (Hansell et al., 2011, Nibbs et al., 2001, Nibbs et al., 1997, Hansell et al., 2020).

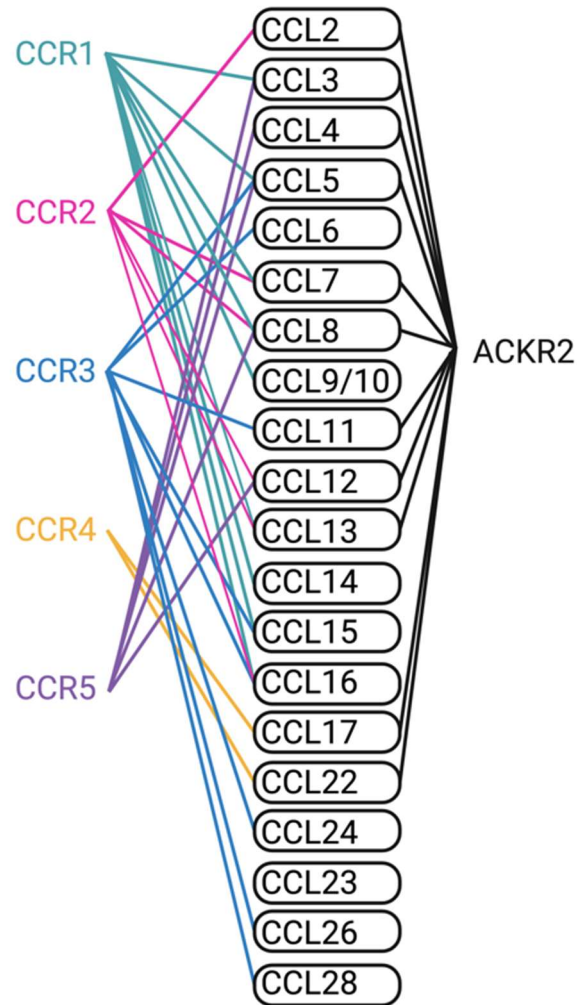


Figure 1-3 Chemokine Receptor Interactions

The interactions between CCR1 (green), 2 (pink), 3 (blue), 4 (yellow), 5 (purple), and ACKR2 (black) with cognate ligands (circled). Colour of lines correspond with receptors to aid visualisation. Note complexity of system, various CC-receptors interact with the same ligands. Created using BioRender.com, adapted from a Figure by Wilson et al., 2020 (Wilson et al., 2020).

ACKR2 is recycled back to the cell surface by endosomal trafficking as chemokines are degraded and this occurs at a differential rate depending on the specific ligand bound to the receptor (different chemokines may exert varying impacts on receptor recycling dynamics and scavenging efficiency) (Savino et al., 2009). Furthermore, it has been reported that ACKR2 surface expression is enhanced in response to ligand binding by activating various pathways dependent on β -arrestin (Galliera et al., 2004). The importance of ACKR2 in mediating leukocyte migration is underpinned as ACKR2 deficiency leads to increased leukocyte accumulation within lymphatic vessels during inflammation (Lee et al., 2019). ACKR2 has been shown to scavenge chemokines at lymphatic endothelial surfaces (Lee et al., 2019, McKimmie et al., 2013) and thus enhance the interaction between CCR7⁺ DCs and CCL21, providing insight into the mechanism for the selective presentation of CCL21 across lymphatic vessels in the inflammatory context where many other chemokines are present (McKimmie et al., 2013).

Furthermore, *in vitro*, ACKR2 on LECs is upregulated in response to inflammatory mediators such as IFN- γ , type -1 interferons and IL-6 (McKimmie et al., 2013). ACKR2 deficiency in challenged mice is associated with increased inflammation across normal ACKR2 expressing tissues and responses to cutaneous inflammatory stimuli (Jamieson et al., 2005) and allergy models are also enhanced (de la Torre et al., 2005, Whitehead et al., 2007). The receptor has also been implicated in the pathogenesis of inflammatory diseases, including psoriasis (Singh et al., 2012), as well as in the progression of tumour formation (Nibbs et al., 2007), for example in thyroid and breast cancer (Sjoberg et al., 2019, Pacifico et al., 2017).

1.10.4 The Role of ACKR2 in the Placenta

Whilst conventional chemokine receptors are expressed across vertebrates, ACKR2 is expressed exclusively by mammals, with particularly high expression on the surface of trophoblast cells (Lee et al., 2019). Trophoblast cells surround the inner cell mass of the blastocyst during early embryogenesis. Trophoblast cells differentiate to form part of the placenta, the first organ to form during pregnancy (Rossant and Cross, 2001). The placenta is the interface between mother and foetus and provides simultaneous nutrient exchange and protection against maternal immune rejection of the partially allogeneic foetus (foetus carries paternal histocompatibility antigens). Specialised immune cells are selectively recruited to the placenta and play a crucial role in the positioning of trophoblastic cells during placental development. However, uncontrolled inflammation at the placenta is

associated with pregnancy complications such as preeclampsia, and preterm birth (Michalczyk et al., 2020, Quinn et al., 2020). Furthermore, alterations in immune cell distribution at the placenta, such as decreases in maternal decidual NK cells or uterine DCs, have been shown to result in loss of pregnancy (Hanna et al., 2006, Plaks et al., 2008).

ACKR2 is expressed in human placenta on the surface of extravillous trophoblast cells which protrude into maternal blood, as well as on syncytiotrophoblast cells, potentially to regulate leukocyte infiltration, and thus inflammation at the site (Martinez de la Torre et al., 2007). ACKR3 and 4 are also expressed by placenta and ACKR3 deficiency in mice is associated with an increase in leukocytes at the interface and subsequent consequences on normal pregnancy and offspring development (Quinn et al., 2020, Fukuoka et al., 2024)

The role of ACKR2 during ontogeny has been demonstrated as ACKR2 in the mouse placenta increases the survival of embryos transferred into allogeneic WT mice (Madigan et al., 2010). WT, heterozygous ACKR2^{+/-} and homozygous ACKR2^{-/-} (ACKR2-deficient) embryos were transferred into allogenic WT pseudo-pregnant female mice. Embryonic ACKR2 deficiency was associated with increased abnormal fetuses and reabsorption plaques (Madigan et al., 2010). This was suggested to be related to an accumulation of leukocytes at the placenta in response to increased levels of inflammatory chemokines, leading to immune attack of the allogenic foetus. In addition, ACKR2 deficiency in both mother and foetus is also associated with increased foetal loss in response to inflammation induced by LPS or anti-phospholipid antibodies from patients with anti-phospholipid syndrome, as chemokine levels and leukocyte infiltration at the placenta cannot be controlled and resolved by the receptor (Teoh et al., 2014, Martinez de la Torre et al., 2007).

Furthermore, ACKR2 deficiency in DB/AJ1 mice leads to changes in the structural development of the placenta. For example, ACKR2 deficiency decreases the percentage volume of the labyrinth zone, the site in which maternal and foetal blood circulate in proximity in the mouse placenta. ACKR2 deficiency also increases the percentage volume of the chorionic plate (situated beneath the labyrinthine zone) (Teoh et al., 2014). This therefore emphasises a link between leukocyte infiltration and successful placental growth. A recent study has demonstrated a link between reduced ACKR2 mRNA expression and preeclampsia in human patients with early onset preeclampsia or preterm birth, whilst no association was found in those with late onset preeclampsia (Yan et al., 2020). In contrast,

investigation by Tersigni et al., did not observe reduced levels of ACKR2 in preeclampsia cases; however, ACKR2 did appear to be functionally impaired in preeclampsia (Tersigni et al., 2016). Therefore, data regarding this association in humans remains conflicted (Yan et al., 2020, Cho et al., 2015, Tersigni et al., 2016).

Ultimately, ACKR2 appears to play a clear role in protecting the developing foetus during both systemic and acute maternal inflammation. ACKR2 at this site has been suggested to reduce leukocyte infiltration to maintain maternal immune tolerance during pregnancy (Madigan et al., 2010, Teoh et al., 2014). However, recent findings have extended the role of ACKR2 in the placenta from protection during maternal inflammation to the regulation of chemokine compartmentalisation, controlling the transfer of chemokines from mother to embryo (Lee et al., 2019). This is visualised by Figure 1-4. Injection of human CCL2 and CCL7 into pregnant mice led to subsequent detection of these ligands in embryos, with considerably higher levels of the chemokines detected in ACKR2-deficient embryos, highlighting both that the ligands can cross the trophoblast and that they are prevented from doing so by ACKR2 (Figure 1-4).

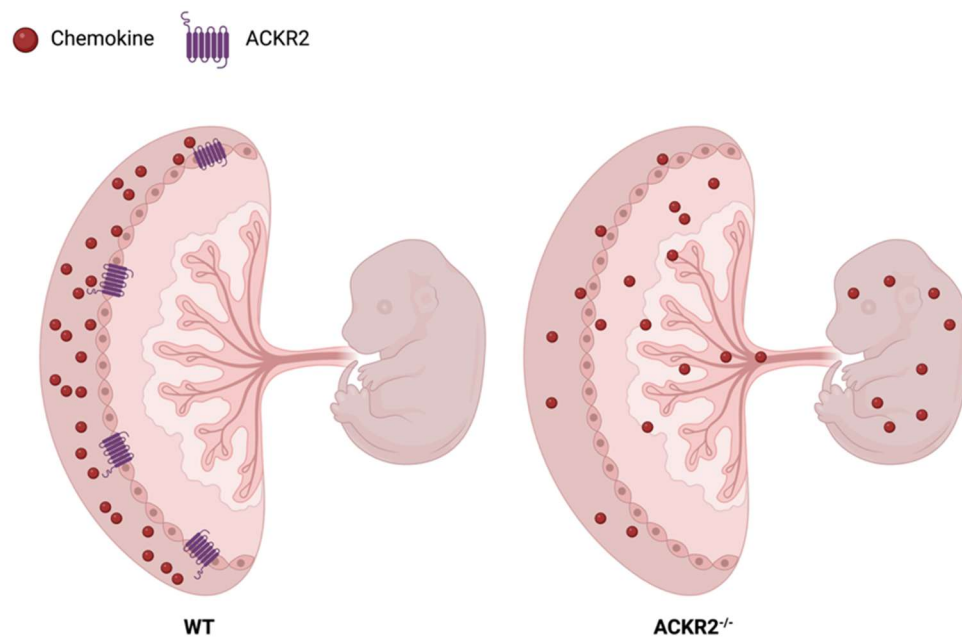


Figure 1-4 Chemokine Compartmentalisation at the Placenta-Embryo Interface

In the wild type (WT), ACKR2 (purple) expressed in the junction zone of the placenta regulates the passage of ACKR2 chemokine ligands (red) from the maternal circulation to the circulation of the developing embryo. This is dysregulated in ARCK2-deficient (ACKR2^{-/-}) mice, leading to maternal chemokine in embryo circulation (Lee et al., 2019). Created using BioRender.com.

Furthermore, ACKR2 deficiency is associated with increased levels of CCL2 in embryonic circulation and subsequent changes in leukocyte populations within embryonic tissues. A novel intermediate macrophage population (F4/80^{int}, CD11b⁺) found in embryonic skin is significantly reduced in number in ACKR2 deficient mice vs wild type (WT) mice (Lee et al., 2019). This reduction has been linked to changes in localised CCL2 concentration gradients essential for the recruitment of this population to the skin. The intermediate macrophages are also reduced in the skin in CCR2-deficient mice during mid gestation, highlighting that these cells are recruited to the skin by CCL2-CCR2 signalling (Lee et al., 2019). Thus, ACKR2 in the placenta plays a significant part in homeostasis during embryogenesis as well as in previously described cases of maternal inflammation (Lee et al., 2019). Furthermore, ACKR2 deficiency has been shown to impact macrophage dynamics in embryonic tissues, as a specific F4/80^{int} macrophage population is reduced in numbers in the E14.5 skin of ACKR2-deficient embryos (Lee et al., 2019).

1.10.5 ACKR2 and Macrophage Distribution and Function

Macrophages express high levels of chemokine receptors CCR1, 2 and 5 (Medina-Ruiz et al., 2022). ACKR2 has been shown to regulate macrophage numbers in a variety of contexts and a link between ACKR2 and CCR1-dependent/CCR2-dependent macrophage migration and lymphatic development has been established (Lee et al., 2014, Wilson et al., 2020). Lymphatic vessel density is increased in ACKR2-deficient embryonic skin as higher levels of CCL2 leads to an increase in CCR2-dependent macrophage migration to the site, bringing the leukocytes much closer to lymphatic endothelium (Lee et al., 2014). CCR2⁺ macrophages produce pro-lymphangiogenic cytokines, leading to accelerated lymphatic development and increased vessel density (Lee et al., 2014). In contrast, an inverse phenotype is observed in CCR2-deficient mice as reduced macrophage infiltration leads to the development of a less dense lymphatic vascular network in inflammatory conditions. Furthermore, a similar relationship between ACKR2 expressing fibroblasts and CCR1⁺ CD206⁺ macrophages and lymphatic development has been elucidated as ACKR2 deficiency increases ductal branching during pubertal mammary gland development (Wilson et al., 2020). In this context, increased CCL7 in the absence of ACKR2 led to greater numbers of CD206⁺ macrophages in the pubertal mammary gland which in turn accelerated branching. Importantly, 17 β -oestradiol was shown to upregulate CCR1 on CD206⁺ pubertal mammary gland macrophages, uncovering a mechanism for mammary gland development in which oestradiol increases CCR1 expression on CD206⁺

macrophages which are localised to the mammary gland ductal epithelium by CCL7 in a tightly regulated manner by stromal ACKR2 (Wilson et al., 2020).

1.10.6 ACKR3

ACKR3, previously referred to as CXCR7/RDC1, has crucial roles in both homeostasis and the resolution of inflammation. ACKR3 is expressed across many embryonic and adult tissues including heart, lung, brain, kidney and bone marrow (Gerrits et al., 2008, Burns et al., 2006, Lasagni et al., 2003, Mazzinghi et al., 2008). The receptor internalises endogenous CXCL12 and CXCL11 but binds CXCL11 with considerably lower affinity than CXCL12 (20-fold) (Burns et al., 2006). ACKR3 has also been reported to bind non-chemokine ligands including chemokine-like molecule macrophage inhibitory factor (MIF) and opioid peptides such as adrenomedullin (Alampour-Rajabi et al., 2015, Comerford and McColl, 2024, Meyrath et al., 2020). ACKR3 is found on the surface of non-immune cells such as trophoblast cells (Fukuoka et al., 2024) and endothelial cells (Ngamsri et al., 2017) as well as leukocyte subsets including marginal zone and memory B cells (Radice et al., 2020, Humpert et al., 2012, Humpert et al., 2014) and myeloid cells (Chatterjee et al., 2015).

ACKR3 is critical for normal cardiac development and ACKR3 deficiency is associated with defective and thick semilunar valves and cardiomyocyte hyperplasia leading to an enlarged heart (Gerrits et al., 2008, Sierro et al., 2007, Yu et al., 2011). ACKR3 is also required for neural stem cell survival (Zhu et al., 2012) as well as adhesion and positioning of interneurons to the cortical plate during brain development (Wang et al., 2011, Sanchez-Alcaniz et al., 2011). In addition, ACKR3 also functions at barrier tissues, this is evidenced as ACKR3 contributes to the control of leukocyte migration across the blood brain barrier (Cruz-Orengo et al., 2011). ACKR3 upregulation during experimental autoimmune encephalomyelitis, a model for MS, is required for leukocyte infiltration to the brain, and blockade of ACKR3 leads to reduced pathogenesis (Cruz-Orengo et al., 2011).

Furthermore, chemokine compartmentalisation on the maternal side of the placenta by ACKRs has also been demonstrated by blockade of ACKR3 during pregnancy. Mouse pups born to females injected with the ACKR3 blocker, CCX771, had elevated CXCL12 in plasma and significant anaemia due to defective haematopoiesis, leading to pups appearing visibly paler and smaller than those born to non-CCX771 treated mothers. In addition, pups born to CCX771 treated females showed an impaired inflammatory response in

response to intraperitoneal injection of sterile *E. coli* particles, as peritoneal immune cells from the offspring of CCX771 treated females showed reduced uptake of the *E. coli* particles in comparison with vehicle treated offspring (Fukuoka et al., 2024).

In addition to leukocyte recruitment, ACKR3 has unique roles in promoting cellular adhesion and survival, and therefore, unlike other atypical chemokine receptors, ACKR3 is significantly upregulated in various solid tumours (Neves et al., 2019). These include breast cancers, malignant lung carcinoma, and brain cancers, where ACKR3 contributes to tumour growth, angiogenesis, metastasis as well as immune evasion and tumour survival (Neves et al., 2019). Small molecule ACKR3 antagonists, such as CCX754 and CCX771 have been shown to reduce tumorigenesis in mouse models (Burns et al., 2006). However, despite some promising results in animal studies, no small molecule drugs targeting ACKR3 are currently used therapeutically for cancer treatment.

1.10.7 ACKR4

ACKR4 is expressed across lymphatic endothelial cells, as well as lung and gut epithelial cells, where it contributes to mucosal immunity (Bonecchi and Graham, 2016, Gosling et al., 2000). The role of ACKR4 has been best defined in the lymph node, where it carves a CCL21 gradient for the guidance of CCR7 expressing DCs from the subcapsular sinus to the T cell zone for antigen presentation (Ulvmar et al., 2014). ACKR4 also internalises the other CCR7 ligand, CCL19, and does not exhibit ligand-induced desensitisation. Research has demonstrated that ACKR4 can continuously internalise and degrade CCL19 over an extended period (Comerford et al., 2006). In addition, ACKR4 also binds CCL25 (Meyrath et al., 2021). CCL25 signals through CCR9, a homeostatic chemokine receptor which homes T-cells to the gut mucosa (Stenstad et al., 2006). ACKR4 can sequester chemokines independently of β -arrestin interaction (Matti et al., 2020b). Furthermore, in contrast to ACKR2 and ACKR3, the recruitment of β -arrestin by ACKR4 following chemokine binding does not trigger downstream signal transduction (Bonecchi and Graham, 2016). Recently, CCL20 has been added to the accepted repertoire of ACKR4 ligands (Matti et al., 2020a). CCL20 binds CCR6, which for example, homes Th17 cells to the intestine and Peyer's patches (Wang et al., 2009). CCL22 has also been identified as a potential partial agonist of ACKR4 (Matti et al., 2020a, Meyrath et al., 2021).

In addition, hematopoietic ACKR4 deficiency has recently been shown to lead to dysregulated migration of self-antigen-bound B cells to splenic follicular zones in response to increased CCL19 and CCL21. This results in early and increased preferential expansion

of plasma blasts in comparison with germinal centre B cells (Kara et al., 2018). Thus, it appears that B cell expression of CCR7 and ACKR4 plays a role in the regulation of migration to splenic follicular zones during earlier stages of T cell-dependent humoral immunity and that this positioning acts to modulate differentiation fate, tipping the balance towards an early plasma blast fate (Kara et al., 2018).

1.10.8 ACKR5

Finally, there has been a recent addition to the ACKR family, GPR182 (ACKR5), which is expressed on lymphatic endothelial and microvascular cells and contributes to hematopoietic stem cell homeostasis (Le Mercier et al., 2021, Schmid et al., 2018). Various studies have aimed to define the ACKR5 ligands, mostly using competitive binding assays. The first chemokines found to bind ACKR5 were human and mouse CXCL12, CXCL13 and CXCL10 and ACKR5-deficient mice demonstrated increased plasma concentrations of all three chemokines, indicating its role as a chemokine scavenging receptor (Le Mercier et al., 2021). The relative binding capacities of other members of the chemokine family have since been inferred. This was assessed based on their ability to displace fluorescently labelled (AlexaFluor-647) human CXCL10 (50nM) from ACKR5 at low (250 nM) to high (1 μ M) ligand concentrations and was measured by flow cytometry (Bonnavion et al., 2023). Both mouse and human CXCL9, CXCL14, CCL11, CCL25, CCL28 as well as human only CXCL3, CXCL11, CXCL16, CCL1, CCL19, CCL26, XCL1 and mouse only CCL22, CCL24, and CCL27 displaced ≥ 50 % of ACKR5 bound CXCL10 at ligand concentrations of 250 nM (Bonnavion et al., 2023). Importantly, ACKR5 may therefore be the first known receptor for CXCL14. In addition, levels of CCL22, CCL24, CCL25 and CCL27 increased in the absence of ACKR5, further supporting the role of ACKR5 as a chemokine scavenger (Bonnavion et al., 2023). Although ACKR5 has been implicated in hematopoietic stem cell homeostasis and the development of splenic marginal zones, its precise *in vivo* function remains to be determined (Le Mercier et al., 2021, Melgrati et al., 2023).

1.11 Aims of the Project

Chemokine signalling is crucial for coordinating cell migration *in vivo*, both during the inflammatory response and in a variety of homeostatic contexts. The finely tuned system is regulated by atypical chemokine receptors, which internalise chemokines from the

extracellular environment and contribute to the establishment and maintenance of chemokine concentration gradients that guide cell migration, as well as to the resolution of inflammation. Direct consequences of disrupted chemokine signalling during embryonic development in the context of ACKR2 and ACKR3 deficiency have previously been demonstrated. However, a comprehensive, spatial and temporal map of chemokine expression throughout embryogenesis is lacking.

It was hypothesised that chemokines would exhibit spatially and temporally restricted expression during embryogenesis, consistent with highly specific roles in tissue patterning and cell migration. The first aim of this project is therefore to systematically map chemokine expression at multiple developmental time points across a range of tissues. This will improve our understanding of the extent to which chemokine signalling may contribute to developmental processes and tissue patterning events in the embryo and produce a resource for the scientific community.

In addition, this thesis will explore whether specific macrophage populations in adult tissues are altered in ACKR2-deficient mice. This approach will help uncover broader roles for ACKR2 in the regulation of myeloid cell populations. Given the well-established roles of macrophages in tissue patterning, immune surveillance, and the orchestration of inflammatory and repair responses, alterations in macrophage number or phenotype are likely to have significant implications for tissue homeostasis and immune competence. It was hypothesised that the loss of ACKR2 would lead to dysregulated chemokine levels, resulting in altered macrophage number and positioning in adult tissues, and that this would impair the immune response to bacterial infection.

2 Materials and Methods

2.1 Mouse Models

Animals were bred at the pathogen-free Wolfson Research Facility (WRF) within the University of Glasgow. All animal experiments conformed to the animal care and welfare protocols approved by the University of Glasgow and were carried out under the UK Home Office Project Licences held by Gerard Graham (PP6655603 and PP1443954). Mice were euthanised by carbon dioxide asphyxiation or cervical dislocation. Both males and females between 6-10 weeks were used; however, across each experiment, mice were matched for age, sex and background.

2.1.1 Wild Type Mice

Wild type C57BL/6J mice were either purchased from Charles River Laboratories or were CD45.1 C57BL/6J mice bred in-house at the WRF.

2.1.2 ACKR2 Knock Out Mice

ACKR2-deficient mice were originally generated on a 129/Sv background and subsequently backcrossed with C57BL/6J WT mice. Heterozygous 129–C57BL/6 mice were then crossed to generate WT and ACKR2-deficient animals (Jamieson et al., 2005).

2.1.3 iCCR-Reporter Mice

iCCR reporter (iREP) mice were generated on a C57BL/6NTac background, through the random integration of an engineered BAC (bacterial artificial chromosome) into the mouse genome. The BAC encodes the iCCR locus, in which the CCR genes have been replaced with genes encoding non-overlapping fluorescent reporter proteins; *CCR1* with Clover, *CCR2* with mRuby2, *CCR3* with mTagBFP2, and *CCR5* with iRFP682 (Medina-Ruiz et al., 2022). Embryos were generated via hemi-hemi crosses between heterozygous iCCR reporter mice to produce litters containing both reporter and WT genotypes. WT littermates served as controls and were matched for age, sex, and genetic background.

2.2 Embryo Age and Sex Determination

2.2.1 Phenotyping Age

Females in timed pregnancies were observed for the presence of plugs and embryo age was calculated from the observed plug date, which was noted as embryonic day 0.5 (E0.5) and confirmed by assessing size and developmental indicators.

2.2.2 Sex Genotyping

Genotyping of embryos for sex (*Rbm31x/y* genes) was carried out by PCR using the forward primer; CACCTTAAGAACAAGCCAATACA, and the reverse primer GGCTTGTCTGAAAACATTTGG (Tunster, 2017). DNA was isolated from the embryo tail by incubation with DNA lysis buffer (5 mM EDTA (Invitrogen, 15575020), 0.1 mg/ml Proteinase K (Invitrogen, 25530-015), 0.2 % w/v SDS, 100 mM Tris.HCl (pH7.5), 200 mM NaCl) at 55 °C and 1000 rpm for 5+ h. Incubation was followed by heating for 5 min at 95 °C. 1 ml of RNase free water (Qiagen, 129115) was then added, and samples were centrifuged for 5 min at 13000 rpm. DNA concentration was analysed using a NanoDrop spectrometer (DeNovix, DS-11+). Simplex PCR was carried out using Phusion Polymerase (NEB, M0530S) with an annealing temperature of 62 °C and extension time of 5 seconds. Genotype was then confirmed by DNA-gel electrophoresis (1.8% agarose gel with SYBR Safe DNA Gel Stain (Invitrogen, S33102), ran at 110 V) product size was measured against a 100 bp DNA ladder (NEB, N3231S). The presence of two bands corresponded with male embryo DNA, and the presence of a single band only indicated female embryo DNA (Figure 2-1). Female embryo tissue only was selected for consistency.

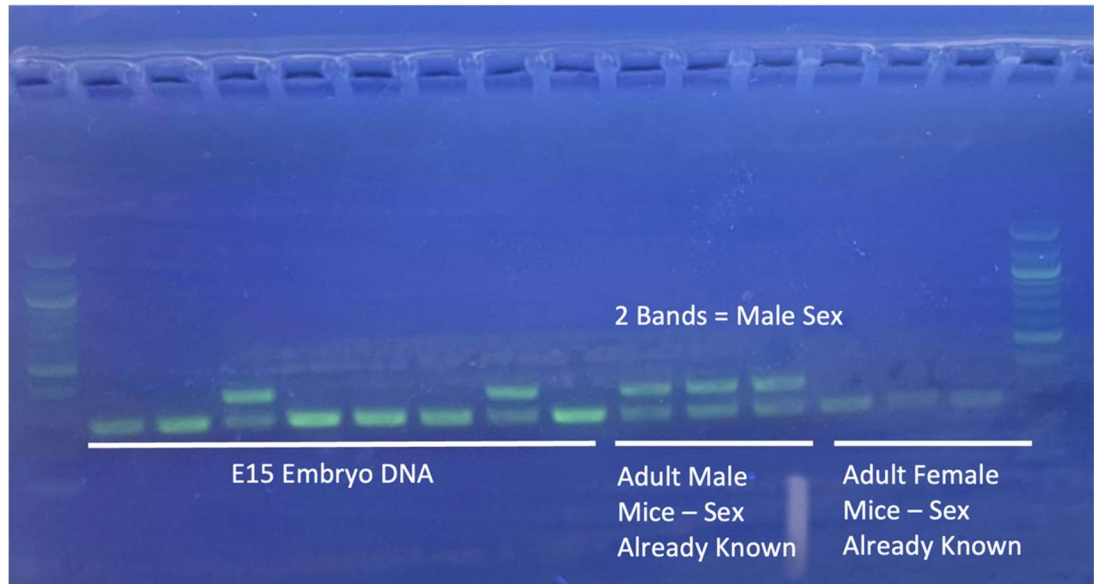


Figure 2-1 Representative Gel: Sex Genotyping E15 Mouse Embryos by Simplex PCR

2 bands indicate male sex; one band indicates female sex. Adult mice of known sex were used as positive controls (Tunster, 2017).

2.3 Transcriptional Analysis

2.3.1 RNA Isolation and Purification

Embryo tissues were harvested at specific time points (E15-E18.5). Tissue (heart, lung, skin, liver and spleen) was obtained and placed in 20 µl of RNAlater (Invitrogen, AM7020) and frozen at -80 °C for subsequent isolation and purification. Samples were homogenised in 1 ml TriZOL (Ambion by life technologies, 15596026) per 50-100 mg of tissue using a TissueLyser (Qiagen) (1 min, 25 Hz x2, 1x 5 mm stainless steel beads) (Qiagen, 69989). Beads were removed and 0.2 ml of chloroform per 1 ml of TRIzol was added and mixed thoroughly to the samples by shaking. Samples were incubated for 2-3 min and then centrifuged at 12000 x g, 4 °C, for 15 min to separate the mixture of aqueous and organic layers. The upper aqueous layer was collected, and one volume of 70 % ethanol was added to each volume of cell homogenate. The samples were vortexed to disperse any visible precipitate following the addition of ethanol. For embryo tissues, RNA purification was then achieved using the Invitrogen PureLink RNA Mini Kit (Invitrogen, 12183018A) according to manufacturer's guidelines and eluted into 30 µl nuclease-free water and stored at -80 °C.

For isolated adult lung macrophages RNA was isolated and purified from cells in RLT buffer using the RNAeasy micro kit (Qiagen, 74004) according to manufacturer's guidelines and eluted into 15 µl nuclease free water (RNAeasy micro kit Qiagen, 74004) and stored at -80 °C. On-column DNase digestion using DNase (RNase free) (Qiagen, 79254) removed DNA according to manufactures guidelines. RNA concentration was measured using a NanoDrop spectrometer (DeNovix, DS-11+).

2.3.2 RNA Quantification

cDNA conversion was undertaken using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4374966) with RNase inhibitor. Where possible, this conversion was undertaken on the same day as RNA extraction. PCR cycle length and temperature settings as per kit protocol. DNA concentration was measured using a NanoDrop spectrometer (DeNovix, DS-11+).

Customised pre-loaded 384-well qPCR arrays (TaqMan Array, ThermoFisher) were run on a QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems enabling 384 simultaneous real-time PCR (qPCR) reactions. Minus reverse transcriptase and no template control plates were also run.

Ct values were analysed using the Δ Ct method, normalised to *GAPDH*, which was selected as the reference gene as it is reported to exhibit stable, constitutive expression across embryonic time points (Hildyard et al., 2021). Expression data were presented as $2^{-\Delta Ct}$ values which represent the difference between the recorded Ct values of the target gene in comparison with the housekeeping gene (i.e. $Ct_GAPDH - Ct_Target\ Gene$). Values were transformed to convert the raw Ct difference into values of gene expression in which higher values indicate greater transcript abundance. To determine relative changes in expression between developmental time points, the $\Delta\Delta$ Ct method was applied. Δ Ct values were normalised to a reference time point (E15 or E17.5, as appropriate). Fold change was calculated as: $\text{mean } 2^{(-\Delta Ct)} \text{ of time point} / \text{mean } 2^{(-\Delta Ct)} \text{ of reference time point}$. $\Delta\Delta$ Ct analysis was restricted to gene targets that exceeded an arbitrarily defined threshold of $2^{-\Delta Ct} > 0.001$ at one or more time points. This threshold is equivalent to a Ct difference of approximately 10 cycles and was applied to exclude lowly expressed genes from statistical analysis, and aiding interpretation of the dataset. Statistical analysis was only performed on genes that showed a fold change of ≥ 2 (upregulation) or ≤ 0.5 (downregulation) relative to E15.0 or E17.5 as this was considered a substantial enough change in expression to report. Statistical analysis was also limited to genes where expression ($2^{-\Delta Ct}$) was ≥ 0.001 for at least one time point.

2.4 Proteomic Analysis

2.4.1 Protein Isolation

2.4.1.1 Embryo Tissue

Embryo tissue was weighed and 1:20 weight to volume of Cell Lysis Buffer 2 (R&D systems, 895347), containing HALT protease inhibitors (Thermo Fisher Scientific, 87786) was added. Tissue was chopped finely, and 2x 5 mm stainless steel beads (Qiagen, 69989) were added for homogenisation of tissue using Qiagen Tissue Lyser LT (Qiagen, 85600). Samples were then agitated at room temperature (RT) for 30 min. Beads were removed and

samples were centrifuged at 10,000 x g for 20 min at 4 °C. Supernatant was collected and stored at -80 °C.

2.4.1.2 Peritoneal Lavage

Peritoneal lavage fluid was centrifuged at 400 x g for 5 min at 4°C and the supernatant was collected for direct downstream proteomic analysis. The cell pellet was then lysed using Cell Lysis Buffer 2 (R&D systems, 895347), containing HALT protease inhibitors (Thermo Fisher Scientific, 87786) for analysis of the cell lysate. Samples were stored at -80 °C.

2.4.1.3 Peritoneal Wall

Peritoneal walls were harvested and snap frozen in liquid nitrogen. A pestle and mortar were used to crush frozen tissue. Tissue was added to dH₂O containing protease inhibitors (Pierce Protease inhibitors (Thermo Fisher Scientific, A32963) and stored at -80 °C.

2.4.2 Protein quantification

Target protein concentration for all tissues was determined using a customised Magnetic Luminex Multiplex assay (R&D Systems), according to manufacturers' instructions, and read using a Bio-Rad Luminex-100 machine. Data was normalised to total protein concentration of tissue samples, determined by BCA assay (Pierce, 23225).

Many of the values obtained were extrapolated from below the lower limit of quantification. This occurs when the median fluorescence intensity (MFI) value recorded for a certain target protein is lower than the MFI observed for the lowest concentration of standard. Extrapolated values are considered unreliable for precise quantification or statistical analysis and should be interpreted with caution. However, they can be used for qualitative analysis of expression patterns (Breen et al., 2016). This primarily affected embryonic E15 samples and may have been due to very low chemokine concentration in tissues, insufficient sample loading concentration, or other technical issues. Analytes with MFI values too far below the lower limit of detection could not be extrapolated and were therefore considered not detected, and these are not represented in the graphs shown throughout this study.

2.5 Tissue Processing for Flow Cytometry

2.5.1 Foetal Tissues

Following the sacrifice of the pregnant female, embryos were culled by decapitation and placed in 2 μ M UltraPure EDTA (Invitrogen, 15575020) in DPBS (Gibco, 10270-106) for dissection. Embryos were dissected using fine spring bow scissors and forceps under an Olympus SZ3 stereomicroscope with transmitted illumination attachment.

2.5.1.1 Spleen

Embryo spleen was removed and pulled up and down through a 23 G needle using 2 ml syringes until physically broken down. Spleen samples were then mashed with a plunger and passed through 70 μ m EASYstrainer mesh filters (Greiner Bio-One, 542070). The mesh filters were washed with 5 ml EDTA (Invitrogen, 15575020) in DPBS (Gibco, 10270-106). Samples were then centrifuged at 400 x g for 5 min. Supernatant was discarded, and the cell pellet was treated with 0.5 ml RBC lysis buffer (Invitrogen, 00-4333-57) at RT for 1 min. 5 ml of EDTA/DPBS was added to end the lysis reaction and the cell pellet was centrifuged at 400 x g for 5 min. Supernatant was discarded, and pellet was resuspended in 500 μ l of FACS buffer ((DPBS (Gibco, 10270-106), 1 % foetal bovine serum (FBS) (Gibco, 10270-106) and 2 mM UltraPure EDTA (Invitrogen, 15575020) and 200 μ l was transferred to a round bottom 96-well plate for staining.

2.5.1.2 Lung, Liver and Placenta

Lung, liver and placenta were removed and chopped into small pieces using fine scissors before incubation in 1 ml HBSS (Ca^{2+} Mg^{2+}) containing 800 μ g/ml dispase-II (Gibco, 17105041), 200 μ g/ml collagenase-P (Roche, 11213857001) and 100 μ g/ml DNase-I (Invitrogen, 18068-015) at 37 °C for 1 h at 600 rpm using a temperature-controlled shaker (Eppendorf). After 45 min tubes were inverted and replaced. 10 μ l of FACS buffer containing FBS was added to samples to stop the enzyme reaction. Tissue digests were then mashed with a plunger and passed through 70 μ m EASYstrainer mesh filters (Greiner Bio-One, 542070). 100 μ m cell strainers (Greiner Bio-One, 542000) were used for placenta to obtain trophoblast cells. The mesh filters were washed with 5 ml EDTA/DPBS. 5 ml of EDTA/DPBS was added to end the lysis reaction and the cell pellet was centrifuged at 400 x g for 5 min. Supernatant was discarded, and pellet was resuspended in

500 µl of FACS buffer and 200 µl was transferred to a round bottom 96-well plate for staining.

2.5.1.3 Skin and Heart

Embryonic skin and heart were removed and chopped into small pieces before incubation in 1 ml of HBSS containing 1 mg/ml collagenase-D (Roche, 66505632) 500 µg/ml dispase-II (Gibco, 17105041), and 100 µg/ml DNase-I (Invitrogen, 18068-015) at 37 °C for 1 h at 1000 rpm using a temperature-controlled shaker (Eppendorf). Tissue digests were then mashed with a plunger and passed through 70 µm EASYstrainer mesh filters (Greiner Bio-One, 542070). The mesh filters were washed with 5 ml EDTA/DPBS. 5 ml of EDTA/DPBS was added to end the lysis reaction and the cell pellet was centrifuged at 400 x g for 5 min. Supernatant was discarded, and pellet was resuspended in 500 µl of FACS buffer and 200 µl was transferred to a round bottom 96-well plate for staining.

2.5.2 Adult Tissues

2.5.2.1 Peritoneal Lavage

Mice were sacrificed by terminal anaesthesia (CO₂) and severing of the femoral artery confirmed death. A peritoneal lavage (PL) was carried out using 10 ml DPBS (10 ml syringe, 25 G needle). Samples were centrifuged at 400 x g for 5 min, supernatant was discarded, and cells were resuspended in 200 µl DPBS. Samples should not require RBC lysis. If samples are very red, blood contamination is likely to hamper analysis of lavage, and any pink/red pellets were discarded.

2.5.2.2 Peritoneal Wall

The entire front of the peritoneal wall was removed and placed in 1 ml HBSS. The wall was then chopped into small pieces and incubated with 35 µl of Liberase (Roche, 05401020001) at 37 °C and 1000 rpm for 1 h. Digested walls were passed through 70 µm EASYstrainer mesh filters (Greiner Bio-One, 542070). Filters were washed with 5 ml DPBS and isolated cells were centrifuged at 400 x g for 5 min. Supernatant was discarded and the cell pellet was treated with 1 ml RBC lysis buffer (Invitrogen, 00-4333-57) for 1 min. 9 ml of DPBS was added to end lysis reaction and centrifuged at 400 x g for 5 min. Supernatant was discarded, and pellet was resuspended in 200 µl of FACS buffer and transferred to a round bottom 96-well plate for staining.

2.5.2.3 Omentum

The Omentum was removed, placed in DMEM (Invitrogen, 21885-025) and chopped into small pieces. The omentum was then digested with 2 mg/ml Collagenase type I (Gibco, 17100-017) and 4 % BSA (Sigma Aldrich, A7906-50G) at 37 °C for 1 h. Digested tissue was passed through 40 µm EASYstrainer mesh (Greiner Bio-One, 542040) and isolated cells were centrifuged at 400 x g for 5 min. Cells were treated with 1 ml RBC lysis buffer (Invitrogen, 00-4333-57) for 1 min. 9 ml of DPBS was added to end lysis reaction and centrifuged at 400 x g for 5 min. Supernatant was discarded, and pellet was resuspended in 200 µl of FACS buffer and transferred to a round bottom 96-well plate for staining.

2.5.2.4 Spleen/Lymph Node

Tissue was obtained and mashed through a 70 µm EASYstrainer mesh (Greiner Bio-One, 542070). Cells pellets were produced by centrifugation at 400 x g 5 min. 1 ml of RBC lysis buffer (Invitrogen, 00-4333-57) was added to spleen samples and following suspension of the reaction cell pellets were produced by centrifugation. Samples were resuspended in 200 µl FACS buffer for subsequent staining in a round bottom 96-well plate for staining.

2.5.2.5 Lung

Lungs were removed and then chopped into fine pieces in 2 ml of HBSS containing DNase I (100 µg/mL, Roche), dispase II (800 µg /mL, Roche) and collagenase P (200 µg/mL, Roche). Chopped lungs were transferred into 15 ml falcon tubes and the volume of digestion enzyme cocktail was made up to 5 ml. Lungs were incubated at 37 °C for 1 h at 800 rpm. Lungs were inverted after 45 min incubation. 20 µl of FBS was added to samples to neutralise enzyme reactions. Tissue was then mashed through a 70 µm EASYstrainer mesh (Greiner Bio-One, 542070) and isolated cells were centrifuged at 400 x g for 5 min. Cells were treated with 1 ml RBC lysis buffer (Invitrogen, 00-4333-57) for 1 min. 9 ml of DPBS was added to end lysis reaction and centrifuged at 400 x g for 5 min. This was repeated several times until RBCs were fully removed. Supernatant was discarded, and pellet was resuspended in 200 µl of FACS buffer and transferred to a round bottom 96-well plate for staining.

2.5.2.6 Skin

The mouse back was shaved, and 5 cm x 5 cm skin was dissected and chopped into fine pieces in 2 ml of HBSS containing collagenase D (1 mg/mL Roche), dispase II (500 µg/mL, Roche) and DNase I (100 µg/mL, Roche). Chopped skin was transferred into 15 ml falcons and the volume of digestion cocktail was made up to 5 ml. Skin was incubated at 37°C for 1 h at 800 rpm and samples were inverted after 45 min incubation. 20 µl of FBS was added to samples to neutralise enzyme reactions. Tissue was then mashed through a 70 µm EASYstrainer mesh filter (Greiner Bio-One, 542070) and isolated cells were centrifuged at 400 g for 5 min. Cells were treated with 1 ml RBC lysis buffer (Invitrogen, 00-4333-57) for 1 min. 9 ml of DPBS was added to end lysis reaction and centrifuged at 400 g for 5 min. This was repeated until RBCs were fully removed. Supernatant was discarded, and pellet was resuspended in 200 µl of FACS buffer and transferred to a round bottom 96-well plate for staining.

2.6 Flow Cytometry

2.6.1 Cell Staining

Cells were stained with 50 µl of fixable viability dye eFluor 506 (1:1000 in DPBS, ThermoFisher) for 20 min at RT. Cells were then washed in FACS buffer (DPBS (Gibco, 10270-106), 1% foetal bovine serum (FBS) (Gibco, 10270-106) and 2 mM EDTA UltraPure EDTA (Invitrogen, 15575020) and centrifuged 400 x g 5 min. Cells were then stained with antibody or fluorescence minus one (FMO) cocktail containing antibodies (dilution 1:200) and Fc block (TruStain FcX, Biolegend) at 1:200 in FACS buffer for 30 min at 4°C. Finally, cells were fixed in 2 % PFA in DPBS for 20 min at 4 °C before being washed and resuspended in FACS buffer.

Single-stain controls were prepared by incubating one drop of Invitrogen™ UltraComp eBeads with 1 µl of the appropriate antibody for 20 min at 4 °C. Following incubation, beads were washed with 1 ml of FACS buffer and centrifuged at 400 × g for 5 min. The supernatant was discarded, and the beads were resuspended in 200 µl of FACS buffer. Samples and controls were run on the Fortessa flow cytometer (BD Biosciences) at the Flow Core Facility within the Institute of Infection and Immunity, University of Glasgow. Data was then analysed using FlowJo version 10.10.0 software. All flow cytometry was performed in accordance with established best practice guidelines (Cossarizza et al., 2021).

Antibody	Clone	Conjugated Fluorophore	Catalogue Number
CD45	30-F11	BV711	BioLegend, 103147
CD11b	M1/70	PerCP-Cy5-5	BioLegend, 101229
F4/80	BM8	BUV805	eBioscience, 368-4801-82
Ly6G	1A8	AF700	BioLegend, 127621
TCR- β	H57-597	AF700	BioLegend, 109223
Ly6C	HK1.4	PE-Cy7	BioLegend, 128017
SiglecF	E50-2440	BUV395	BD Biosciences, 740280
MHCII	M5/114.15.2	BV785	BioLegend, 107645
CD19	1D3	BV605	BioLegend, 152425
CD11c	N418	APC-Cy7	BioLegend, 117317

Table 2-1. Antibodies Used for Flow Cytometry of Myeloid Cells Obtained from Embryonic Tissues

Antibody target, clone, conjugated fluorophore, supplier and catalogue number are listed. All antibodies were used at a dilution of 1/200 in FACS buffer (DPBS, 1 % foetal bovine serum (FBS), and 2 mM EDTA (Thermo Fisher Scientific)).

Antibody	Clone	Conjugated Fluorophore	Supplier, Catalogue Number
CD45	30-F11	BUV395	BioLegend, 103191
CD11b	M1/70	BV785	BioLegend ,101243
F4/80	BM8	PE-Cy7	BioLegend, 123113
Ly6G	1A8	PerCP-Cy5-5	BioLegend, 127615
Ly6G	1A8	BUV395	BD Biosciences, 563978
Ly6C	HK1.4	BV711	BioLegend, 128037
SiglecF	S17007L	PE	BioLegend, 155505
MHCII	M5/114.15.2	AF700	BioLegend, 107621
MHCII	M5/114.15.2	Qdot-605-A	eBioscience, 406-5321-82
CD206	C068C2	BV605	BioLegend, 141721
CD19	1D3	BUV805	BD Biosciences 568287
CD19	1D3	PerCP-Cy5-5	BioLegend, 152405
CD19	1D3	BV421	BioLegend, 1524215
CD226	TX42.	APC	BioLegend, 133619
CD11c	N418	APC-Cy7	BioLegend, 117323

Table 2-2 Antibodies Used for Flow Cytometry of Myeloid Cells Obtained from Adult Tissues

Antibody target, clone, conjugated fluorophore, supplier and catalogue number are listed. All antibodies were used at a dilution of 1/200 in FACS buffer (DPBS, 1 % foetal bovine serum (FBS), and 2 mM EDTA (Thermo Fisher Scientific).

2.6.2 Cell Counts

Total concentration (cells/ml) of single cell suspensions was determined by loading cells stained with acridine orange (LabTech, F23001) into cartridges for counting using a Luna cell counter (Logos Biosystems).

2.7 Fluorescence Activated Cell Sorting

Lung was digested and stained according to the described method of tissue processing and fluorescent staining for flow cytometry. In this instance, sterile technique for working with RNA was used throughout, including use of *RNaseZap* (Invitrogen, AM9780). The entire volume of digested lung was stained. Following staining, lungs from WT or ACKR2-deficient mice ($n = 4$) were resuspended in FACS buffer for processing and pooled to produce one pooled sample per group. Cells were filtered through nytex membrane and ran through FACSaria II or III cell sorter (BD Biosciences) treated with *RNaseZap* (Invitrogen, AM9780) at the Flow Core Facility within the Institute of Infection and Immunity, University of Glasgow. Sorted cells were recovered directly into 350 μ l of RLT buffer (RNAeasy micro kit Qiagen, 74004).

2.8 Imaging

2.8.1 Wholemout Imaging of the Peritoneal Wall

The whole peritoneal wall was placed in 2 % PFA for 2 h and then washed 3 times with 1X PBS. 1 ml of 0.1% TritonX-100 (Merk-Sigma Aldrich T8787) in PBS was added to samples which were then incubated for 10 min. Samples were washed 3 times with PBS. 1 ml of DAPI (Invitrogen, D21490) at 0.005 mg/ml in PBS was added to appropriate samples. Samples were then transferred to glass bottom dishes (Thermo Fisher Scientific, 150680) and for imaging an aluminium weight was placed on top of the tissue to flatten the samples and press them against the bottom of the dish (Ferriz et al., 2023). Samples were imaged at 20X magnification using a Nikon inverted AX-R at the Glasgow Imaging Facility at the Institute of Infection and Immunity, University of Glasgow. Image analysis was carried out using ImageJ Version 1.54.

2.8.2 RNAScope

The peritoneal wall was fixed with 4 % PFA for 24 h. The walls were then cryopreserved by tissue immersion in sucrose (in 1x PBS) gradient; 10 % for 24 h, 20 % for 24 h and 30 % for 24-48 h at 4 °C until tissue sunk to the bottom of the well. Tissue was then frozen in Optimal Cutting Temperature (OCT) embedding media (TissueTek, Sakura 45831) using dry ice and stored at -80 °C until sectioning. Tissue was sectioned onto Superfrost plus slides (Immedge VWR 631-0108) at 7 µm using a Microtome.

Slides were washed with PBS for 5 min to remove OCT and baked for 45 min at 60 °C. Slides were dehydrated at RT using 50 % ethanol for 5 min, 70 % ethanol and twice with 100% for 5 min. Tissues were then incubated with RNAScope Multiplex FL v2 hydrogen peroxide (Advanced Cell Diagnostics, 322381) for 10 min at RT. Boiled (99-102 °C) in antigen-retrieval buffer for 15 min. Slides were rinsed for 15 s in dH₂O and then transferred to 100 % ethanol for 3 min. Slides were dried at RT and tissues were circled using a hydrophobic pen (VWR, 101098-065). 1 drop of protease III (Advanced Cell Diagnostics, 322381) was added to each section and incubated for 30 min at 40 °C in an RNAScope oven (ACD HybEZ™ II Hybridization System (110v) (Advanced Cell Diagnostics, 321711)). Slides were rinsed twice in dH₂O for 2 min. Slides were then hybridised using the RNAScope Mm-ACKR2 probes (Advanced Cell Diagnostics, 323110). Positive and negative probe controls were also used (Advanced Cell Diagnostics, 323110). The appropriate probes were added to each section and slides were incubated for 2 h at 40 °C. Slides were then rinsed 2x in wash buffer for 2 min.

Signal amplification was then performed through a series of sequential hybridization steps following the Advanced Cell Diagnostics RNAScope Multiplex Fluorescent Assay V2 protocol (Advanced Cell Diagnostics, 323110). One drop of hybridisation amp 1 was added to sections and slides were incubated for 30 min at 40 °C. Slides were rinsed 2x in wash buffer for 2 min. One drop of hybridisation amp 2 was added to sections and slides were incubated for 15 min at 40 °C. Slides were rinsed 2x in wash buffer for 2 min. One drop of hybridisation amp 3 was added to sections and slides were incubated for 30 min at 40 °C. Slides were rinsed 2x in wash buffer for 2 min. One drop of RNAScope Multiplex FL v2 HRP-C1 was then added to sections and slides were incubated at 40 °C for 15 min. Slides were then washed 2x in wash buffer for 2 min.

2.8.3 Co-staining with Antibodies

Slides were treated with 50 µl per sample of 0.1 % TritonX-100 (Merk-Sigma Aldrich T8787) in PBS for 10 min and then blocked with 1 % BSA (Sigma Aldrich, A7906-50G) in PBS containing 0.1% Tween-20 (Sigma) for 30-45 min at RT. Samples were stained with Lyve-1 Alexa Fluor 488 (clone 223322, R&D systems, FAB2125G) at 1:200 dilution in 1 % BSA in PBS containing 0.1 % Tween-20 (Sigma) overnight at 4 °C. Samples were then washed 3 times with PBS for 10 min and mounted with ProLong™ Gold Antifade Mountant with DAPI (Invitrogen, P36931). Slides were dried at 4 °C.

Images were visualised at 20X magnification using an Axioimager (Zeiss) microscope.

2.9 Peritoneal Infection of Mice with *E. coli*

Mice were injected intraperitoneally with 5×10^6 Colony forming units (CFU) of *E. coli* (UPEC isolate CFT073) and monitored for weight loss and clinical signs of infection before being culled 18 h post-infection (Wilson et al., 2024, Wilson et al., 2022). CFU of liver, peritoneal wall and spleen tissue were determined by tissue homogenisation using a TissueLyser (Qiagen) (10 min, rate of 50s^{-1} , 1x 5 mm stainless steel bead (Qiagen, 69989) per sample) and subsequent streaking of the supernatant to form single colonies (measured by serial dilution). CFU of peritoneal cavity fluid was determined by centrifugation of lavage fluid at $13000 \times g$ followed by resuspension of the cell pellet in 50 µl PBS and plating for serial dilution (Wilson et al., 2024).

2.10 Bioinformatics

2.10.1 Bulk-RNA sequencing

RNA sequencing was performed by Novogene Genomics (Beijing, China). This included RNA quality control assessment, mRNA library preparation via poly(A) enrichment, SMARTer-based cDNA amplification, and sequencing on the Illumina high-throughput platform using 150 bp paired-end reads (PE150). Sequencing reads were aligned to the *Mus musculus* reference genome (mm10), and differential gene expression analysis was performed using Searchlight 2 (version 2.0.3) (Cole et al., 2021).

2.11 Statistical Analysis

All statistical analyses were performed using GraphPad Prism (version 10.1.2). Normality of data distribution was assessed using the Shapiro-Wilk and Kolmogorov-Smirnov tests. For comparisons between two groups, two-tailed unpaired t-tests were used for normally distributed data, and the Mann-Whitney U test was applied to non-normally distributed data. For comparisons of three or more independent groups, one-way ANOVA was used to assess differences in group means. Tukey's post hoc test was applied for normally distributed data and Dunnet's post hoc test was applied for non-normally distributed data. Statistical significance was defined as $p < 0.05$. Error bars represent the standard error of the mean (SEM) unless otherwise stated. Parametric tests were used even when sample size was small if data was normally distributed as literature has suggested that parametric tests should be applied when assumptions of normality are met (Grech and Calleja, 2018). When multiple independent statistical tests were performed, the Benjamini-Hochberg (BH) test was applied to control the false discovery rate. Adjusted p-values (q-values) were significant if $q < 0.05$.

3 Chemokine Ligand and Receptor Expression in the Developing Embryo

3.1 Introduction

The significance of chemokine signalling in embryonic development has previously focused on the role of homeostatic chemokines. For example, CXCL12 is essential for life in mammals and has well characterised functions during organogenesis (Sierro et al., 2007, Belmadani et al., 2005, Gerrits et al., 2008). However, less is known about the role of inflammatory chemokines during embryonic development. It has previously been shown that CCL2-CCR2 signalling in the developing embryo controls the migration of a specific subpopulation of F4/80^{int} macrophages to the skin at mouse embryonic day 14.5 (E14.5) (Lee et al., 2019). This mechanism is regulated by ACKR2 expressed on placental trophoblasts, which restricts and localises chemokine activity to the maternal circulation and decidua, preventing passage into the embryonic circulation (Lee et al., 2019, Fukuoka et al., 2024). In line with this, other ACKR2 ligands, including CCL7, 12 and 22 are elevated in the absence of ACKR2, whilst non-ACKR2 ligands such as CXCL13 are unaffected (Lee et al., 2019). These findings raise the possibility that ACKR2 at the maternal-foetal interface regulates additional chemokine dependent migratory dynamics in the embryo and that inflammatory chemokine signalling and wider chemokine functions in the embryo may be more extensive than previously understood.

Understanding the timing of immune cell emergence in the embryo is essential for interpreting chemokine expression patterns and their potential roles in guiding immune cell positioning during late gestation. Mouse embryonic days begin from conception, and gestation typically lasts between 19 and 21 days. In the developing mouse embryo, primitive haematopoiesis begins in the yolk sac at day E7.5/E8 with megakaryocyte and macrophage lineage markers being observed by E9.5, marking the beginning of definitive erythro-myeloid progenitor (EMP) differentiation (Figure 1-1) (McGrath et al., 2015). Infiltration of yolk sac derived macrophages into embryonic tissues occurs between E10.5 and E12.5 (Syeda et al., 2024). At E10.5 definitive hematopoietic stem cells (HSC) arise in the mesodermal aorto-gonado-mesonephros region and then migrate to and colonise the foetal liver (Figure 1-1) (Bertrand et al., 2010). The foetal liver becomes the major site for HSC differentiation and expansion at this stage (Gao et al., 2018) with a population of circulating neutrophils observable by E11.5 (Gao et al., 2018, McGrath et al., 2015). HSCs

are then seeded in the spleen at E15.5 and finally in the bone marrow between E16.5 and E17.5, where they persist throughout adult life (Figure 1-1) (Gao et al., 2018).

Understanding of leukocyte origin and development in the embryo is continuously expanding. For example, a population of cardiac macrophages has recently been shown to be intrinsic to the heart, developing within the tissue from endocardial cells and playing an important role in the modelling and formation of valves (Shigeta et al., 2019).

Importantly, evidence suggests that the inflammatory response becomes more functionally active in mouse embryos around E14 (Takaya et al., 2022). This was demonstrated by a study that assessed wound healing in embryonic skin following full-thickness incisions across developmental time points. Prior to E13, the skin was capable of complete regeneration without scarring; however, at E14, the embryonic skin was no longer able to regenerate fully, with changes in the morphology of the epidermal structure observed following repair processes. By E17, incisions resulted in fibrotic scar formation (Takaya et al., 2022), indicating an activated inflammatory response. This finding, in combination with the observation that disruption of chemokine signalling at E14.5 alters macrophage populations (Lee et al., 2019) identifies a developmental timepoint of interest for the study of inflammatory chemokines in the embryo from E14.5-E15.

The spatiotemporal expression patterns of inflammatory chemokines during embryonic development remain poorly characterised. Given the importance of chemokines in guiding immune and stromal cell positioning, this chapter aims to characterise chemokine expression in WT mouse embryos and to pair this with a profiling of inflammatory chemokine receptor expression on the surface of myeloid cell populations across embryonic tissues.

3.2 Chemokine Ligand Expression in Embryonic Tissues at E15, E17.5 and E18.5

To identify chemokine expression patterns across embryonic development, RNA expression levels of selected chemokines listed in Table 3-1 were measured across multiple embryonic tissues (heart, lung, skin, liver) at embryonic days 15, 17.5, and 18.5 (E15, E17.5, E18.5). It is important to note that RNA expression levels of different chemokines throughout this chapter cannot be directly quantitatively compared to one another, as each target gene is measured using unique primer and probe sequences with potentially differing amplification efficiencies. Furthermore, the transcript expression values obtained represent relative expression, meaning that data are normalised within each sample and reflect changes within genes, rather than absolute expression levels across genes. However, expression of the housekeeping gene (*GAPDH*) can vary to some extent between tissue types, so interpretation of results should focus on relative expression patterns within the same tissue rather than making absolute comparisons between different organs. An additional reference gene, *18S rRNA*, was included; however, its extremely low Ct values in this dataset made it unsuitable for accurate normalisation in this context (Table 3-1). As transcript expression does not always reflect protein expression, the concentration of selected CC and CXC chemokines outlined in Table 3-1 was also measured at the protein level in heart, lung, skin, and liver at E15, E17.5, and E18.5 by Luminex assay.

Notably, CCL4 and CCL20 were not detected at any time point in any of the tissues analysed. CCL19, CCL22 and CXCL10 were only detected as extrapolated values below the standard curve across all time points. In contrast, values within the quantifiable range were successfully obtained for multiple chemokine analytes from E17.5 and E18.5 samples across all tissues. Extrapolated values are represented as transparent data points on graphs and only analytes with at least one time point showing a quantifiable mean value (without extrapolation across samples) are represented in the graphs for each tissue.

Ultimately, this dataset provides a temporal and tissue-specific map of chemokine expression during late gestation in mice, highlighting a role for chemokines during embryo development, and informing future studies investigating how maternal inflammation or chemokine dysregulation may impact embryonic immune development and tissue patterning.

Transcript Targets	Exclusion Rationale	Protein Targets	Exclusion Rationale
CCL1	No amplification	CCL2	
CCL2		CCL3	
CCL3		CCL4	
CCL4		CCL5	
CCL5		CCL7	
CCL6		CCL11	
CCL7		CCL12	
CCL8		CCL19	
CCL9		CCL20	
CCL11		CCL21	Extremely high values across every sample analysed.
CCL12		CCL22	
CCL17		CXCL1	
CCL19		CXCL2	
CCL20		CXCL10	
CCL22		CXCL12	
CCL24		CXCL13	No Matching RNA data
CCL25		CXCL16	
CCL26			
CCL28			
CXCL1			
CXCL2			
CXCL3			
CXCL4			
CXCL5			
CXCL6			
GAPDH			
CXCL9			
CXCL10			
CXCL11			
CXCL12			
CXCL13	Contamination in no reverse transcription controls		
CXCL14			
CXCL15			
CXCL16			
CXCL17			
CX3CL1			
XCL1			
18S	Very low Ct values (<10).		

Table 3-1 qPCR and Luminex Targets with Rationale for Target Exclusion

List of qPCR selected TaqMan array card gene targets and Luminex analyte targets, including justification for exclusion of specific targets from analysis. Targets highlighted in red were excluded.

3.2.1 Chemokine Expression in Embryonic Heart

As shown in Figure 3-1 most CC-chemokines were expressed at very low, to undetectable, transcript levels in the embryonic heart from E15 to E18.5. The relative expression values remained at or near background levels across all time points ($2^{-\Delta Ct} < 0.001$), and large standard error margins suggest high variability among samples Figure 3-1A and Figure 3-1B. The expression value $2^{-\Delta Ct} = 0.001$ is equivalent to a Ct difference of approximately 10 cycles between the target gene and the housekeeping gene, *GAPDH*. In this chapter, a $2^{-\Delta Ct}$ value of greater than 0.001 is used as an arbitrarily defined threshold for detection and for downstream $\Delta\Delta Ct$ analysis. This threshold was applied to exclude extremely lowly expressed genes from statistical analysis, improving biological relevance and aiding interpretation of the dataset given the large number of targets assessed. In the heart, CC-chemokine expression did not exceed $2^{-\Delta Ct} = 0.001$ at one or more time points across any of the targets measured, and therefore no downstream statistical analysis comparing CC-chemokine expression in the heart across time points was performed.

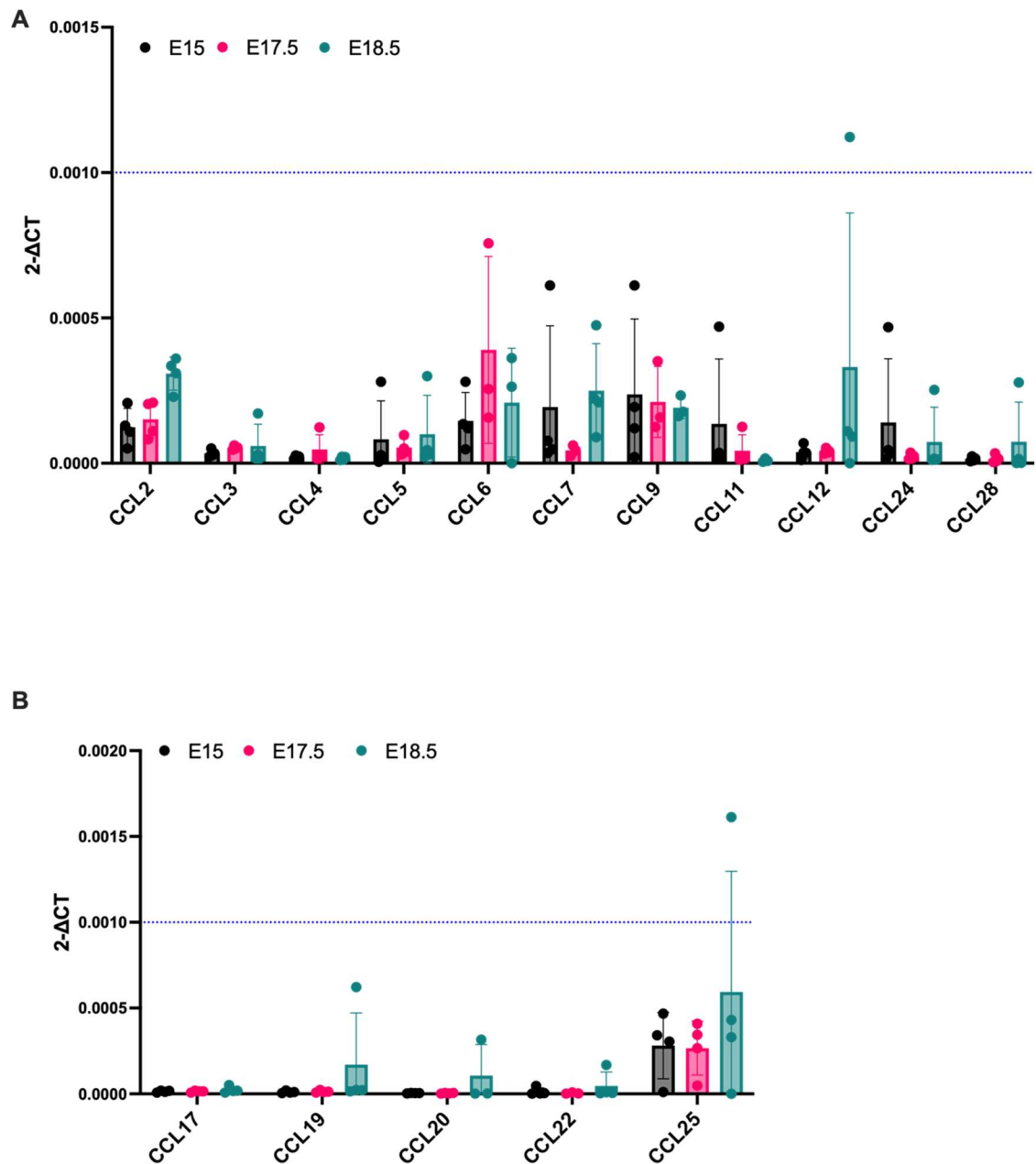


Figure 3-1 CC-Chemokine Transcription in Embryonic Heart

RT-qPCR analysis of CC-chemokine transcript expression in embryo heart at E15 (black) E17.5 (pink) and E18.5 (teal). Expression of iCCR chemokine ligands (**A**) and non-iCCR chemokine ligands (**B**) was measured relative to *GAPDH* using the $2^{-\Delta C_t}$ method. Error bars represent SEM. $n = 4$ biological replicates per group. The blue dotted line represents a threshold of $2^{-\Delta C_t} = 0.001$, corresponding to a ΔC_t of ~ 10 between the housekeeping gene and target gene. This threshold was used to indicate robust detection of RNA expression. It should be noted that expression values below this level are not zero and may still reflect true low transcript expression. Statistical analysis details are provided in the Methods and in corresponding table legends. No CC-chemokine targets in the embryonic heart met the criteria for downstream statistical analysis.

In the heart, the low transcript expression of CC-chemokines was also reflected at protein level (Figure 3-2). CCL4 and CCL20 were not detected and CCL5, CCL7, CCL19 and CCL22 were detected at values that were extrapolated from the bottom of the standard curve for all time points and were therefore excluded from analysis. CCL3 was consistently detected at all time points but at very low values of less than 20 pg/mg of protein, which could represent background expression, or release by a small subset of cells (Figure 3-2). No changes in CCL3 protein expression across developmental timepoints were observed. CCL11 and CCL12 proteins were detected at slightly higher protein concentrations than that observed for CCL3 in the embryonic heart at E15, E17.5 and E18.5, despite not being observed at RNA level (Figure 3-1A and Figure 3-2). CCL11 showed highest expression at E17.5 with a mean concentration of 107.5 pg/mg decreasing to 43.4 pg/mg at E18.5 ($p = 0.0159$). CCL12 was not detected at E15 but increased to 66.7 pg/mg at E17.5 and sustained this expression at E18.5 (mean = 49.7 pg/mg) (Figure 3-2).

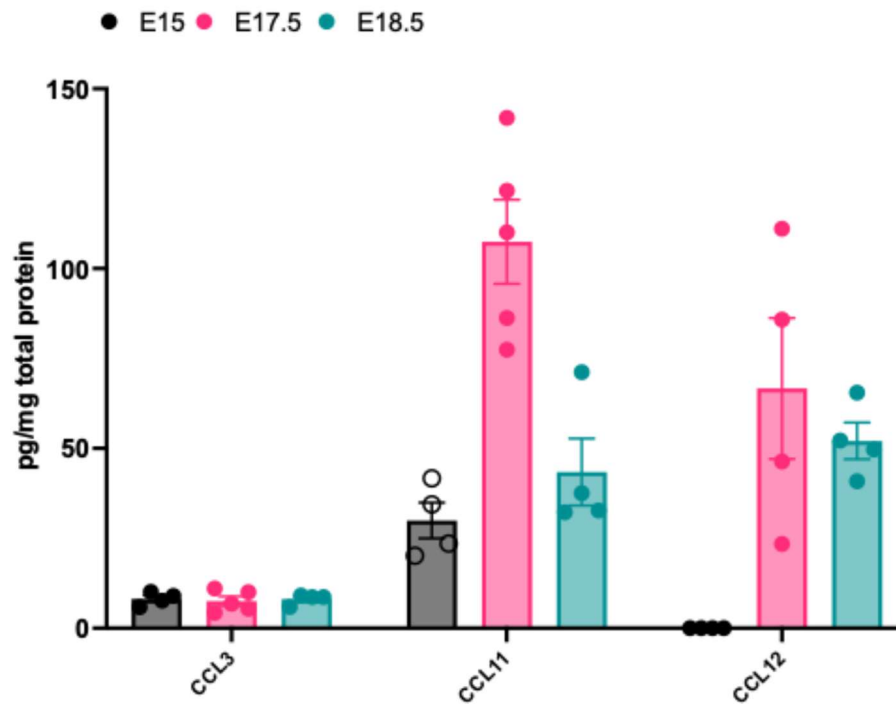


Figure 3-2 CC-Chemokine Protein Expression in Embryonic Heart

CC-chemokine protein in total wild type embryonic heart at E15 (black), E17.5 (pink) and E18.5 (teal) was determined by Luminex assay and normalised to total protein (mg), as measured by BCA assay. Each group included $n = 4$ biological replicates for E15 and E18.5, $n = 5$ biological replicates for E17.5. Outliers were identified and excluded using Grubb's test. Transparent data points were extrapolated from below the standard curve. These data were excluded from statistical analysis due to low quantification reliability. Statistical comparisons were performed based on the number of valid, quantifiable time points per analyte. For three valid time points: One-Way ANOVA with Tukey's or Dunnett's post hoc test depending on distribution. Two valid time points: Unpaired t-test or Mann Whitney depending on distribution. One valid time point: No statistical comparison. Asterisks indicate adjusted p values from post hoc tests or p values from unpaired t tests/Mann Whitney: $p/p_{adj} < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****).

In contrast, data in Figure 3-3 show that CXCL12 RNA was detectable at relatively high $2^{-\Delta Ct}$ values in the embryonic heart throughout all three developmental time points measured (Figure 3-3). Although CXCL12 RNA expression appeared to decrease between E15-E18.5 this was not statistically significant ($p = > 0.05$). The high detection of expression of CXCL12 RNA was recapitulated at the protein level, in which CXCL12 remained the most highly expressed CXC chemokine across E15, E17.5 and E18.5 (Figure 3-4). However, it should be noted that the absolute quantification of CXCL12 at E15 should be interpreted with caution due to extrapolated samples contributing to the mean. At the protein level, CXCL12 decreased in concentration from 969.37 pg/mg at E17.5 to 379.44 pg/mg at E18.5 ($p = 0.0039$). At E15, CX3CL1 was also detected at the transcript level ($2^{-\Delta Ct} = > 0.001$) in the embryonic heart (Figure 3-3).

Given the large number of targets assessed, additional parameters were applied to help distil this dataset and these criteria are described in the Methods as well as in the table legends throughout this chapter. Data have been collated into tables which highlight fold-change (calculated using the $\Delta\Delta Ct$ method) across time points, as well as statistical significance. In the heart only CX3CL1 met the criteria for downstream analysis and Table 3-2 shows that there is a significant decrease in CX3CL1 RNA expression in the embryonic heart between E15 and E18.5 by a fold change (FC) of 0.42 ($\log_2 FC = -1.27$), ($p = < 0.0001$).

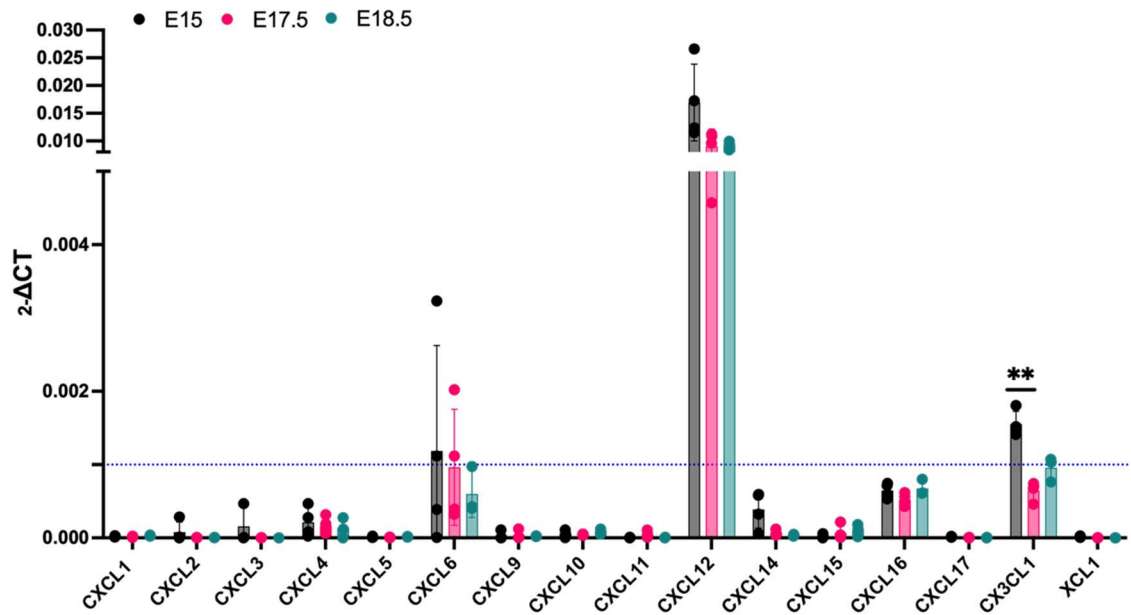


Figure 3-3 CXC/CX3C/XC-Chemokine Transcription in Embryonic Heart

RT-qPCR analysis of CXC/CX3C/XC-chemokine transcript expression in total embryo heart at E15 (black), E17.5 (pink) and E18.5 (teal). Expression was measured relative to *GAPDH* using the $2^{-\Delta C_t}$ method. Error bars represent SEM. $n = 4$ biological replicates per group. The blue dotted line represents a threshold of $2^{-\Delta C_t} = 0.001$, corresponding to a ΔC_t of ~ 10 between the housekeeping gene and target gene. This threshold was used to indicate robust detection of RNA expression. It should be noted that expression values below this level are not zero and may still reflect true low transcript expression. Statistical analysis details are provided in the Methods and in corresponding table legends. Briefly, normality was assessed, and significance was determined using one-way ANOVA with Tukey's post hoc test for normally distributed data, or Dunn's test for non-normally distributed data. The Benjamini-Hochberg method was used to control the false discovery rate. $p_{\text{adj}} < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****).

Gene	Comparison	Fold Change	Log2 FC	<i>p</i> .adj value	q-value	Significant (FDR < 0.05)
CX3CL1	E15-E17.5	0.42	-1.27	<0.0001	0.0043	Yes

Table 3-2 Differential expression of CX3CL1 in the Embryonic Heart Across Developmental Stages

Fold changes in gene expression of CX/CX3C/XC-chemokines in the embryonic heart between embryonic timepoints E15, E17.5 and E18.5. Only genes that showed a fold change ≥ 2 (upregulation) or ≤ 0.5 (downregulation) relative to E15.0 or E17.5 and had expression ($2^{-\Delta C_t}$) ≥ 0.001 for at least one time point were included. Log₂-transformed fold change values (log₂FC) are also presented to facilitate interpretation of upregulation and downregulation on a symmetric scale. For example, a 2-fold upregulation becomes +1 ($\log_2(2) = 1$) and a 2-fold downregulation becomes -1 ($\log_2(0.5) = -1$). Normality was assessed and statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test for normally distributed data ($p < 0.05$) or Dunn's test for non-normally distributed data. As multiple independent statistical tests were performed across different genes, the Benjamini-Hochberg (BH) test was applied to control the false discovery rate. Adjusted p-values (q-values) were significant if $q < 0.05$. Significant q values are indicated as 'YES'; non-significant results are marked 'NO'.

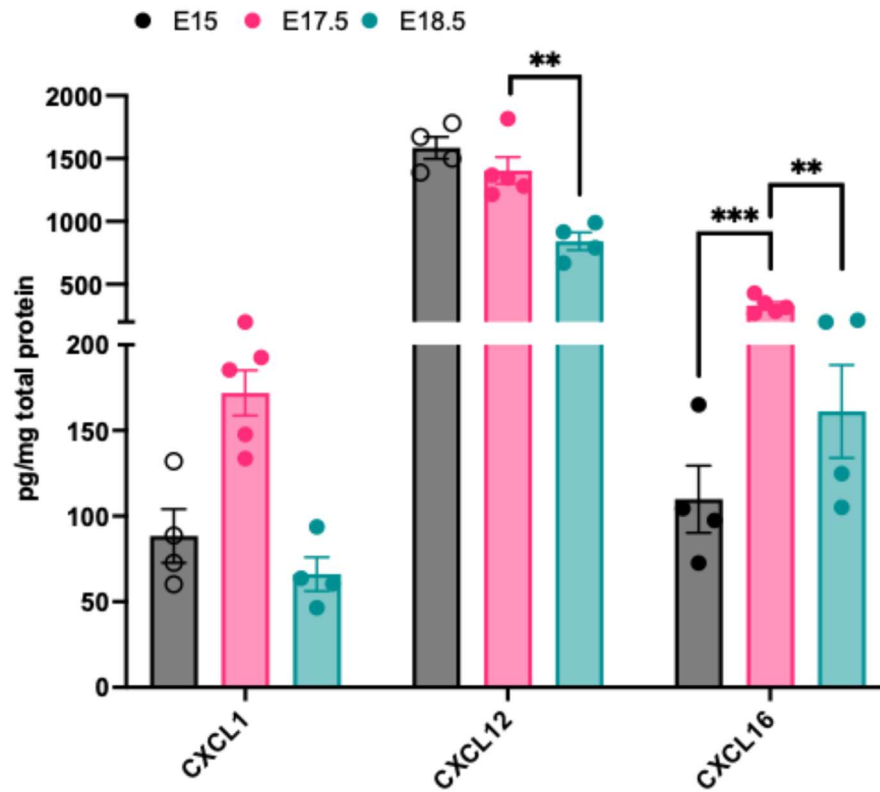


Figure 3-4 CXC-Chemokine Protein Expression in Embryonic Heart

CXC-chemokine protein in total wild type embryonic heart at E15 (black), E17.5 (pink) and E18.5 (teal) was determined by Luminex assay and normalised to total protein (mg), as measured by BCA assay. Each group included $n = 4$ biological replicates for E15 and E18.5, $n = 5$ biological replicates for E17.5. Outliers were identified and excluded using Grubb's test. Transparent data points were extrapolated from below the standard curve. These data were excluded from statistical analysis due to low quantification reliability. Statistical comparisons were performed based on the number of valid, quantifiable time points per analyte. For three valid time points: One-Way ANOVA with Tukey's or Dunnett's post hoc test depending on distribution. Two valid time points: Unpaired t-test or Mann Whitney depending on distribution. One valid time point: No statistical comparison. Asterisks indicate adjusted p values from post hoc tests or p values from unpaired t tests/Mann Whitney: $p/p.adj < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****).

At the protein level, CXCL2 and CXCL10 were not detected in the embryonic heart (Figure 3-4). In contrast, CXCL1 was present at low levels across all three measured timepoints. Expression was most highly detected at E17.5 with a mean concentration of 172.0 pg/mg. Expression at E17.5 was significantly higher than at E18.5 ($p = 0.0005$), indicating that E17.5 may represent a window for neutrophil recruitment, as CXCL2 is a potent neutrophil chemoattractant. CXCL16 was consistently detected at E15, E17.5, and E18.5 in embryonic heart (Figure 3-4). Its expression significantly increased from 110.0 pg/mg at E15 to 429.5 pg/mg at E17.5 ($p = 0.003$), followed by a notable decrease to 161.2 pg/mg at E18.5 ($p = 0.0024$). Although CXCL16 RNA expression did not exceed a ΔCT of 0.001, values approached the detection threshold with low variability (Figure 3-3) suggesting that ΔCT values below 0.001 may still be biologically meaningful, despite statistical comparisons not being included in this thesis.

Overall, while some CC-chemokine proteins were detectable in the embryonic heart, CXC chemokines demonstrated higher expression patterns at E15, E17.5, and E18.5, indicating that CXC-chemokines likely play a more prominent role than CC-chemokines in heart development during these stages.

3.2.2 Chemokine Expression in the Embryonic Lung

Chemokine ligand expression was also analysed in the embryonic lung at E15, E17.5, and E18.5. Figure 3-5A shows that of the iCCR ligands measured, CCL3, CCL4, CCL5, CCL7, CCL24, were not expressed at levels considered detectable levels across any of the time points measured ($2^{-\Delta\text{Ct}} < 0.001$). At E15, CCL2 expression in the lung exceeded the threshold (Figure 3-5A) ($2^{-\Delta\text{Ct}} = 0.00124$) and CCL9 RNA expression was detected at E15 ($2^{-\Delta\text{Ct}} = 0.001476$) and E17.5 ($2^{-\Delta\text{Ct}} = 0.001824$) (Figure 3-5A). CCL6 was detected at all time points, showing a sequential increase in expression between E15–E17.5 (fold change = 6.68, $\log_2\text{FC} = 2.74$, $p = 0.0404$) and E17.5–E18.5 (fold change = 2.43, $\log_2\text{FC} = 1.28$, $p = 0.0034$) (Table 3-3). Overall, CCL6 expression increased significantly by a fold change of 16.22 from E15 to E18.5 ($p = 0.003$, one-way ANOVA) (Table 3-3). Additionally, Figure 3-5A shows that CCL11 was the most highly detected CC-chemokine in the lung at E15. CCL11 peaked at E17.5, increasing from a $2^{-\Delta\text{Ct}}$ of 0.0156 to a $2^{-\Delta\text{Ct}}$ of 0.0225. As the fold change was below 2, statistical analysis was not conducted on this comparison. However, CCL11 expression significantly decreased by a fold change of 0.21 ($\log_2\text{FC} = -2.23$) between E17.5 and E18.5 ($p < 0.0001$), with an overall decrease of 0.31 ($\log_2\text{FC} = -1.70$) from E15 to E18.5 ($p = 0.0009$) (Table 3-3).

Furthermore, whilst CCL12 and CCL28 transcripts were not expressed at E15, both increased to levels above the detection threshold by E18.5 Figure 3-5A. CCL12 shows a fold change of 9.65 ($\log_2\text{FC} = 3.27$) between E17.5 and E18.5 ($p < 0.0001$), rising from below threshold level expression at E15 and E17.5. Similarly, CCL28 demonstrated a fold change of 23.74 ($\log_2\text{FC} = 4.57$) between E17.5 and E18.5 ($p < 0.0001$), indicating upregulation during this developmental window (Table 3-3).

Finally, among the non-iCCR ligands, CCL17 also showed increased expression at E18.5 with RNA levels rising by a fold change of 29.05 ($\log_2\text{FC} = 4.86$) between E17.5 and E18.5 ($p < 0.0001$), from previously undetectable levels ($2^{-\Delta\text{Ct}} < 0.001$) (Figure 3-5B and Table 3-5). Overall, across both iCCR and non-iCCR ligands, CC-chemokine RNA expression in the embryonic skin was higher after E15 (Figure 3-5), potentially reflecting that, as previously mentioned, the inflammatory response in embryonic skin becomes switched on around E14 (Takaya et al., 2022). Figure 3-5B indicates that the CCL25 transcript was expressed during a short developmental window at E17.5 as relative gene expression increased from E15 by a fold change of 248592.39 ($\log_2\text{FC} = 17.92$, $p = 0.0379$) to above threshold expression level and then dropped by a fold change decrease of 0.05 ($\log_2\text{FC} = -4.38$) back to below $2^{-\Delta\text{Ct}} = 0.001$ by E18.5 (Table 3-3).

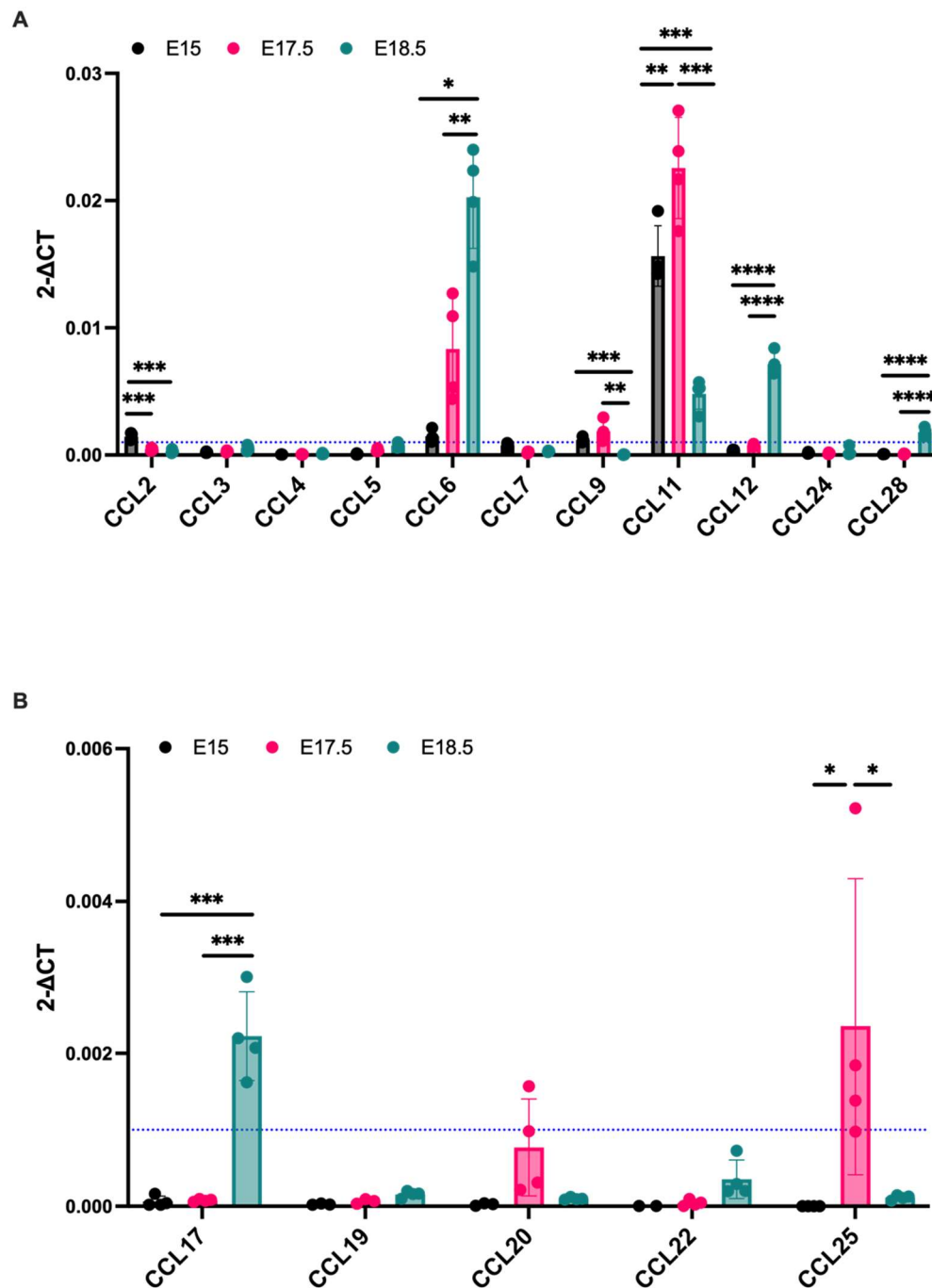


Figure 3-5 CC-Chemokine Transcription in Embryonic Lung

RT-qPCR analysis of CC-chemokine transcript expression in total embryo lung at E15 (black) E17.5 (pink) and E18.5 (teal). Expression of iCCR chemokine ligands (**A**) and non-iCCR chemokine ligands (**B**) was measured relative to *GAPDH* using the $2^{-\Delta C_t}$ method. Error bars represent SEM. $n = 4$ biological replicates per group. The blue dotted line represents a threshold of $2^{-\Delta C_t} = 0.001$, corresponding to a ΔC_t of ~ 10 between the housekeeping gene and target gene. This threshold was used to indicate robust detection of RNA expression. It should be noted that expression values below this level are not zero and may still reflect true low transcript expression. Statistical analysis details are provided in the Methods and in corresponding table legends. Briefly, normality was assessed, and significance was determined using one-way ANOVA with Tukey's post hoc test for normally distributed data, or Dunn's test for non-normally distributed data. The Benjamini-Hochberg method was used to control the false discovery rate. $p_{\text{adj}} < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****).

Gene	Comparison	Fold Change	Log2FC	p.adj value	q-value (BH)	Significant (FDR < 0.05)
CCL2	E15-E17.5	0.31	-1.69	0.0001	0.0002	Yes
CCL2	E15-E18.5	0.19	-2.4	<0.0001	0.0002	Yes
CCL6	E15-E17.5	6.68	2.74	0.0682	0.0798	No
CCL6	E15-E18.5	16.22	4.02	0.0003	0.0010	Yes
CCL6	E17.5-E18.5	2.43	1.28	0.0034	0.0070	Yes
CCL9	E15-E18.5	0.02	-5.5	0.0183	0.0277	Yes
CCL9	E17.5-E18.5	0.01	-6.16	0.0010	0.0024	Yes
CCL11	E15-E18.5	0.31	-1.7	0.0009	0.0024	Yes
CCL11	E17.5-E18.5	0.21	-2.23	<0.0001	0.0001	Yes
CCL12	E15-E18.5	18.12	4.18	<0.0001	<0.0001	Yes
CCL12	E17.5-E18.5	9.65	3.27	<0.0001	<0.0001	Yes
CCL17	E15-E18.5	36.52	5.19	<0.0001	0.0001	Yes
CCL17	E17.5-E18.5	29.05	4.86	<0.0001	0.0001	Yes
CCL25	E15-E17.5	248592.39	17.92	0.0379	0.0496	Yes
CCL25	E17.5-E18.5	0.05	-4.38	0.0473	0.0594	No
CCL28	E15-E18.5	24.45	4.61	<0.0001	<0.0001	Yes
CCL28	E17.5-E18.5	23.74	4.57	<0.0001	<0.0001	Yes

Table 3-3 Differential Expression of CC-Chemokine Genes in the Embryonic Lung Across Developmental Stages

Fold changes in gene expression of CC-chemokine gene expression in the embryonic lung between embryonic timepoints E15, E17.5 and E18.5. Only genes that showed a fold change ≥ 2 (upregulation) or ≤ 0.5 (downregulation) relative to E15.0 or E17.5 and had expression ($2^{-\Delta Ct}$) was ≥ 0.001 for at least one time point were included. Log₂-transformed fold change values (log₂FC) are also presented to facilitate interpretation of upregulation and downregulation on a symmetric scale. For example, a 2-fold upregulation becomes +1 ($\log_2(2) = 1$) and a 2-fold downregulation becomes -1 ($\log_2(0.5) = -1$). Normality was assessed and statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test for normally distributed data ($p < 0.05$) or Dunn's test for non-normally distributed data. As multiple independent statistical tests were performed across different genes, the Benjamini-Hochberg (BH) test was applied to control the false discovery rate. Adjusted p-values (q-values) were significant if $q < 0.05$. Significant q values are indicated as 'YES'; non-significant results are marked 'NO'.

Of the CC-chemokines analysed at the protein level, CCL4 and CCL20 were not detected. CCL5, CCL19, CCL22 were detected at non quantifiable levels, and therefore analysis of these targets across time points was not possible. Figure 3-6 shows that CCL3 and CCL7 were both detected in the developing lung but at very low levels, CCL7 showed its highest concentration at E17.5 with a mean of 47.1 pg/mg. However, CCL11 protein expression mirrored the expression observed at transcript level. CCL11 was most highly detected at E17.5, rising from a mean of 148.8 pg/mg at E15 to a mean of 891.9 pg/mg at E17.5 ($p = 0.0091$) and then falling to 168.8 pg/mg at E18.5 ($p = 0.0089$), following the pattern observed at the RNA expression level (Figure 3-6). This may indicate a role for CCL11 in the developing lung. The peak at E17.5 corresponds with a switch from the canalicular to the saccular stage of lung development, potentially indicating that the spatial positioning CCR3⁺ leukocytes may contribute to this process. Finally, the CCR2 ligand CCL12 was also detected at the protein level in the embryonic lung, with a concentration of 240.3 pg/mg detected at E17.5, this expression was sustained at E18.5, with a mean concentration of 157.0 pg/mg (Figure 3-6), indicating that monocyte and macrophage recruitment to or within the lung may be particularly important at this time point.

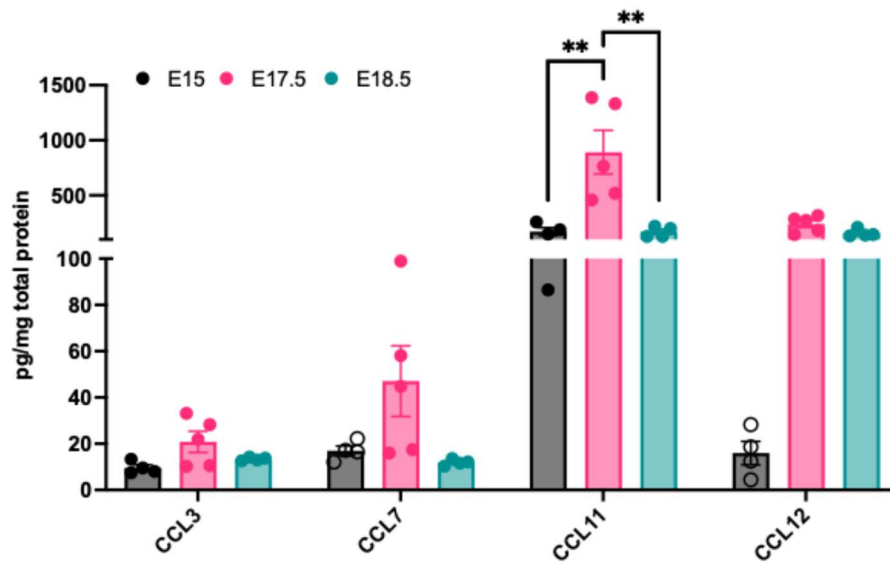


Figure 3-6 CC-Chemokine Protein Expression in Embryonic Lung

CC-chemokine protein in total wild type embryonic lung at E15 (black), E17.5 (pink) and E18.5 (teal) was determined by Luminex assay and normalised to total protein (mg), as measured by BCA assay. Each group included $n = 4$ biological replicates for E15 and E18.5, $n = 5$ biological replicates for E17.5. Outliers were identified and excluded using Grubb's test. Transparent data points were extrapolated from below the standard curve. These data were excluded from statistical analysis due to low quantification reliability. Statistical comparisons were performed based on the number of valid, quantifiable time points per analyte. For three valid time points: One-Way ANOVA with Tukey's or Dunnett's post hoc test depending on distribution. Two valid time points: Unpaired t-test or Mann Whitney depending on distribution. One valid time point: No statistical comparison. Asterisks indicate adjusted p values from post hoc tests or p values from unpaired t tests/Mann Whitney/Mann Whitney: $p/p_{adj} < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****).

Furthermore, various CXC chemokine ligands and CX3CL1 were measured at RNA and protein level in the embryonic lung at E15, E17.5 and E18.5 (Figure 3-7). However, insufficient amplification (amplification in < 3 samples) occurred for the targets CXCL11 and XCL1. Figure 3-7 illustrates that CXCL1, 2, 3, 5, 9 and 17 were not detected in the lung above threshold values for RNA expression ($2^{-\Delta Ct} < 0.001$) at any of the time points measured. Although CXCL4 was expressed at $2^{-\Delta Ct} < 0.001$ at E15 and E17.5 expression was significantly increased at E18.5, with a log2fold change of 2.25 between E17.5 and E18.5 ($p = 0.0120$) (Table 3-4). CXCL6 was expressed in embryonic lung at E15, E17.5 and E18.5 (Figure 3-7) and CXCL6 increased significantly at E18.5, rising by a log2FC of 2.12 from E15 ($p = 0.0097$) and a log2FC of 1.36 from E17.5 ($p = 0.0040$) (Table 3-4). As was observed in the heart, CXCL12 showed high RNA expression in the lung at all three time points (Figure 3-7) and of the CXC chemokine ligands measured at the protein level, CXCL12 was found to be expressed most highly, reaching 1308.5 pg/mg at E17.5 and falling to 552.4 pg/mg at E18.5 ($p = 0.0159$) (Figure 3-8).

Furthermore, CXCL14, CXCL15 and CX3CL1 were expressed at relatively high levels across all time points in the lung and were upregulated at RNA level in the lung between E15 and E17.5 (Figure 3-7 and Table 3-4). CXCL15 was originally termed ‘lungkine’ due to its particularly high expression in the lung and was detected at particularly high transcript levels in the embryonic lung between E17.5 and E18.5. CXCL15 increased sequentially across the time points measured with a substantial overall fold change increase of 138.07 (log2FC = 7.11) between E15 and E18.5 ($p = < 0.0001$) (Figure 3-7 and Table 3-4), indicating that this chemokine may have a specific role close to birth, potentially in preparation for alveolarization which occurs postnatally in mice. In addition, CXCL2 protein was not detected in the lung during late development, mirroring the lack of expression observed at RNA level. Figure 3-8 shows that CXCL1 was detected at below 200 pg/mg across all time points, with unquantifiable detection at E15 and highest concentration of 164.5 pg/mg at E17.5, which then fell to 82.8 pg/mg at E18.5 ($p = 0.0020$). A similar pattern across time points was observed for CXCL16 protein expression; however, the protein was detected at higher levels and was quantifiable at E15 (Figure 3-8). Between E15 and E17.5 CXCL16 increased from 85.7 pg/mg to 257.8 pg/mg ($p = 0.0010$) and then between E17.5 and E18.5 expression fell to 136.6 pg/mg ($p = 0.0069$) (Figure 3-8). In the lung, CXCL16 is secreted by resident alveolar macrophages (Morgan et al., 2005) and therefore these cells may be the source of CXCL16 in the lung between E15 and E18.5. In addition, CXCL16 is constitutively expressed at high levels by human bronchial epithelial cells. Bronchial tree formation is completed at around E16.5 in mice,

therefore the sequential increase in CXCL16 from E15-E17.5 could alternatively reflect an increase in bronchial epithelial cells in the tissue (Day et al., 2009).

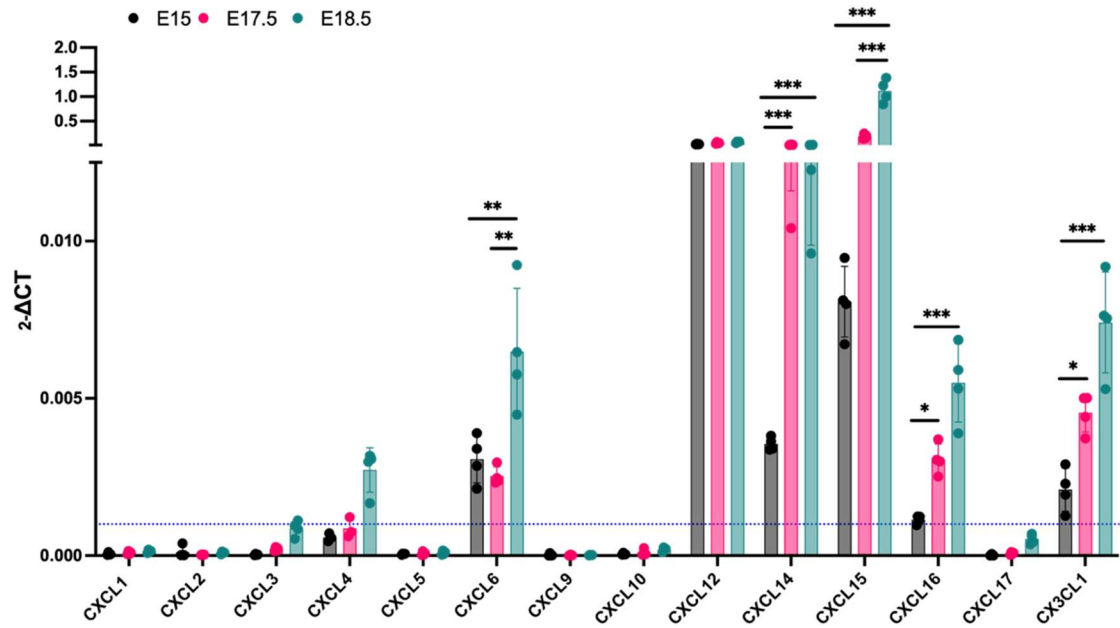


Figure 3-7 CXC/CX3C-Chemokine Transcription in Embryonic Lung

RT-qPCR analysis of CXC/CX3C-chemokine transcript expression in total embryo lung at E15 (black) E17.5 (pink) and E18.5 (teal). Expression was measured relative to *GAPDH* using the $2^{-\Delta C_t}$ method. Error bars represent SEM. $n = 4$ biological replicates per group. The blue dotted line represents a threshold of $2^{-\Delta C_t} = 0.001$, corresponding to a ΔC_t of ~ 10 between the housekeeping gene and target gene. This threshold was used to indicate robust detection of RNA expression. It should be noted that expression values below this level are not zero and may still reflect true low transcript expression. Statistical analysis details are provided in the Methods and in corresponding table legends. Briefly, normality was assessed, and significance was determined using one-way ANOVA with Tukey's post hoc test for normally distributed data, or Dunn's test for non-normally distributed data. The Benjamini-Hochberg method was used to control the false discovery rate. $p_{\text{adj}} < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****).

Gene	Comparison	Fold Change	Log2FC	p.adj value	q-value (BH)	Significant (FDR < 0.05)
CXCL4	E15-E17.5	4.43	2.15	0.3804	0.3375	No
CXCL4	E15-E18.5	4.75	2.25	0.3217	0.3010	No
CXCL6	E15-E18.5	2.12	1.08	0.0097	0.0166	Yes
CXCL6	E17.5-E18.5	2.56	1.36	0.0040	0.0079	Yes
CXCL12	E15-E18.5	2.28	1.19	0.0016	0.0036	Yes
CXCL14	E15-E17.5	4.59	2.20	0.0010	0.0024	Yes
CXCL14	E15-E18.5	3.68	1.88	0.0069	0.0123	Yes
CXCL15	E15-E17.5	23.13	4.53	0.2227	0.2204	No
CXCL15	E15-E18.5	138.07	7.11	<0.0001	<0.0001	Yes
CXCL15	E17.5-E18.5	5.97	2.58	<0.0001	0.0001	Yes
CXCL16	E15-E17.5	2.69	1.43	0.0164	0.0255	Yes
CXCL16	E15-E18.5	4.82	2.27	<0.0001	0.0003	Yes
CX3CL1	E15-E17.5	2.16	1.11	0.0249	0.0347	Yes
CX3CL1	E15-E18.5	3.53	1.82	0.0002	0.0006	Yes

Table 3-4 Differential Expression of CXC/CX3C-Chemokine Genes in the Embryonic Lung Across Developmental Stages

Fold changes in gene expression of CXC and CX3C-chemokines in the embryonic lung between embryonic timepoints E15, E17.5 and E18.5. Only genes that showed a fold change ≥ 2 (upregulation) or ≤ 0.5 (downregulation) relative to E15.0 or E17.5 and had expression ($2^{-\Delta Ct}$) was ≥ 0.001 for at least one time point were included. Log₂-transformed fold change values (Log2FC) are also presented to facilitate interpretation of upregulation and downregulation on a symmetric scale. For example, a 2-fold upregulation becomes +1 ($\log_2(2) = 1$) and a 2-fold downregulation becomes -1 ($\log_2(0.5) = -1$). Normality was assessed and statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test for normally distributed data ($p < 0.05$) or Dunn's test for non-normally distributed data. As multiple independent statistical tests were performed across different genes, the Benjamini-Hochberg (BH) test was applied to control the false discovery rate. Adjusted p-values (q-values) were significant if $q < 0.05$. Significant q values are indicated as 'YES'; non-significant results are marked 'NO'.

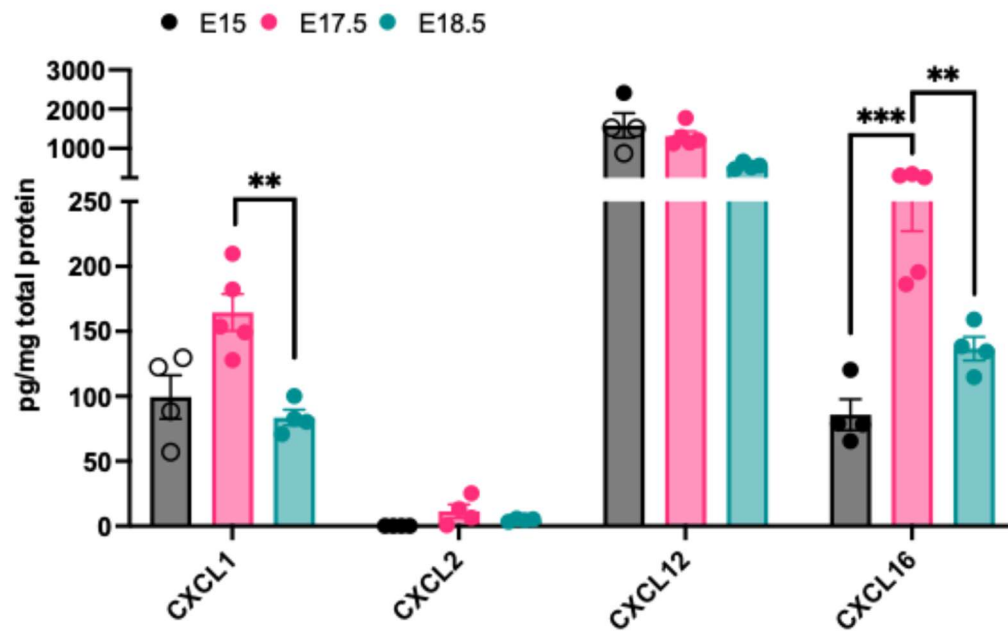


Figure 3-8 CXC-Chemokine Protein Expression in Embryonic Lung

CXC-chemokine protein in total wild type embryonic lung at E15 (black), E17.5 (pink) and E18.5 (teal) was determined by Luminex assay and normalised to total protein (mg), as measured by BCA assay. Each group included $n = 4$ biological replicates for E15 and E18.5, $n = 5$ biological replicates for E17.5. Outliers were identified and excluded using Grubb's test. Transparent data points were extrapolated from below the standard curve. These data were excluded from statistical analysis due to low quantification reliability. Statistical comparisons were performed based on the number of valid, quantifiable time points per analyte. For three valid time points: One-Way ANOVA with Tukey's or Dunnett's post hoc test depending on distribution. Two valid time points: Unpaired t-test or Mann Whitney depending on distribution. One valid time point: No statistical comparison. Asterisks indicate adjusted p values from post hoc tests or p values from unpaired t tests/Mann Whitney: $p/p_{adj} < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****).

3.2.3 Chemokine Expression in the Embryonic Skin

Embryonic skin was also analysed for chemokine expression across the embryonic time points. No RNA expression above the threshold level was detected for CCL2, CCL3, CCL4, CCL5, CCL8, CCL24, or CCL28 in embryonic skin at E15, E17.5, or E18.5 (Figure 3-9A). In contrast, CCL6 was relatively highly expressed at E18.5, increasing from E17.5 by 76.46-fold ($\log_2\text{FC} = 6.26$, $p < 0.0001$) (Table 3-5) and overall, from E15 by 5.61-fold ($p < 0.0001$) (Table 3-5). This suggests that CCL6 may play a prominent role in skin from E18.5 onwards. CCL6 was not included in the Luminex panel due to the unavailability of a validated antibody for this target within the chosen assay kit. As a result, its protein expression in E18.5 skin could not be evaluated.

CCL7 was upregulated at E17.5 with a $\log_2\text{FC}$ increase of 4.24 from E15 ($p = < 0.0001$) (Table 3-5). In addition, CCL9 was expressed in embryonic skin at E15 and E18.5 but appeared to drop significantly by a $\log_2\text{FC}$ of -6.00 from E15 to E17.5 ($p = 0.0008$) (Figure 3-9A and Table 3-5). CCL11 also followed this pattern of expression, with CCL11 RNA being detected above threshold expression at E15 and E18.5, but no expression being observed above the threshold at E17.5. However, wide variation in expression across samples prevented this from being statically significant. In addition, CCL12 began to be expressed above $2^{-\Delta\text{Ct}} = 0.001$ at E17.5 and this was sustained at E18.5 (Figure 3-9A and Table 3-5). Of the non-iCCR ligand CC-chemokines, CCL17, CCL19, CCL20 and CCL22 were not detected above threshold expression values across any of the measured time points (Figure 1-2 and Figure 3-9B). In contrast, CCL25 was expressed at levels above the detection threshold at E15 and E18.5, with a marked downregulation at E17.5, corresponding to a $\log_2\text{FC}$ of -2.29 ($p < 0.0001$) (Table 3-5). This suggests a tightly regulated temporal expression pattern, potentially reflecting recruitment of CCR9⁺ cells in the developing skin. Overall, CC-chemokine RNA levels in the skin were relatively low, especially when compared to the relative expression levels of CXC-chemokines in the skin or CC-chemokine expression in the lung. While absolute direct quantitative comparisons between targets and tissues cannot be made, this observation may indicate a less prominent role for CC-chemokines in the skin, or alternatively, that their expression may be restricted to small, spatially localised cell populations.

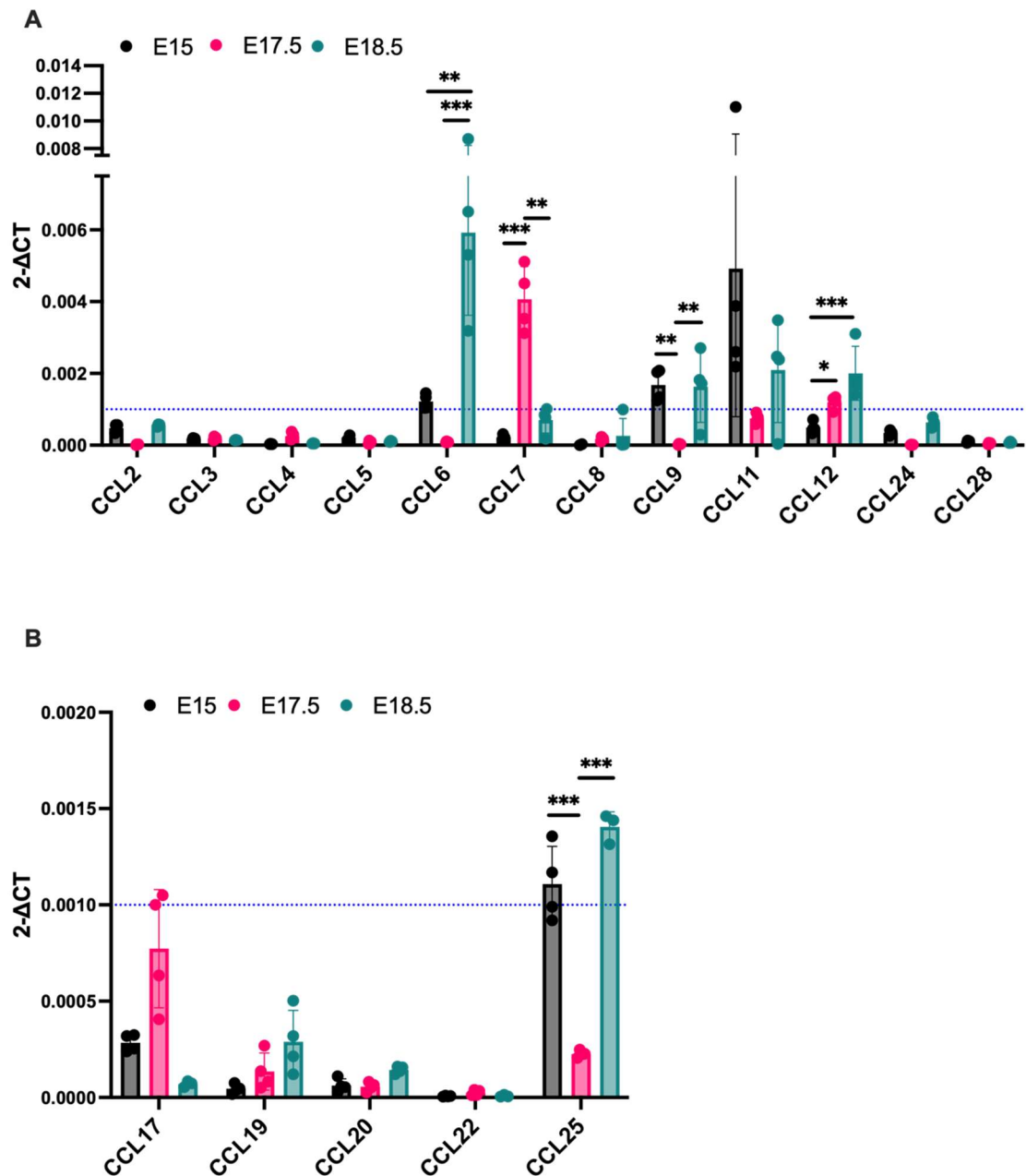


Figure 3-9 CC-Chemokine Transcription in Embryonic Skin

RT-qPCR analysis CC-chemokine transcript expression in embryo skin at E15 (black) E17.5 (pink) and E18.5 (teal). Expression of iCCR chemokine ligands (**A**) and non-iCCR chemokine ligands (**B**) was measured relative to *GAPDH* using the $2^{-\Delta C_t}$ method. Error bars represent SEM. $n = 4$ biological replicates per group. The blue dotted line represents a threshold of $2^{-\Delta C_t} = 0.001$, corresponding to a ΔC_t of ~ 10 between the housekeeping gene and target gene. This threshold was used to indicate robust detection of RNA expression. It should be noted that expression values below this level are not zero and may still reflect true low transcript expression. Statistical analysis details are provided in the Methods and in corresponding table legends. Briefly, normality was assessed, and significance was determined using one-way ANOVA with Tukey's post hoc test for normally distributed data, or Dunn's test for non-normally distributed data. The Benjamini-Hochberg method was used to control the false discovery rate. $p_{\text{adj}} < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****).

Gene	Comparison	Fold Change	Log2FC	Tukey's <i>p</i> .adj value	q-value (BH)	Significant (FDR < 0.05)
CCL6	E15-E17.5	0.07	-3.77	0.2189	0.3522	No
CCL6	E15-E18.5	5.61	2.49	<0.0001	0.0011	Yes
CCL6	E17.5-E18.5	76.46	6.26	0.0004	0.0006	Yes
CCL7	E15-E17.5	18.91	4.24	<0.0001	0.0006	Yes
CCL7	E17.5-E18.5	0.22	-2.22	0.0002	0.0021	Yes
CCL9	E15-E17.5	0.02	-6.00	0.0008	0.0067	Yes
CCL9	E17.5-E18.5	79.30	6.31	0.0003	0.0028	Yes
CCL11	E15-E17.5	0.15	-2.73	0.1101	0.2035	No
CCL11	E17.5-E18.5	3.74	1.90	0.5704	0.6596	No
CCL12	E15-E17.5	2.44	1.29	0.0016	0.0108	Yes
CCL12	E15-E18.5	3.34	1.74	0.0001	0.0013	Yes
CCL25	E15-E17.5	0.20	-2.29	<0.0001	0.0006	Yes
CCL25	E17.5-E18.5	6.21	2.63	<0.0001	0.0004	Yes

Table 3-5 Differential Expression of CC-Chemokine Genes in the Embryonic Skin Across Developmental Stages

Fold changes in gene expression of CC-Chemokines in the embryonic skin between embryonic timepoints E15, E17.5 and E18.5. Only genes that showed a fold change ≥ 2 (upregulation) or ≤ 0.5 (downregulation) relative to E15.0 or E17.5 and had expression ($2^{-\Delta Ct}$) was ≥ 0.001 for at least one time point were included. Log₂-transformed fold change values (Log2FC) are also presented to facilitate interpretation of upregulation and downregulation on a symmetric scale. For example, a 2-fold upregulation becomes +1 ($\log_2(2) = 1$) and a 2-fold downregulation becomes -1 ($\log_2(0.5) = -1$). Normality was assessed and statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test for normally distributed data ($p < 0.05$) or Dunn's test for non-normally distributed data. As multiple independent statistical tests were performed across different genes, the Benjamini-Hochberg (BH) test was applied to control the false discovery rate. Adjusted p-values (q-values) were significant if $q < 0.05$. Significant q values are indicated as 'YES'; non-significant results are marked 'NO'.

This pattern was reflected at the protein level. CCL4 and CCL20 were not detected. CCL5, CCL7 and CCL22 were detected at values below the standard curve and so were not included in analysis for the skin. CCL3 was detected at very low but quantifiable concentrations of below 50 pg/mg in the lung across all measured time points (Figure 3-10). In contrast, CCL11 and CCL12, both of which showed relatively high RNA expression, at levels above the detection threshold, were detected at higher protein levels in embryonic skin (Figure 3-10). CCL11 protein concentration was detected at 69.0 pg/mg at E15 and 67.2 pg/mg at E17.5 but then significantly decreased between E17.5 and E18.5 to 12.0 pg/mg ($p = 0.0230$) ($p = 0.0255$ E15 vs. E18.5) (Figure 3-10).

Notably, CCL12 protein concentration increased significantly from E15 to E17.5, rising from a mean of 50.6 pg/mg to 445.4 pg/mg ($p < 0.0001$), mirroring the onset of CCL12 transcript expression after E15 demonstrated in Figure 3-9A. However, by E18.5, CCL12 protein levels declined to a mean of 169.1 pg/mg ($p = 0.0008$), indicating a peak in expression at E17.5 (Figure 3-10).

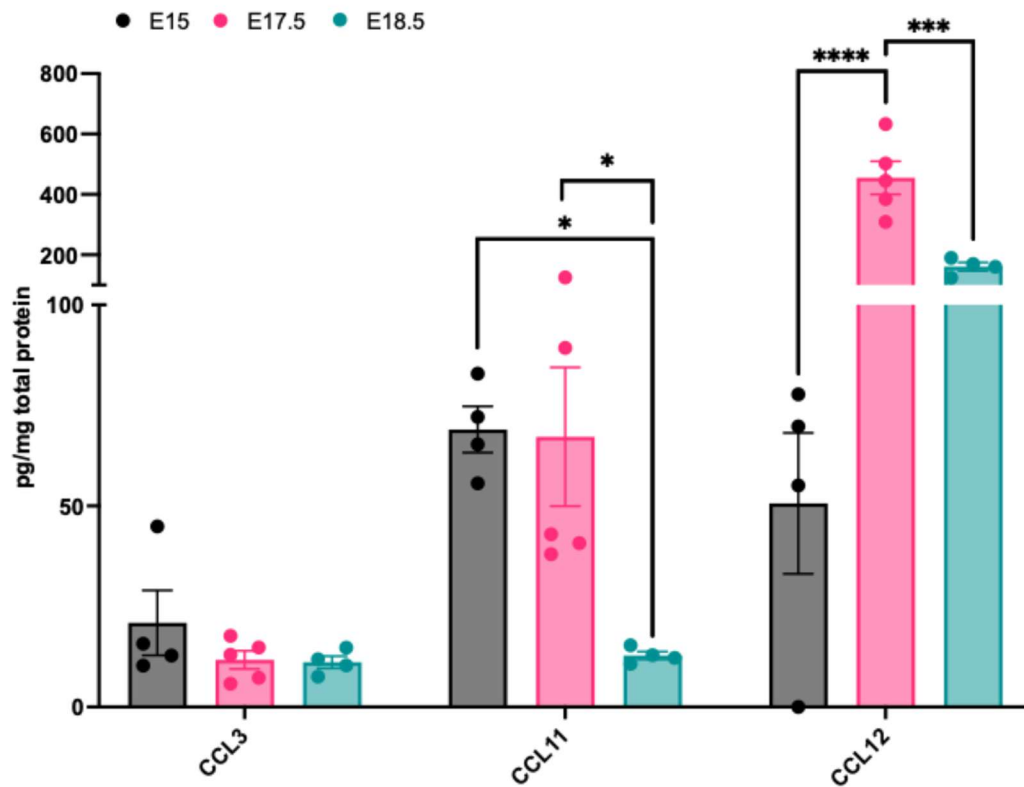


Figure 3-10 CC-Chemokine Protein Expression in Embryonic Skin

CC-chemokine protein in wild type embryonic skin at E15 (black), E17.5 (pink) and E18.5 (teal) was determined by Luminex assay and normalised to total protein (mg), as measured by BCA assay. Each group included $n = 4$ biological replicates for E15 and E18.5, $n = 5$ biological replicates for E17.5. Outliers were identified and excluded using Grubb's test. Statistical comparisons were performed based on the number of valid, quantifiable time points per analyte. For three valid time points: One-Way ANOVA with Tukey's or Dunnett's post hoc test depending on distribution. Two valid time points: Unpaired t-test or Mann Whitney depending on distribution. One valid time point: No statistical comparison. Asterisks indicate adjusted p values from post hoc tests or p values from unpaired t tests/Mann Whitney: $p/p_{adj} < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****).

Figure 3-11 highlights that in embryonic skin, CXCL1, CXCL2, CXCL3, CXCL5, CXCL9, CXCL10, CXCL11, CXCL15, CXCL17, and XCL1 were not expressed above the threshold of $2^{-\Delta Ct} = 0.001$. In contrast, CXCL12 and CXCL14 were consistently detected at relatively high levels across embryonic days E15, E17.5, and E18.5 (Figure 3-11). This underscores the importance of these highly conserved and evolutionarily ancient chemokines in regulating tissue morphology and maintaining homeostasis during embryonic development (Huising et al., 2004).

CX3CL1 and CXCL4 were also expressed at levels above the detection threshold throughout all time points (Figure 3-11). CX3CL1 sustained relatively high transcript expression between E15 and E18.5. CXCL4 expression increased at E17.5 and E18.5, with an overall fold change of 3.62 between E15 and E18.5 ($\log_2FC = 1.86$, $p = 0.0015$) (Table 3-6). Similarly, CXCL15 and CXCL16 showed increased expression between E17.5 and E18.5. CXCL15 exhibited a 14.98-fold increase at E17.5 ($\log_2FC = 3.91$, $p = 0.0039$), while CXCL16 increased by 2.58-fold ($\log_2FC = 1.37$, $p = 0.0083$) (Table 3-6). Despite these increases, expression levels remained relatively low.

At the protein level, CXCL2 and CXCL10 were not detected at quantifiable levels across any of the time points measured. In contrast, Figure 3-12 highlights that CXCL12 was detected at quantifiable levels of 840.2 pg/mg at E17.5, consistent with transcript level data, with mean concentrations of 1140.0 pg/mg at E15, 840.2 pg/mg at E17.5. CXCL16 was also detected at both transcript and protein levels, with protein expression peaking at E17.5, increasing from 73.6 pg/mg to 199.7 pg/mg ($p = 0.0009$) before declining to 102.3 pg/mg at E18.5 ($p = 0.0082$) (Figure 3-12). CXCL1 was detected at low but measurable protein concentrations despite being undetectable at the transcript level. The highest protein concentration for this chemokine analyte was observed at E17.5 (mean = 176.2 pg/mg), significantly higher than at E15 ($p = 0.0369$) and E18.5 ($p = 0.0011$) (Figure 3-12). This discrepancy between transcript and protein expression may be due to limitations in the CXCL1 primers or probes used in the TaqMan array. In contrast, CXCL2 was not detected at any time point at either the transcript or protein level in embryonic skin (Figure 3-11 and Figure 3-12).

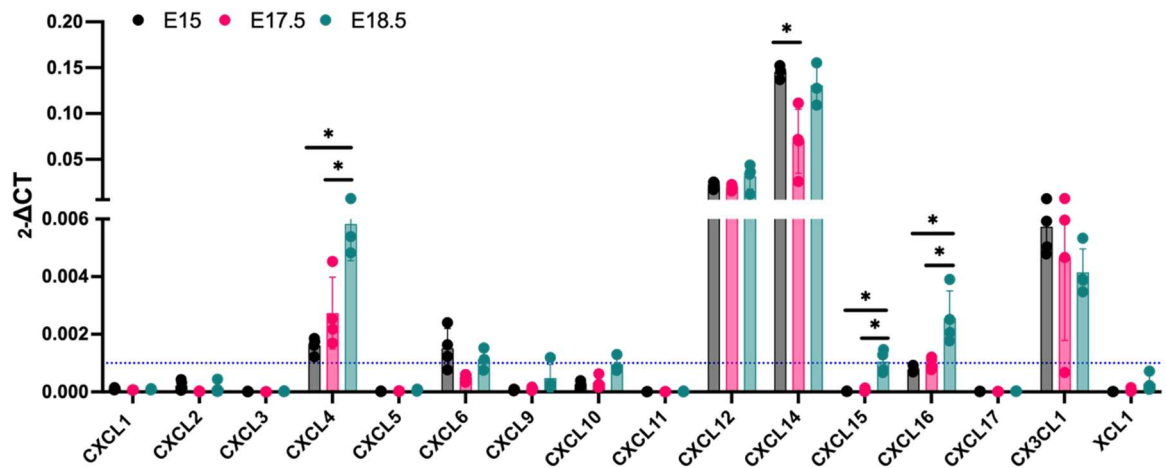


Figure 3-11 CXC/CX3C/XC-Chemokine Transcription in Embryonic Skin

RT-qPCR analysis of CXC/CX3C/XC-chemokine transcript expression in embryo skin at E15 (black) E17.5 (pink) and E18.5 (teal). Expression was measured relative to *GAPDH* using the $2^{-\Delta Ct}$ method. Error bars represent SEM. $n = 4$ biological replicates per group. The blue dotted line represents a threshold of $2^{-\Delta Ct} = 0.001$, corresponding to a ΔCt of ~ 10 between the housekeeping gene and target gene. This threshold was used to indicate robust detection of RNA expression. It should be noted that expression values below this level are not zero and may still reflect true low transcript expression. Statistical analysis details are provided in the Methods and in corresponding table legends. Briefly, normality was assessed, and significance was determined using one-way ANOVA with Tukey's post hoc test for normally distributed data, or Dunn's test for non-normally distributed data. The Benjamini-Hochberg method was used to control the false discovery rate. $p_{adj} < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****).

Gene	Comparison	Fold Change	Log2FC	Tukey's <i>p</i> .adj value	q-value (BH)	Significant (FDR < 0.05)
CXCL4	E15-E18.5	3.62	1.86	0.0015	0.0108	Yes
CXCL4	E17.5-E18.5	2.13	1.09	0.0096	0.0320	Yes
CXCL6	E15-E17.5	0.34	-1.57	0.0409	0.0987	No
CXCL6	E17.5-E18.5	2.23	1.16	0.2505	0.3752	No
CXCL14	E15-E17.5	0.48	-1.06	0.0173	0.0485	Yes
CXCL15	E15-E17.5	3.33	1.73	0.9705	0.9044	No
CXCL15	E15-E18.5	49.85	5.64	0.0030	0.0172	Yes
CXCL15	E17.5-E18.5	14.98	3.91	0.0039	0.0189	Yes
CXCL16	E15-E18.5	3.33	1.73	0.0037	0.0189	Yes
CXCL16	E17.5-E18.5	2.58	1.37	0.0083	0.0288	Yes

Table 3-6 Differential Expression of CXC-Chemokine Genes in the Embryonic Skin Across Developmental Stages

Fold changes in gene expression of CXC-chemokines in the embryonic skin between embryonic timepoints E15, E17.5 and E18.5. Only genes that showed a fold change ≥ 2 (upregulation) or ≤ 0.5 (downregulation) relative to E15.0 or E17.5 and had expression ($2^{-\Delta C_t}$) was ≥ 0.001 for at least one time point were included. Log₂-transformed fold change values (Log2FC) are also presented to facilitate interpretation of upregulation and downregulation on a symmetric scale. For example, a 2-fold upregulation becomes +1 ($\log_2(2) = 1$) and a 2-fold downregulation becomes -1 ($\log_2(0.5) = -1$). Normality was assessed and statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test for normally distributed data ($p < 0.05$) or Dunn's test for non-normally distributed data. As multiple independent statistical tests were performed across different genes, the Benjamini-Hochberg (BH) test was applied to control the false discovery rate. Adjusted p-values (q-values) were significant if $q < 0.05$. Significant q values are indicated as 'YES'; non-significant results are marked 'NO'

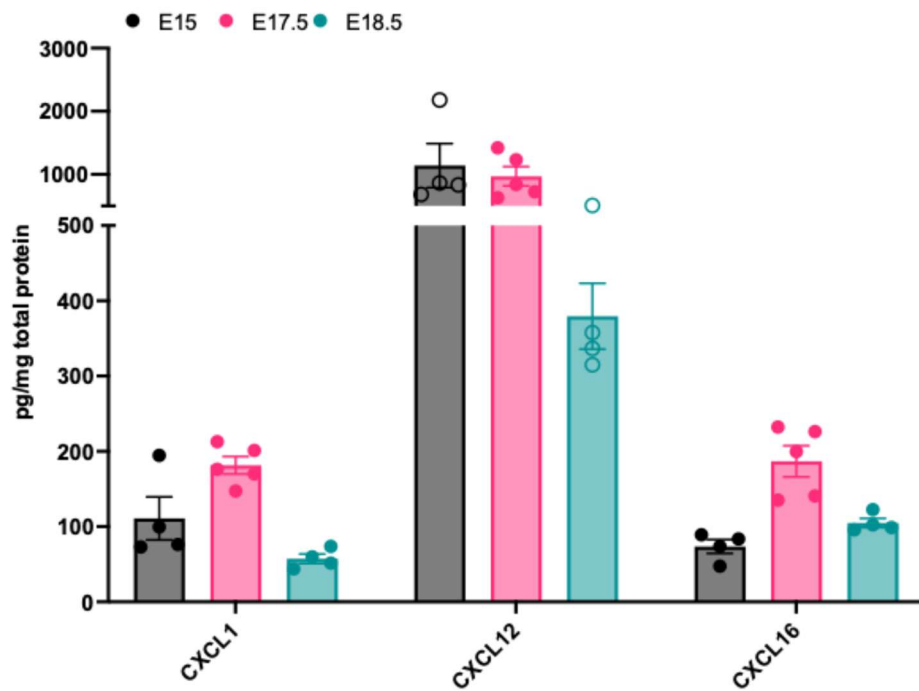


Figure 3-12 CXC-Chemokine Protein Expression in Embryonic Skin

CXC-chemokine protein in wild type embryonic skin at E15 (black), E17.5 (pink) and E18.5 (teal) was determined by Luminex assay and normalised to total protein (mg), as measured by BCA assay. Each group included $n = 4$ biological replicates for E15 and E18.5, $n = 5$ biological replicates for E17.5. Outliers were identified and excluded using Grubb's test. Transparent data points were extrapolated from below the standard curve. These data were excluded from statistical analysis due to low quantification reliability. Statistical comparisons were performed based on the number of valid, quantifiable time points per analyte. For three valid time points: One-Way ANOVA with Tukey's or Dunnett's post hoc test depending on distribution. Two valid time points: Unpaired t-test or Mann Whitney depending on distribution. One valid time point: No statistical comparison. Asterisks indicate adjusted p values from post hoc tests or p values from unpaired t tests/Mann Whitney: $p/p.adj < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****).

3.2.4 Chemokine Expression in the Embryonic Liver

CCL2, CCL3, CCL4, CCL7, CCL11, CCL12, and CCL28 RNA was not detectable in the embryonic liver at E15, E17.5, or E18.5. Among the iCCR ligands with mean expression values above the threshold, large standard deviations were observed, suggesting that these signals may represent background noise rather than true gene expression (Figure 3-13A). No statistically significant changes in any iCCR chemokine ligand expression were detected across the three time points in the embryonic liver (Figure 3-13A and Table 3-7). Additionally, none of the non-iCCR ligands measured were expressed at levels above the detection threshold at any of the analysed stages in the embryonic liver (Figure 3-13B).

This was mostly recapitulated at the protein level as CCL4 and CCL20 were not detected and CCL7, CCL19 and CCL22 could not be detected at quantifiable levels across any of the analysed time points in this tissue. In addition, CCL3 was detected at below 50 pg/mg across all time points and although CCL11 displayed a mean concentration of 92.9 pg/mg at E17.5, high variation between samples suggests that this expression may not be robust (Figure 3-14). These levels are also relatively lower than CCL11 expression for example in the embryonic lung which reached 891.9pg/mg (Figure 3-14). Figure 3-14 also indicates that CCL12 appears to become expressed at E17.5 increasing from 7.4 pg/mg at E15 127.8 pg/mg ($p = 0.0008$) then decreases to 61.5 pg at E18.5 ($p = 0.0343$). However, CCL5 was detected in the embryonic liver at 332.8 pg/mg at E17.5 and 242.9 pg/mg at E18.5 (Figure 3-14). The liver was the only tissue analysed in which quantifiable values were obtained for CCL5 expression.

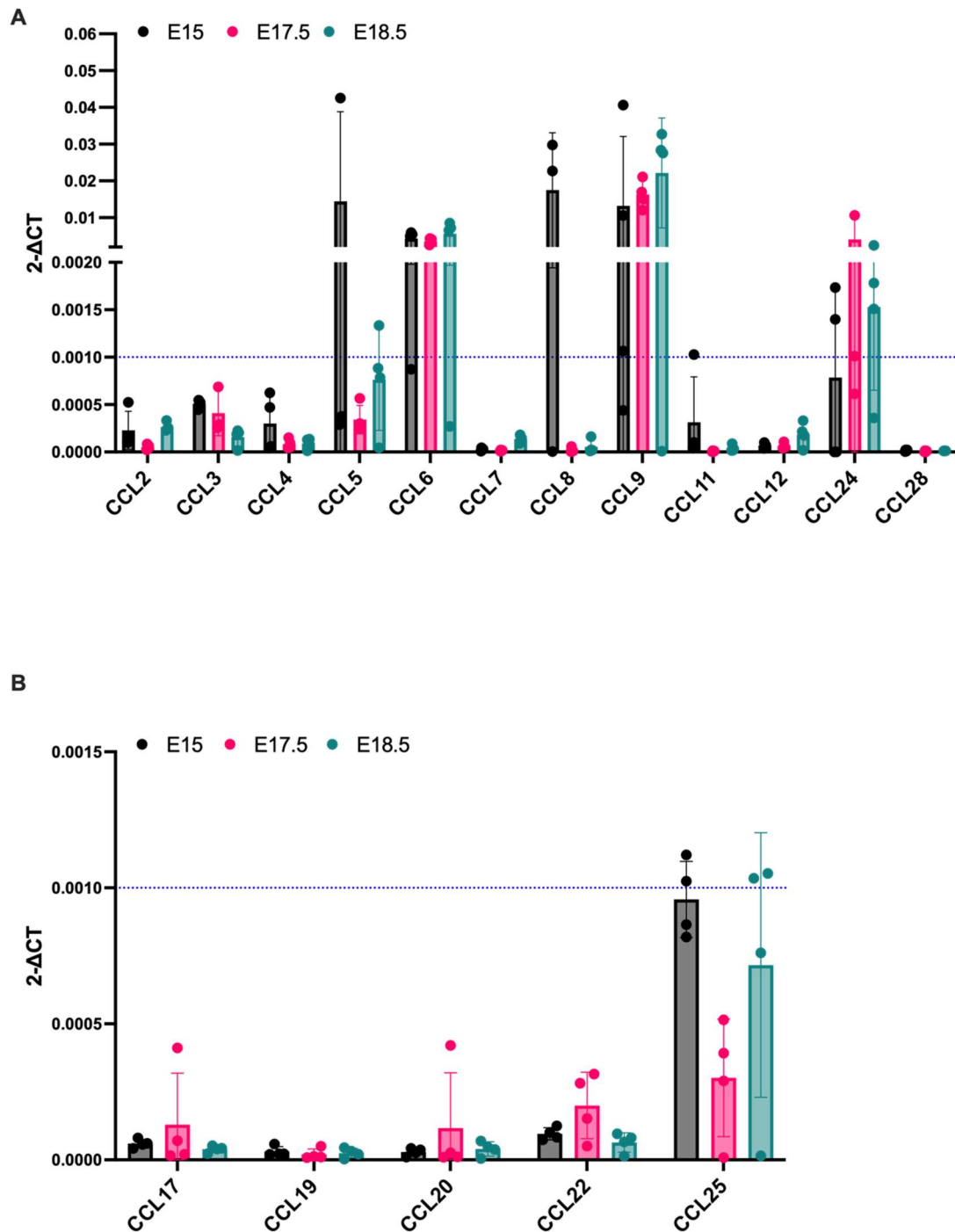


Figure 3-13 CC-Chemokine Transcription in Embryonic Liver

RT-qPCR analysis of CC-chemokine transcript expression in embryo liver at E15 (black) E17.5 (pink) and E18.5 (teal). Expression of iCCR chemokine ligands (**A**) and non-iCCR chemokine ligands (**B**) was measured relative to *GAPDH* using the $2^{-\Delta C_t}$ method. Error bars represent SEM. $n = 4$ biological replicates per group. The blue dotted line represents a threshold of $2^{-\Delta C_t} = 0.001$, corresponding to a ΔC_t of ~ 10 between the housekeeping gene and target gene. This threshold was used to indicate robust detection of RNA expression. It should be noted that expression values below this level are not zero and may still reflect true low transcript expression. Statistical analysis details are provided in the Methods and in corresponding table legends. Briefly, normality was assessed, and significance was determined using one-way ANOVA with Tukey's post hoc test for normally distributed data, or Dunn's test for non-normally distributed data. The Benjamini-Hochberg method was used to control the false discovery rate. $p_{\text{adj}} < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****).

Gene	Comparison	Fold Change	Log2FC	Tukey's <i>p</i> .adj value	q-value (BH)	Significant (FDR < 0.05)
CCL8	E15-E17.5	2.74	1.45	1.0000	1.0000	No
CCL8	E15-E18.5	0.0011	-9.80	0.0442	0.2004	No
CCL8	E17.5-E18.5	0.0031	-8.35	0.0445	0.2004	No
CCL24	E15-E18.5	5.21	2.38	0.3538	1.0000	No

Table 3-7 Differential Expression of CC-Chemokine Genes in the Embryonic Liver Across Developmental Stages

Fold changes in gene expression of CC-Chemokines in the embryonic liver between embryonic timepoints E15, E17.5 and E18.5. Only genes that showed a fold change ≥ 2 (upregulation) or ≤ 0.5 (downregulation) relative to E15.0 or E17.5 and had expression ($2^{-\Delta Ct}$) was ≥ 0.001 for at least one time point were included. Log₂-transformed fold change values (Log2FC) are also presented to facilitate interpretation of upregulation and downregulation on a symmetric scale. For example, a 2-fold upregulation becomes +1 ($\log_2(2) = 1$) and a 2-fold downregulation becomes -1 ($\log_2(0.5) = -1$). Normality was assessed and statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test for normally distributed data ($p < 0.05$) or Dunn's test for non-normally distributed data. As multiple independent statistical tests were performed across different genes, the Benjamini-Hochberg (BH) test was applied to control the false discovery rate. Adjusted p-values (q-values) were significant if $q < 0.05$. Significant q values are indicated as 'YES'; non-significant results are marked 'NO'

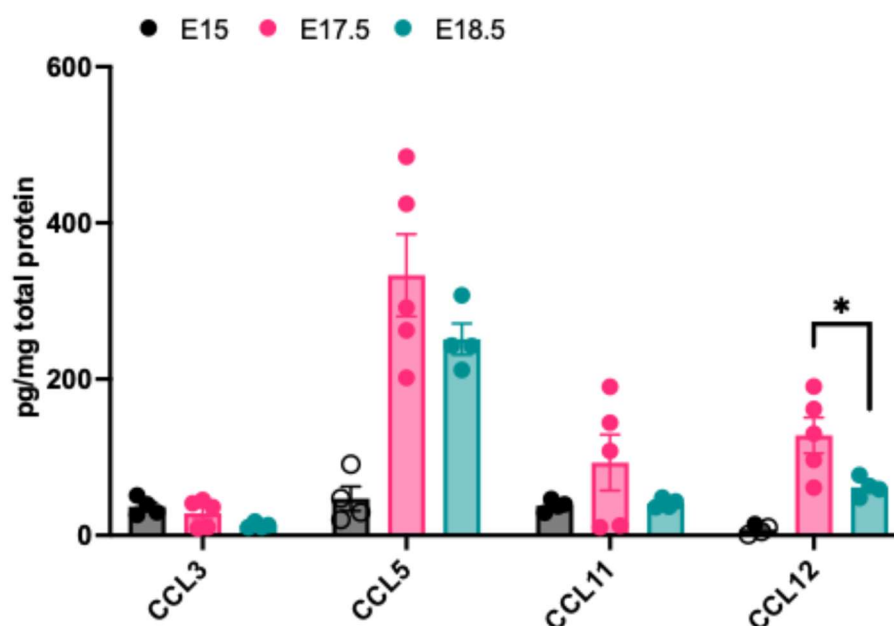


Figure 3-14 CC-Chemokine Protein Expression in Embryonic Liver

CC-chemokine protein in total wild type embryonic liver at E15 (black), E17.5 (pink) and E18.5 (teal) was determined by Luminex assay and normalised to total protein (mg), as measured by BCA assay. Each group included $n = 4$ biological replicates for E15 and E18.5, $n = 5$ biological replicates for E17.5. Outliers were identified and excluded using Grubb's test. Transparent data points were extrapolated from below the standard curve. These data were excluded from statistical analysis due to low quantification reliability. Statistical comparisons were performed based on the number of valid, quantifiable time points per analyte. For three valid time points: One-Way ANOVA with Tukey's or Dunnett's post hoc test depending on distribution. Two valid time points: Unpaired t-test or Mann Whitney depending on distribution. One valid time point: No statistical comparison. Asterisks indicate adjusted p values from post hoc tests or p values from unpaired t tests/Mann Whitney: $p/p_{adj} < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****).

In contrast, various CXC chemokines were expressed in the embryonic liver across the three developmental stages analysed (E15, E17.5, and E18.5 (Figure 3-15)). Insufficient amplification (amplification in only < 3 samples) occurred for CXCL9, CXCL11, CXCL15 and XCL1. In contrast, CXCL6 and CXCL10 consistently showed $2^{-\Delta Ct}$ expression levels greater than 0.001 across all time points (Figure 3-15). CXCL6 signals via interaction with receptors CXCR1 and CXCR2 to regulate neutrophil migration (Wuyts et al., 1997). In the liver, CXCL6 is predominantly secreted by liver sinusoidal endothelial cells (LSECs), specialized endothelial cells (Liu et al., 2021a). LSECs also secrete CXCL1, 3, 5, in adult mice but release much less CXCL2 than hepatocytes in the liver (Liu et al., 2021a). Therefore, LSECs represent a potential source for the CXCL6 measured in the liver throughout the developmental time frame. The absence of detectable CXCL2 expression in the skin at embryonic days E15, E17.5, and E18.5 suggests that liver sinusoidal endothelial cells (LSECs) may be the principal source of CXC chemokines during this developmental window.

CXCL4 and CXCL12 were also expressed throughout, exhibiting a reduction in expression at E17.5, before returning to E15 levels by E18.5 (Table 3-8). CXCL12 decreased by a fold change of 0.48 ($\log_2FC = -1.06$) between E15 and 17.5 ($p = 0.0002$) before increasing again at E18.5 ($p = <0.0001$). Between E15 and E17.5, CXCL4 expression decreased by a fold change of 0.29 ($\log_2FC = -1.8$, $p < 0.0001$), while CXCL12 decreased by a fold change of 0.48 ($\log_2FC = -1.06$, $p = 0.0002$) (Figure 3-15 and Table 3-8). Furthermore, CXCL16 increased by a fold change of 4.05 ($\log_2FC = 2.02$, $p < 0.0001$) between E17.5 and E18.5, with $2^{-\Delta Ct}$ expression levels exceeding the 0.001 threshold at E18.5 (Figure 3-15 and Table 3-8). Conversely, CXCL1, CXCL2, CXCL3, CXCL5, and CXCL14 transcripts were not expressed in the embryonic liver at any of the analysed stages (Figure 3-15).

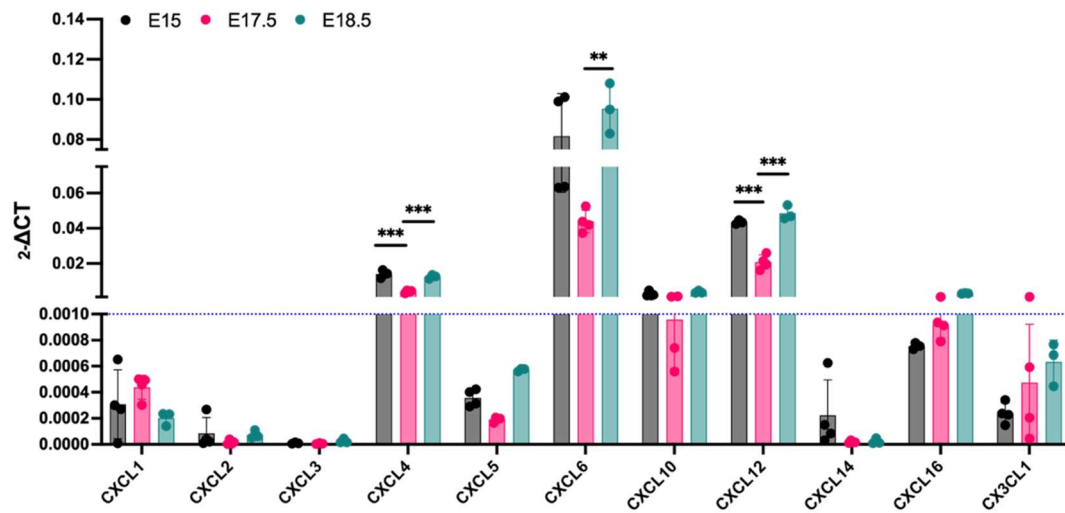


Figure 3-15 CXC/CX3C-Chemokine Transcription in Embryonic Liver

RT-qPCR analysis of CXC/CX3C-chemokine transcript expression in embryo liver at E15 (black) E17.5 (pink) and E18.5 (teal). Expression was measured relative to *GAPDH* using the $2^{-\Delta C_t}$ method. Error bars represent SEM. $n = 4$ biological replicates per group. The blue dotted line represents a threshold of $2^{-\Delta C_t} = 0.001$, corresponding to a ΔC_t of ~ 10 between the housekeeping gene and target gene. This threshold was used to indicate robust detection of RNA expression. It should be noted that expression values below this level are not zero and may still reflect true low transcript expression. Statistical analysis details are provided in the Methods and in corresponding table legends. Briefly, normality was assessed, and significance was determined using one-way ANOVA with Tukey's post hoc test for normally distributed data, or Dunn's test for non-normally distributed data. The Benjamini-Hochberg method was used to control the false discovery rate. $p_{\text{adj}} < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****).

Gene	Comparison	Fold Change	Log2FC	Tukey's <i>p</i> .adj value	q-value (BH)	Significant (FDR < 0.05)
CXCL4	E15-E17.5	0.29	-1.80	<0.0001	0.0003	Yes
CXCL4	E17-E18.5	3.10	1.63	0.0001	0.0013	Yes
CXCL6	E17-E18.5	2.17	1.12	0.0050	0.337	No
CXCL10	E15-E17.5	0.36	-1.46	0.0813	0.3291	No
CXCL10	E17.5-E18.5	4.01	2.00	0.0097	0.0602	No
CXCL12	E15-E17.5	0.48	-1.06	0.0002	0.5991	No
CXCL12	E17-E18.5	2.33	1.22	<0.0001	0.0016	Yes
CXCL16	E15-E18.5	4.05	2.02	<0.0001	<0.0001	Yes
CXCL16	E17.5-E18.5	3.33	1.74	<0.0001	<0.0001	Yes

Table 3-8 Differential Expression of CXC-Chemokine Genes in the Embryonic Liver Across Developmental Stages

Fold changes in gene expression of CXC-Chemokines in the embryonic skin between embryonic timepoints E15, E17.5 and E18.5. Only genes that showed a fold change ≥ 2 (upregulation) or ≤ 0.5 (downregulation) relative to E15.0 or E17.5 and had expression ($2^{-\Delta Ct}$) was ≥ 0.001 for at least one time point were included. Log₂-transformed fold change values (Log2FC) are also presented to facilitate interpretation of upregulation and downregulation on a symmetric scale. For example, a 2-fold upregulation becomes +1 ($\log_2(2) = 1$) and a 2-fold downregulation becomes -1 ($\log_2(0.5) = -1$). Normality was assessed and statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test for normally distributed data ($p < 0.05$) or Dunn's test for non-normally distributed data. As multiple independent statistical tests were performed across different genes, the Benjamini-Hochberg (BH) test was applied to control the false discovery rate. Adjusted p-values (q-values) were significant if $q < 0.05$. Significant q values are indicated as 'YES'; non-significant results are marked 'NO'

At the protein level, CXCL2 was not detected at levels above the standard curve across any time point and CXCL10 did not surpass the LLOQ in embryonic liver across any time points. In contrast, Figure 3-16 shows that CXCL1 was detected at E15, E17.5 and E18.5, increasing sequentially from 75.9 pg/mg at E15 to 176.6 pg/mg at E17.5 ($p = 0.0134$) and then to 230.5 pg/mg at E18.5 ($p = 0.0011$). However, expression was still relatively low. In addition, CXCL12 was detected at the protein level across E15, E17.5, and E18.5 (Figure 3-16); however, the pattern of change in expression levels observed at the RNA level was not mirrored at the protein level and the concentration of CXCL12 protein decreased in the embryonic liver (Figure 3-15 and Figure 3-16). CXCL12 was found at a lower protein concentration in the liver at E17.5 and E18.5 compared to tissues such as the heart and the lung, with highest expression at E17.5 reaching 565.7 pg/mg which decreased to 290.6 pg/mg at E18.5 (Figure 3-16). Finally, expression of CXCL16 was also detected at the protein level in the embryonic liver at E17.5, where it peaks at 272.1 pg/mg rising significantly from E15 ($p = 0.0055$) and falling again at E18.5 ($p = 0.0225$) (Figure 3-16).

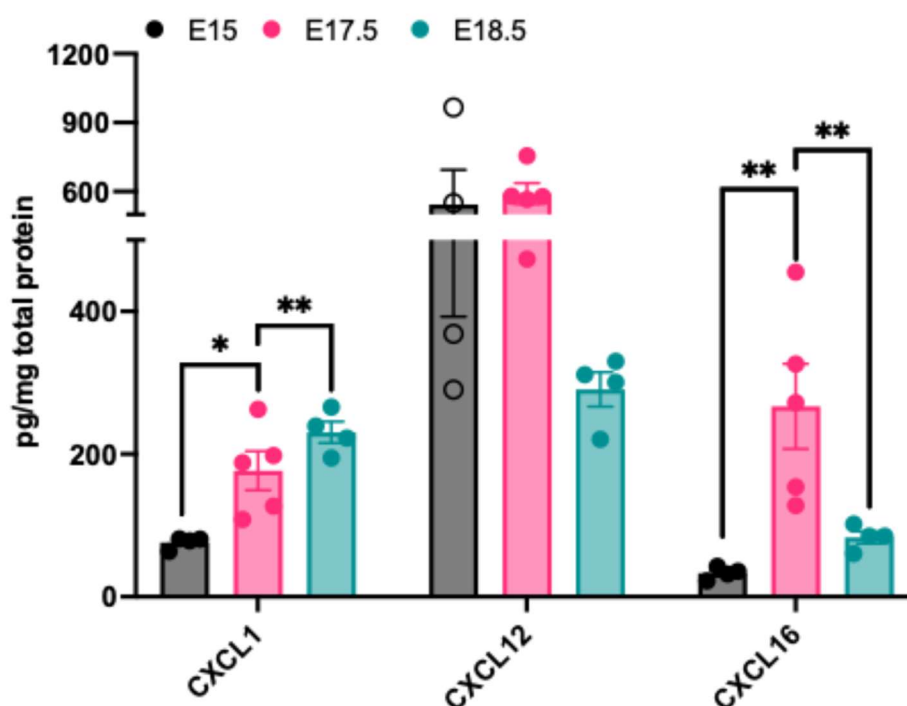


Figure 3-16 CXC-Chemokine Protein Expression in Embryonic Liver

CXC-chemokine protein in total wild type embryonic liver at E15 (black), E17.5 (pink) and E18.5 (teal) was determined by Luminex assay and normalised to total protein (mg), as measured by BCA assay. Each group included $n = 4$ biological replicates for E15 and E18.5, $n = 5$ biological replicates for E17.5. Outliers were identified and excluded using Grubb's test. Transparent data points were extrapolated from below the standard curve. These data were excluded from statistical analysis due to low quantification reliability. Statistical comparisons were performed based on the number of valid, quantifiable time points per analyte. For three valid time points: One-Way ANOVA with Tukey's or Dunnett's post hoc test depending on distribution. Two valid time points: Unpaired t-test or Mann Whitney depending on distribution. One valid time point: No statistical comparison. Asterisks indicate adjusted p values from post hoc tests or p values from unpaired t tests/Mann Whitney: $p/p.adj < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****).

Overall, chemokines appear to be selectively expressed in specific tissues at precise developmental time points during embryogenesis. The key findings are summarised in Figure 3-17. CXCL12 was consistently highly expressed across all tissues and time points whereas other chemokines displayed tissue-specific expression. For example, in the heart, CX3CL1 decreased from E15 to E18.5, whilst CXCL12 remained stable (Figure 3-3). In the lung, CCL6, CCL12, CXCL14, CXCL15 increased toward E18.5 (Figure 3-5 and Figure 3-7) whilst CCL11 peaked sharply at E17.5 (Figure 3-5 and Figure 3-6). In the skin, CCL7 and CXCL4 were transiently upregulated at E17.5 (Figure 3-9 and Figure 3-11) and in the liver, CXCL6 peaked at E18.5. Overall, these data highlight that chemokine expression is both tissue-specific and temporally regulated during late embryonic development.

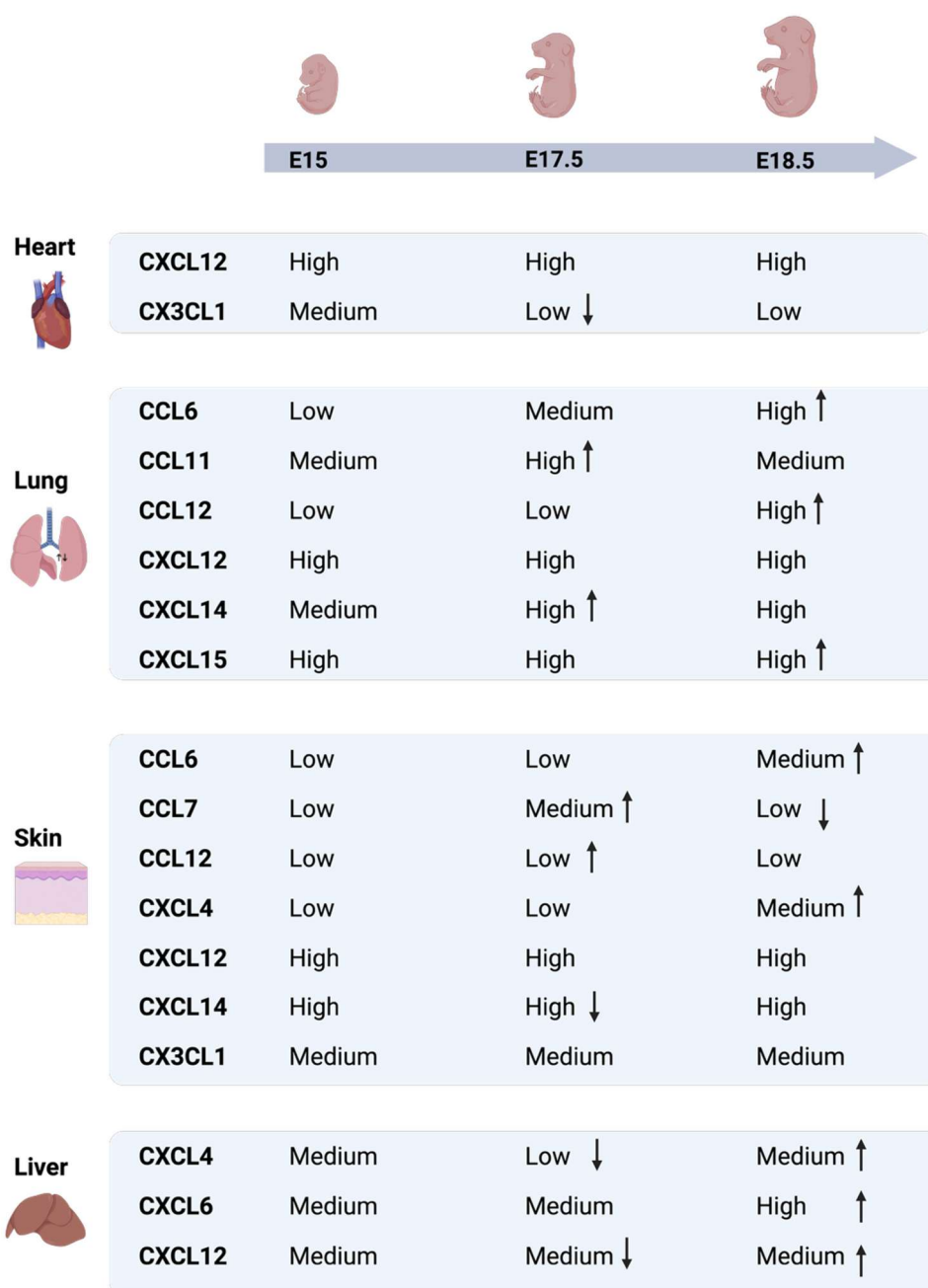


Figure 3-17 Summary of Chemokine Expression Pattern in Embryonic Heart, Lung, Liver and Skin Across E15, E17.5 and E18.5

Summary of the relative expression level of selected chemokines in embryonic heart, lung, skin, and liver across E15, E17.5 and E18.5. Expression level is categorised as low, medium, or high based on relative abundance within each tissue. Arrows represent statistically significant changes in transcript expression in comparison with the preceding time point. Created using BioRender.com.

3.3 Chemokine Receptor Expression

The chemokine expression data presented throughout this chapter so far reflects whole tissue analysis. Therefore, to align chemokine ligand expression with the presence of specific CCR-expressing leukocyte populations, we next examined inflammatory chemokine receptor expression (iCCRs: CCR1, CCR2, CCR3, and CCR5) at the level of individual cell types within embryonic tissues (lung, skin, liver and spleen). Given the previous finding that ACKR2 regulates the migration of a distinct macrophage subpopulation in embryonic skin (Lee *et al.*, 2019), this analysis focused on myeloid cells, which are key expressers of iCCRs and respond to iCCR ligands (Medina-Ruiz *et al.*, 2022). iCCR expression in the embryonic lung, skin, liver, and spleen at embryonic day 17.5 (E17.5) was evaluated using engineered fluorescent reporter mice. These reporter mice carry a construct encoding the iCCR locus, in which the *Ccr* genes have been replaced with spectrally-distinct fluorescent reporter genes: *CCR1* with Clover, *CCR2* with mRuby2, *CCR3* with mTagBFP2, and *CCR5* with iRFP682 (Medina-Ruiz *et al.*, 2022), allowing for the detection of inflammatory chemokine receptors on individual cell subsets by flow cytometry. The embryonic heart was not included in this analysis due to the low number of myeloid cells isolated.

3.3.1 Chemokine Receptor Expression in E17.5 Lung Myeloid Populations

Figure 3-18 depicts the gating strategy used to identify myeloid cell populations in the E17.5 lung. All cells were gated as single cells, live cells, CD45⁺, CD19⁻, Ly6G⁻, TCR⁻, SiglecF⁻. Monocytes were CD11b⁺ F4/80⁻ and classified as either Ly6C^{low} or Ly6C^{high}. Non-alveolar macrophages were Ly6C^{low/-} and CD11b⁺ F4/80⁺.

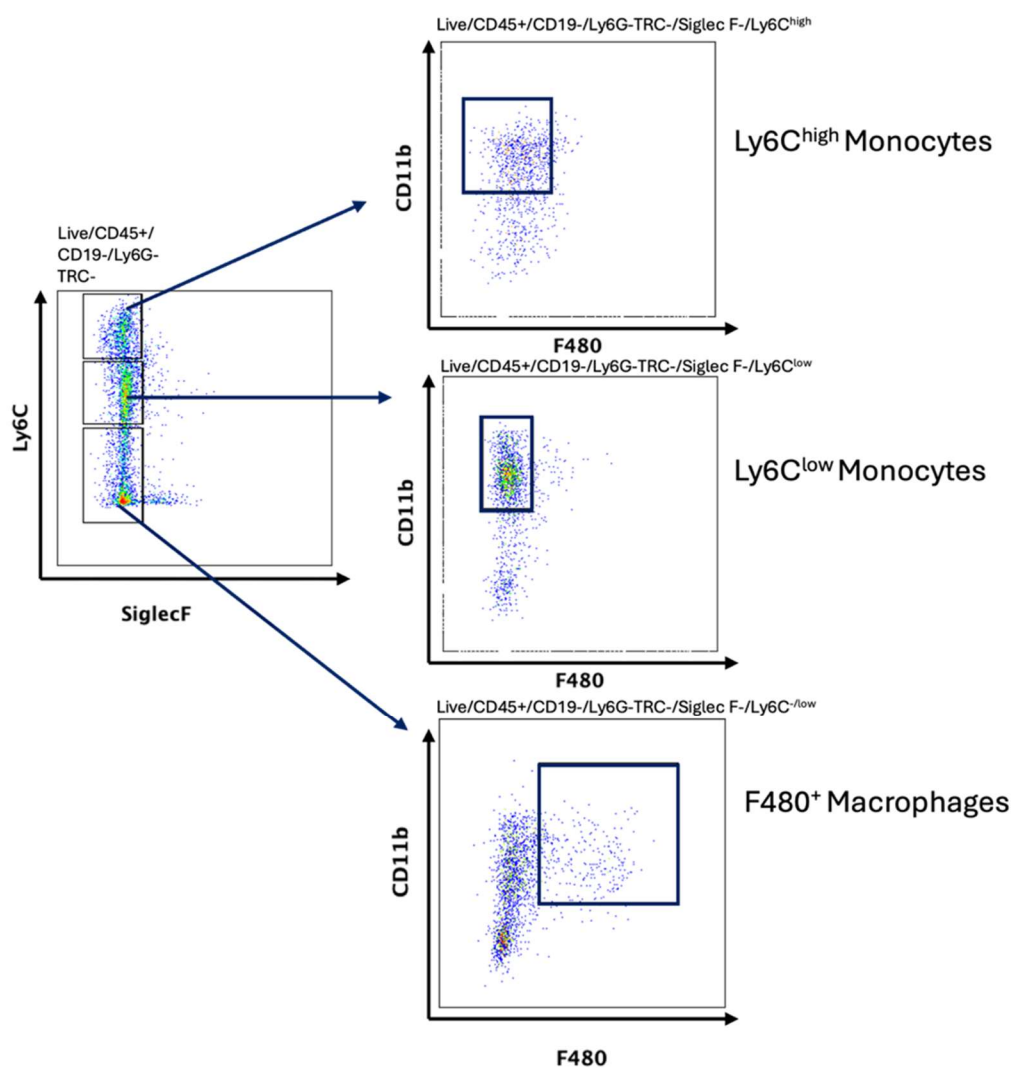


Figure 3-18 Representative Gating Strategy of Myeloid Cells in Embryonic Lung at E17.5

All cells were gated as single cells, live cells, CD45⁺, CD19⁻, Ly6G⁻, TCR⁻, SiglecF⁻. Monocytes were CD11b⁺, F4/80⁻ and classified as either Ly6C^{low} or Ly6C^{high}. Non-alveolar, F4/80⁺ macrophages were Ly6C^{low/-} and CD11b⁺ F4/80⁺.

In the E17.5 lung, 74.1 % of Ly6C^{high} monocytes were CCR2⁺ (Figure 3-19A.i) including 71.4 % that expressed only CCR2 (Figure 3-19A.ii). Only 2.2 % of Ly6C^{high} monocytes co-expressed CCR2 and CCR5, with CCR5 expression occurring almost exclusively in CCR2⁺ cells (Figure 3-19A.i). This expression pattern reflects the classical monocyte subset. In contrast, the majority of Ly6C^{low} monocytes in the embryonic lung lacked expression of all tested chemokine receptors (82.2 % iCCR⁻). Among Ly6C^{low} monocytes, 17.2 % were CCR2⁺ (Figure 3-19B.i) and 4.1 % co-expressed CCR2 and CCR5 (Figure 3-19B.ii), again with all CCR5 expression co-occurring with CCR2. Whilst CCR2 was the most highly expressed iCCR in embryonic lung monocytes, CCR5 was the predominant receptor expressed by F4/80⁺ E17.5 lung macrophages, as 53.3 % of lung macrophages were CCR5⁺ (Figure 3-19C.i). Nonetheless, 30.5 % of macrophages expressed CCR2 (Figure 3-19C.i), including 17.9 % that co-expressed both CCR2 and CCR5 (Figure 3-19C.ii). CCR3 was not expressed across any of the cell types in the lung at E17.5 (Figure 3-19) and throughout iCCR analysis across all embryonic tissues was not included in combinatorial analysis due to consistently low detection.

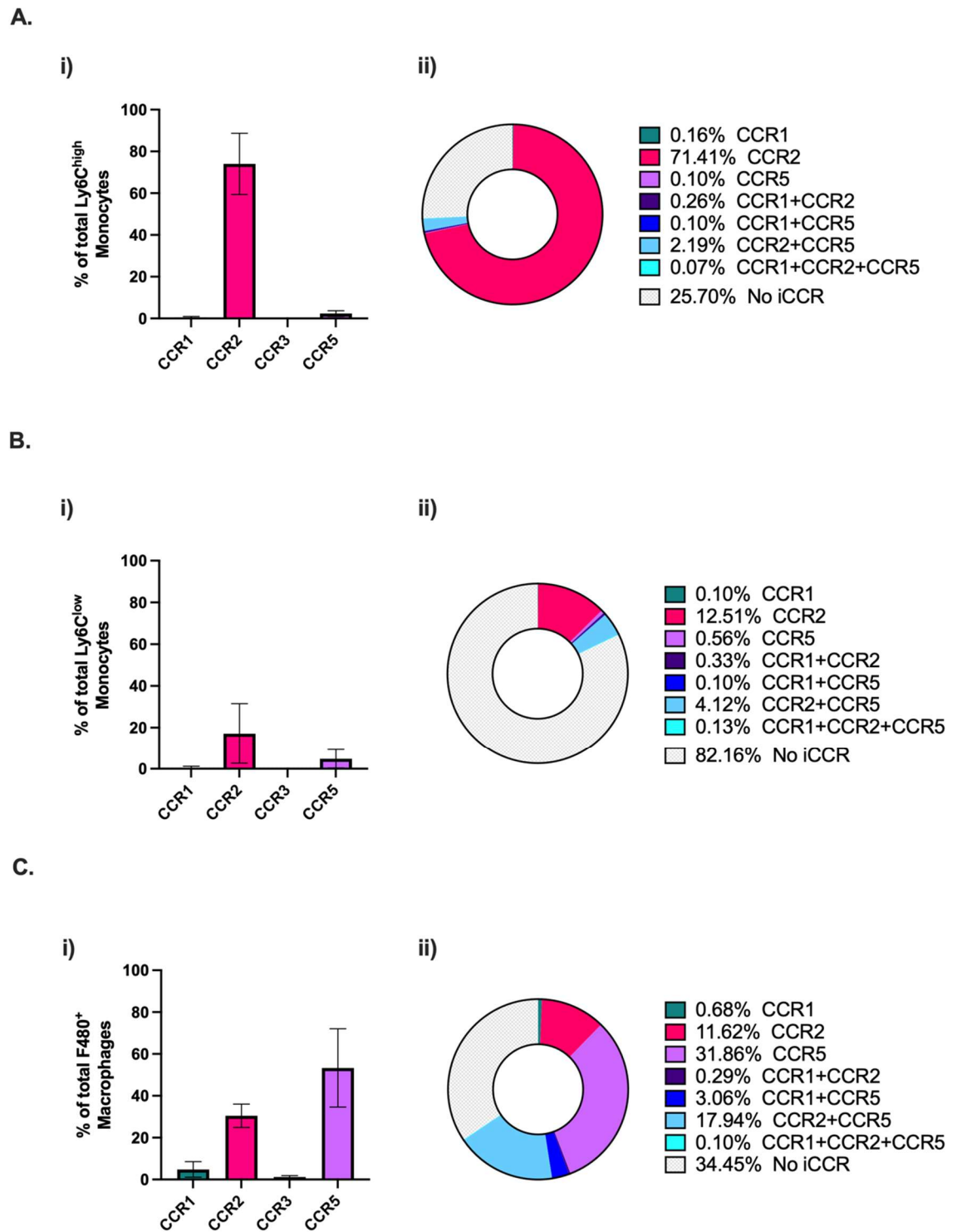


Figure 3-19 iCCR Expression Across Myeloid Cells in Total Embryonic Lung at E17.5

The percentage of Ly6C^{high} monocytes (Ai), Ly6C^{low} monocytes (Bi) and F4/80^{high} macrophages (Ci) expressing CCR1 (teal), CCR2 (pink), CCR3 (black) and CCR5 (purple) in embryonic lung at E17.5 was determined by flow cytometry using iCCR fluorescent reporter embryo mice, wild type embryos were used as FMOs controls (reporter embryos $n = 4$, wild type embryos, $n = 1$). Error bars show SD. iCCR co-expression across Ly6C^{high} monocytes (Aii), Ly6C^{low} monocytes (Bii) and F4/80^{high} macrophages (Cii) in embryonic lung at E17.5 is summarised using doughnut charts, key indicates colour corresponding with co-expression patterns.

Although CCL11, a high affinity ligand for CCR3, was detected at both the RNA and protein levels in the E17.5 lung (Figure 3-5 and Figure 3-5), CCR3 surface expression could not be detected by flow cytometry using reporter mice (Figure 3-19). Therefore, iCCR transcript expression in the E17.5 lung, including CCR3 expression, was analysed to determine whether CCR3 is expressed in the total E17.5 lung (Figure 3-20). However, CCR3 transcript was not detected above a $2^{-\Delta CT}$ value of 0.001, with a mean $2^{-\Delta CT}$ of 7.47×10^{-5} , suggesting that CCL11 may be interacting with an alternative receptor in the embryonic lung at E17.5. Among the iCCRs, CCR2 was the most highly expressed, followed by CCR1, which was detected at higher levels than CCR5 (Figure 3-20). Figure 3-19 showed that a greater percentage of F4/80⁺ macrophages in the E17.5 lung expressed surface CCR5 than CCR1. This disparity could reflect CCR1 expression by other leukocyte subsets within the lung or could be explained by the repeated observation by our group that there is a discrepancy between CCR1 transcript and protein levels (data not shown), likely due to post-transcriptional regulation.

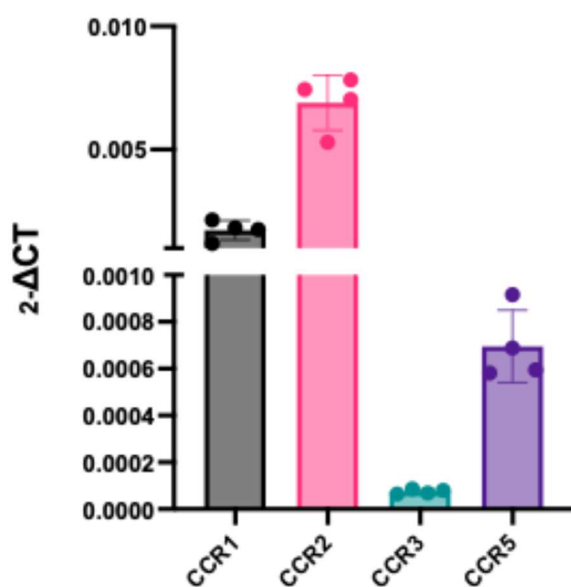


Figure 3-20 qRT-PCR Analysis of iCCR Gene Expression in Total Embryo Lung at E17.5

Expression of CCR1 (black), CCR2 (pink), CCR3 (teal) and CCR5 (purple) was measured relative to *GAPDH* expression using the ΔC_t method. Error bars represent SEM. $n = 4$ biological replicates per group.

3.3.2 Chemokine Receptor Expression in E17.5 Skin Myeloid Populations

iCCR expression was also analysed in embryonic skin. The gating strategy used to distinguish Ly6C^{high} monocytes, F4/80^{high} and F4/80^{low} macrophages, as well as CD11b^{low} macrophages, is shown in Figure 3-21. Similar to observations in the lung, Ly6C^{high} monocytes in E17.5 skin were primarily CCR2⁺, with 89.6 % of total monocytes expressing this receptor (Figure 3-22A.i). The most common expression pattern was CCR2 only, found in 65.2 % of Ly6C^{high} monocytes (Figure 3-22A.ii). Co-expression of other iCCRs was also observed. A total of 8.9 % of cells were CCR1⁺ and CCR2⁺, while 13.7 % were positive for both CCR2 and CCR5 (Figure 3-22A.iii). In comparison, F4/80^{high} skin macrophages retained high total CCR2 expression (76.9 %), although CCR2-only expression was much lower than in monocytes, at 18.1% (Figure 3-22B). In addition, F4/80^{high} skin macrophages showed high CCR5 expression (69.2 %), and 41.3 % of cells co-expressed CCR2 and CCR5 (Figure 3-22B). CCR1 was present on 22.8 % of F4/80^{high} macrophages, and 15.8 % of cells were positive for CCR1, CCR2, and CCR5 together (Figure 3-22B.iii).

In both F4/80^{high} and F4/80^{low} macrophages CCR2 and CCR5 co-expression was the predominant pattern observed. In F4/80^{low} macrophages 92.6 % of cells expressed CCR2, 66.5 % expressed CCR5 (Figure 3-22C.i) and 59.2 % expressed both the receptors simultaneously (Figure 3-22C.ii). Additionally, 7.9 % of these macrophages were CCR1⁺ (Figure 3-22C.i) and 6.5 % of the total population expressed CCR1, CCR2 and CCR5 (Figure 3-22C.ii). Finally, CD11b^{low} F4/80⁺ macrophages in embryonic skin at E17.5 demonstrated very different expression compared with the other two macrophage subsets explored, with 52 % of cells expressing no-iCCRs at all (Figure 3-22D.ii). Furthermore, total CCR2 expression was considerably lower (15.4 %) and CCR5 was the highest expressed chemokine receptor measured (40.2 %) of CD11b^{low} macrophages. Although CCR3 was expressed at close to 0 % for all other subsets analysed in embryonic skin 6.6 % of CD11b^{low} macrophages were CCR3⁺ (Figure 3-22D.i).

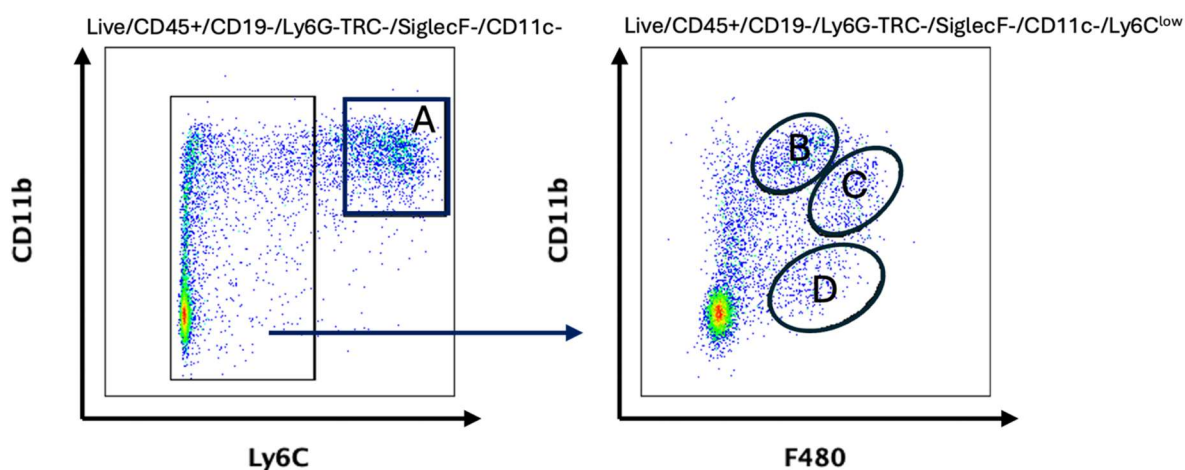


Figure 3-21 Representative Gating Strategy of Myeloid Cells in Embryonic Skin at E17.5

All cells were gated as single cells, live cells, CD45⁺, CD19⁻, Ly6G⁻, TCR⁻, SiglecF⁻, CD11c⁻. Ly6C^{high} monocytes (**A**) were CD11b⁺, Ly6C^{high}. Macrophage populations were divided into F4/80^{low} (**B**) F4/80^{high} (**C**) and CD11b^{low}, F4/80⁺ (**D**).

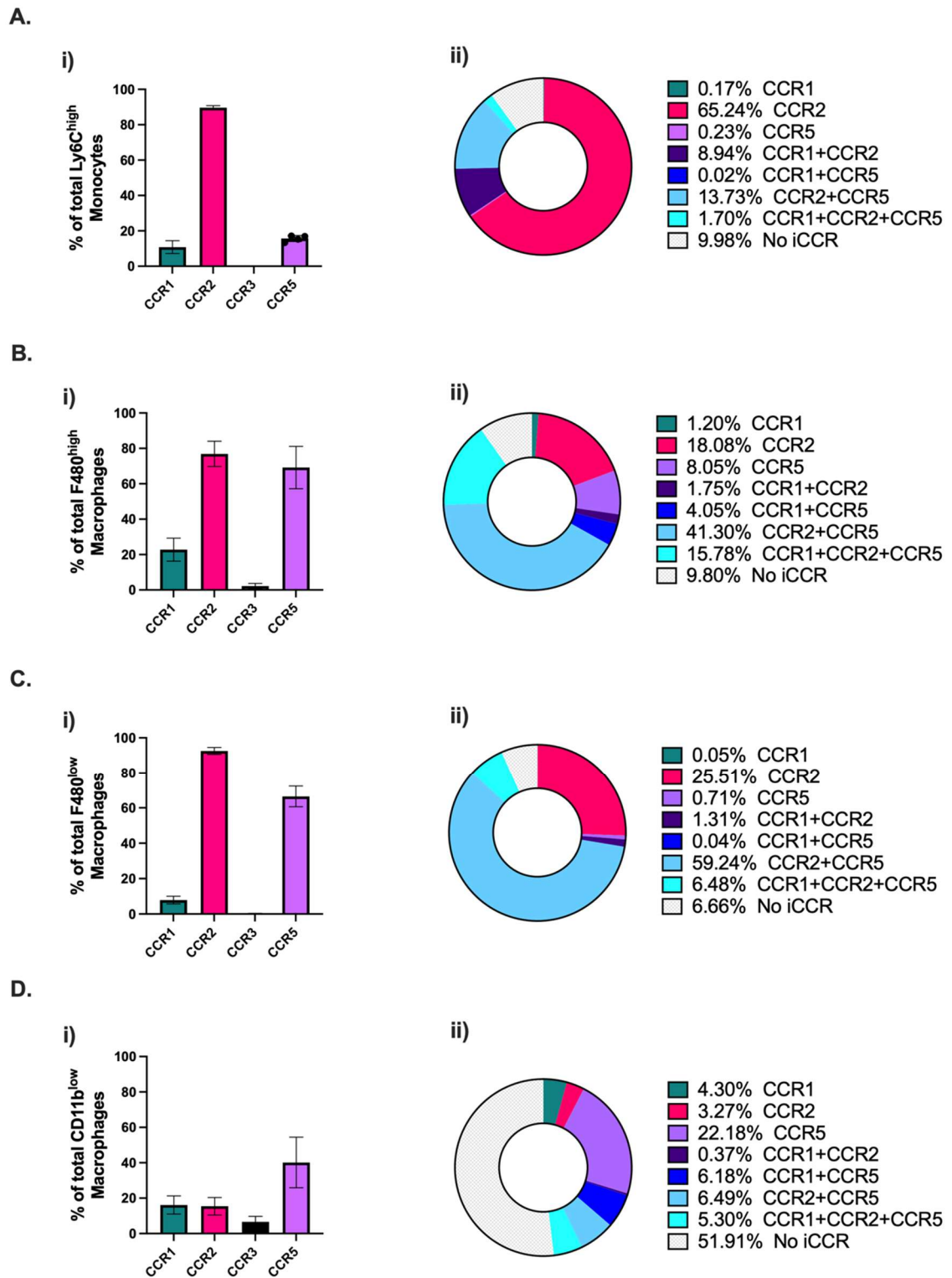


Figure 3-22 iCCR Expression Across Myeloid Cells in Embryonic Skin at E17.5

The percentage of Ly6C^{high} monocytes (Ai), F4/80^{low} macrophages (Bi), F4/80^{high} macrophages (Ci) and CD11b^{low} macrophages (Di) expressing CCR1 (teal), CCR2 (pink), CCR3 (black) and CCR5 (purple) in embryonic skin at E17.5 was determined by flow cytometry using fluorescent reporter embryo mice, wild type embryos were used as FMOs controls (reporter embryos $n = 4$, wild type embryos, $n = 1$). Error bars show SD. iCCR co-expression across Ly6C^{high} monocytes (Aii), F4/80^{low} macrophages (Bii), F4/80^{high} macrophages (Cii) and CD11b^{low} macrophages (Dii) in embryonic skin at E17.5 is summarised using doughnut charts, key indicates colour corresponding with co-expression patterns.

3.3.3 Chemokine Receptor Expression in E17.5 Liver Myeloid Populations

The expression of CCR1, CCR2, CCR3, and CCR5 was then explored across myeloid populations in the E17.5 foetal liver using the gating strategy shown in Figure 3-23. Unlike the other tissues analysed, iCCR expression was relatively low across all cell types analysed. Among these, Ly6C^{high} monocytes exhibited the highest iCCR expression, with 58.4 % expressing CCR2 and 17.5 % expressing CCR1 (Figure 3-24). Figure 3-24A.ii highlights that 11.1 % of these monocytes co-expressed both CCR1 and CCR2. In adult bone marrow, CCR1 and CCR2 co-expression in inflammatory monocytes has been associated with a distinct, non-overlapping transcriptional profile associated with a more neutrophilic phenotype (Medina-Ruiz et al., 2024). This suggests that the CCR1⁺CCR2⁺ monocytes in the foetal liver may represent a unique monocyte subset. In contrast, over 99 % of Ly6C^{low} monocytes did not express any of the four iCCRs (Figure 3-24B). Similarly, in both CD11b⁺ F4/80⁺ macrophages and CD11b^{low} F4/80⁺ macrophages in the E17.5 foetal liver, over 95 % of cells did not express iCCRs (Figure 3-24C and Figure 3-24D). Although most Kupffer cells (liver resident macrophages) are CD11b⁻, a subset of Kupffer cells expresses CD11b (Kinoshita et al., 2010). The low iCCR expression observed by foetal liver CD11b⁺ F4/80⁺ macrophages at E17.5 (Figure 3-24C) suggests that these cells are likely to represent resident CD11b⁺ Kupffer cells.

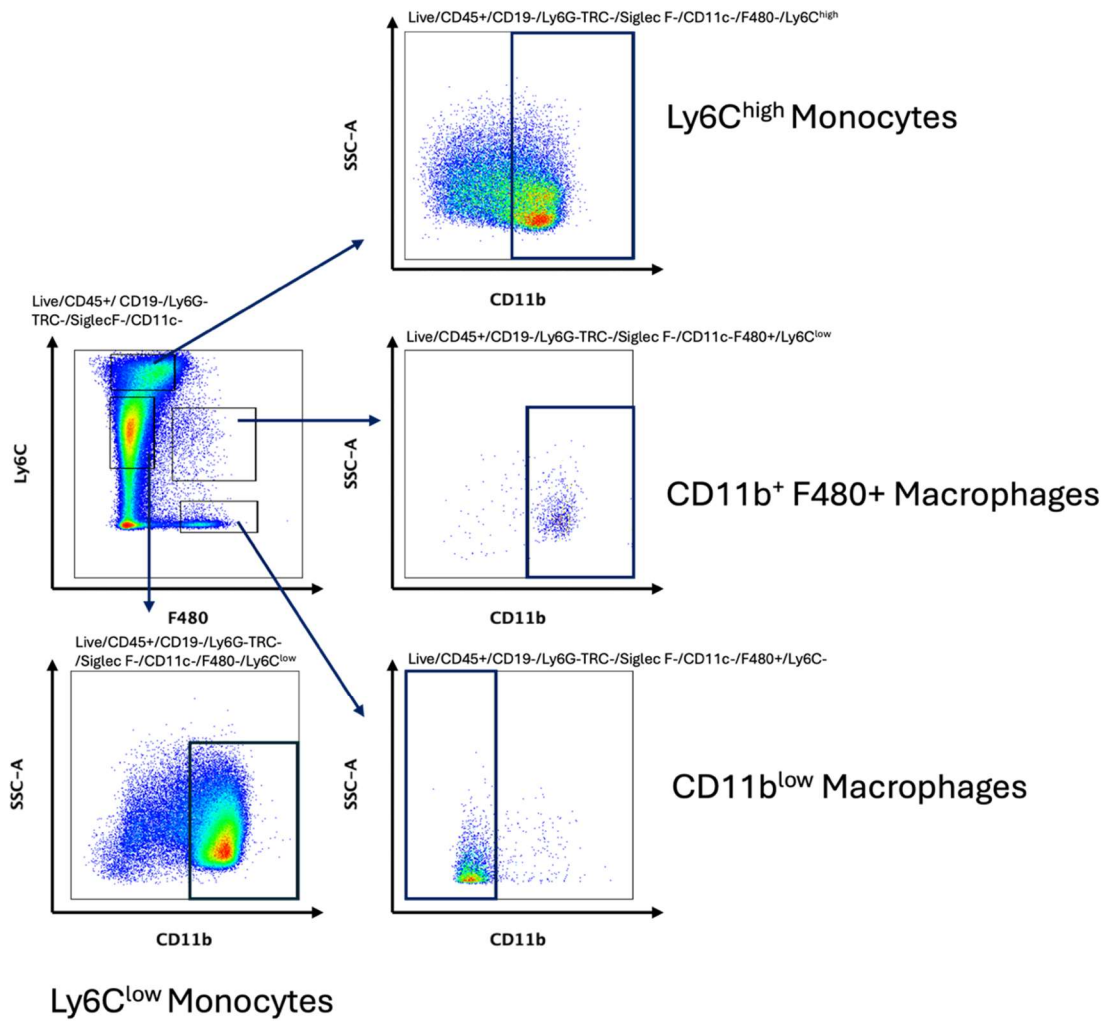


Figure 3-23 Representative Gating Strategy of Myeloid Cells in Embryonic Liver at E17.5

All cells were gated as single cells, live cells, CD45⁺, CD19⁻, Ly6G⁻, TCR⁻, SiglecF⁻, CD11c⁻. Cell types were distinguished based on LY6C and F4/80 expression. Ly6C^{high} and Ly6C^{low} monocytes were F4/80⁻ and CD11b⁺. CD11b⁺, F4/80⁺ macrophages were Ly6C^{int} and Ly6C⁻, F4/80⁺ cells were mostly CD11b⁻ (CD11b^{low} macrophages).

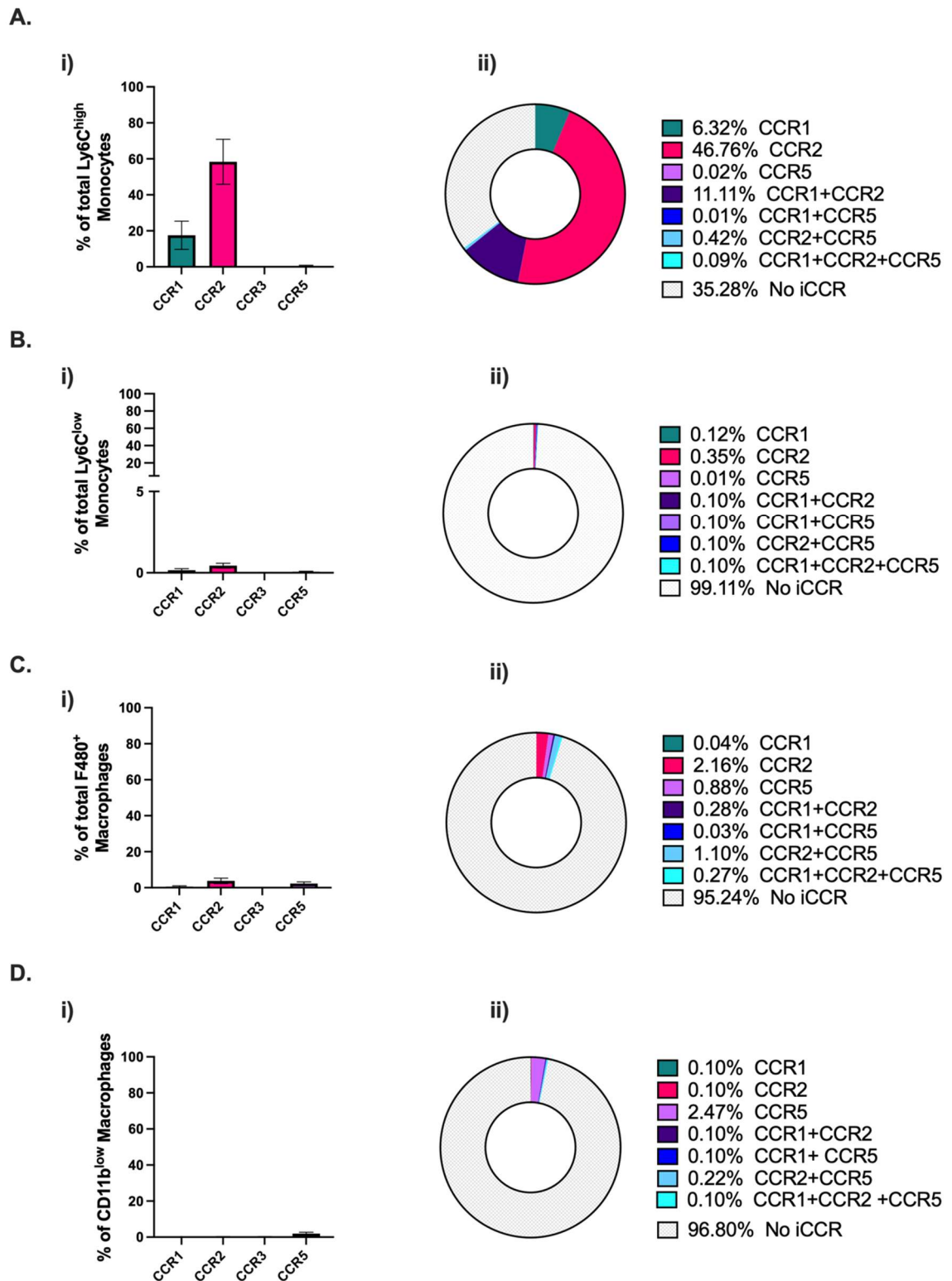


Figure 3-24 iCCR Expression Across Myeloid Cells in Embryonic Liver at E17.5

The percentage Ly6C^{high} monocytes (Ai), Ly6C^{low} monocytes (Bi), F4/80⁺ macrophages (Ci) and CD11b^{low} macrophages (Di) expressing CCR1 (teal), CCR2 (pink), CCR3 (black) and CCR5 (purple) in the embryonic liver at E17.5 was determined by flow cytometry using fluorescent reporter embryo mice, wild type embryos were used as FMOs controls (reporter embryos $n = 4$, wild type embryos, $n = 1$). Error bars show SD. iCCR co-expression across Ly6C monocytes (Aii), Ly6C^{low} monocytes (Bii), F4/80⁺ macrophages (Cii) and CD11b^{low} macrophages (Dii) in the embryonic liver at E17.5 is summarised using doughnut charts, key indicates colour corresponding with co-expression patterns.

3.3.4 Chemokine Receptor Expression in E17.5 Spleen Myeloid Populations

Inflammatory chemokine receptor expression in the embryonic spleen at E17.5 was also examined in Ly6C^{high} monocytes, CD11b⁺ F4/80⁺ macrophages, and CD11b^{low}, red pulp macrophages, based on the gating strategy shown in Figure 3-25. Corresponding chemokine ligand data for the spleen is not presented in this study, due to difficulty dissecting the E15 spleen and low yields of RNA. Among Ly6C^{high} monocytes, 59.9 % of cells expressed CCR2 and 13.8 % expressed CCR1 (Figure 3-26A.i) with 12.4 % of the total population co-expressing both receptors (Figure 3-26A.ii). CCR5 expression was also detected at low levels within this monocyte population (Figure 3-26A.i) largely in combination with CCR2 and 2.9 % of these monocytes co-expressed both receptors (Figure 3-26A.ii). Additionally, CCR2 and CCR5 showed the highest levels of expression in CD11b⁺ F4/80⁺ macrophages in the spleen, found in 49.3 % and 32.0 % of cells respectively (Figure 3-26B.i). CCR1 and CCR3 were expressed at lower levels, 3.5 % of CD11b⁺ F4/80⁺ macrophages expressed CCR1 and 7.3 % expressed CCR3 (Figure 3-26B.i). Among the CCR2⁺ and CCR5⁺, CD11b⁺ F4/80⁺ macrophages, most cells co-expressed both receptors, representing 26.5% of the total population (Figure 3-26B.ii). A smaller subset of 2.5 % of cells simultaneously expressed CCR1, CCR2, and CCR5 (Figure 3-26B.ii). In contrast, the majority of CD11b^{low}, splenic red pulp macrophages (72 %) did not express any of the iCCR receptors analysed, consistent with their identity as resident macrophages (Figure 3-26C.i and Figure 3-26C.ii).

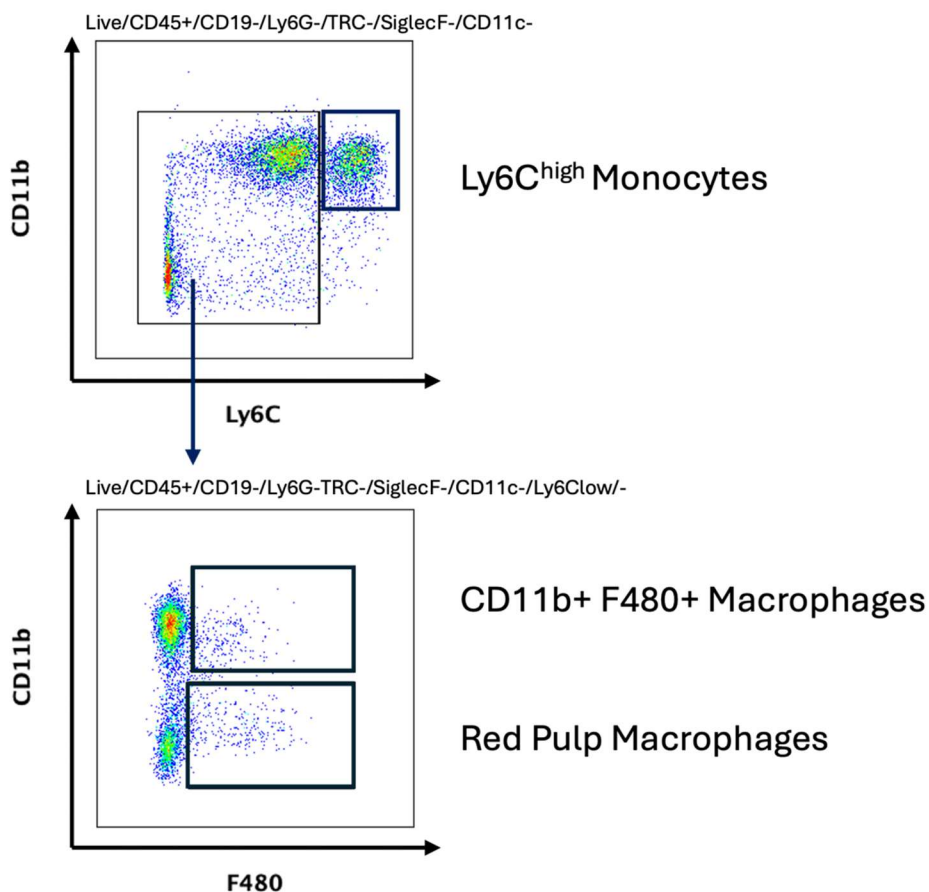


Figure 3-25 Representative Gating Strategy of Myeloid Cells in Embryonic Spleen at E17.5

All cells were gated as single cells, live cells, CD45⁺, CD19⁻, Ly6G⁻, TCR⁻, SiglecF⁻, CD11c⁻. Ly6C^{high} monocytes were CD11b⁺ and Ly6C^{high}. The Ly6C^{low} gate was used to identify CD11b⁺, F4/80⁺ macrophages and CD11b⁻, F4/80⁺ macrophages, which are splenic red pulp macrophages.

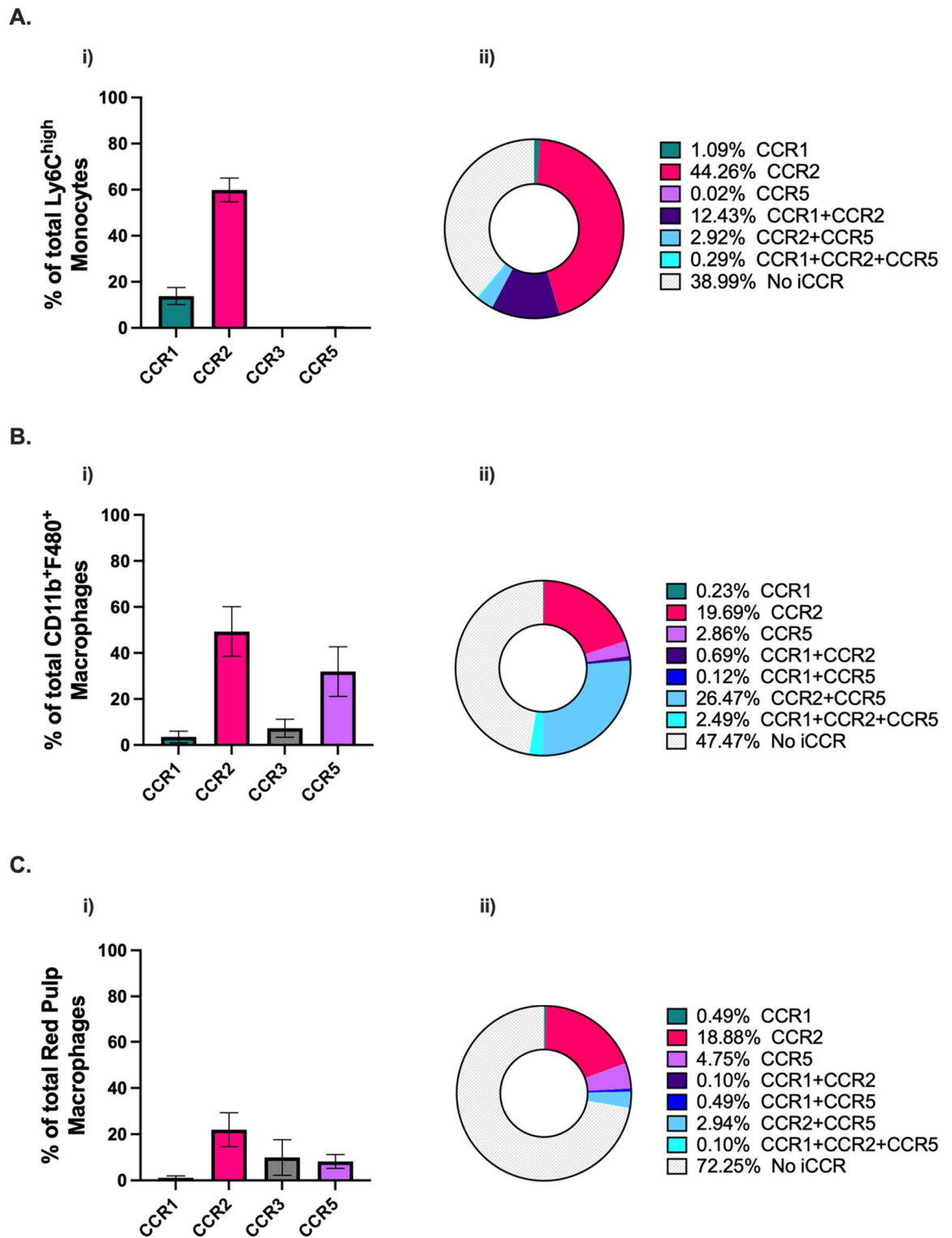


Figure 3-26 iCCR Expression Across Myeloid Cells in Embryonic Spleen at E17.5

The percentage of Ly6C^{high} monocytes (Ai), F4/80⁺ macrophages (Bi) and CD11b^{low} Red Pulp macrophages (Ci) expressing CCR1 (teal), CCR2 (pink), CCR3 (black) and CCR5 (purple) in the embryonic spleen at E17.5 was determined by flow cytometry using fluorescent reporter embryo mice, wild type embryos were used as FMOs controls (reporter embryos $n = 4$, wild type embryos, $n = 1$). Error bars show SD. iCCR co-expression across Ly6C^{high} monocytes (Aii), F4/80⁺ macrophages (Bii), and CD11b^{low} macrophages (Cii) in embryonic spleen at E17.5 is summarised using doughnut charts, key indicates colour corresponding with co-expression patterns.

3.4 Discussion

Whilst individual chemokines have established roles in embryogenesis, such as CXCL12, which coordinates the migration of neural progenitors during brain development (Belmadani et al., 2005), a systematic overview of chemokine expression across developing tissues was lacking. This chapter provides analysis of chemokine ligand expression across multiple embryonic tissues (heart, liver, lung, and skin) from E15 to E18.5 as well as an overview of inflammatory chemokine receptor expression on embryonic myeloid populations within multiple tissues. This work highlights the dynamic, tissue-specific expression of chemokines during late embryonic development.

CXCL12 and CXCL14 are the most highly conserved chemokines throughout evolution. They have key roles in homeostasis (Huisin et al., 2004), and both chemokines were detected across the time points and tissues analysed. There is not yet a clearly defined receptor, or well-established function, for CXCL14 and therefore the function of CXCL14 in these tissues during embryogenesis is difficult to elucidate. CXCL12 was consistently expressed at RNA and protein level by all of the embryonic tissues analysed, reflecting its key role in coordinating the migration of hematopoietic stem cells (HSCs) during development (Sugiyama et al., 2006). For example, CXCL12 was detected at relatively high RNA levels and at the protein level in the foetal liver. During embryo development, the foetal liver acts as a haematopoietic niche, and secretion of CXCL12, for example by hepatic stellate cells (Yin et al., 2013), holds HSCs at this site. Interestingly, all of the CXC chemokine ligands expressed at the transcript level in the foetal liver showed a decrease in expression between E15 and E17.5 which was then restored by E18.5. This reduction corresponds with the timing of HSC emergence in the embryonic bone marrow, which takes place around E16.5 (Gao et al., 2018). Potentially, CXC chemokines in the foetal liver decrease between E15 and E17.5 to release HSCs to the spleen and bone marrow for the next wave of haematopoiesis. Furthermore, CXCL12 was the highest detected CXC-chemokine ligand in the heart, paralleling its essential role during cardiac morphogenesis (Cavallero et al., 2015). It is possible that high CXCL12 expression across tissues and time points may reflect a broader role for CXCL12 in the positioning of endothelial progenitors, as this has previously been observed in the lung (Chandrasekaran et al., 2022).

In addition, CX3CL1 was detected in multiple tissues between E15-E18.5. In the embryonic heart, CX3CL1 was the only chemokine that showed a significant change in

transcript expression, with a decrease observed between E15 and E17.5 (Table 3-2).

CX3CL1 is the ligand for CX3CR1, which is expressed by EMP-derived macrophages that seed early in embryonic tissues and develop into resident macrophages. Trafficking of EMP macrophages from the yolk sac ends around E14.5, which may be reflected by the decrease in CX3CL1 observed in the embryonic heart after E15 (Stremmel et al., 2018). However, as CX3CR1 is also expressed across other leukocytes, such as non-classical monocytes, the CX3CL1 in the heart may be controlling the recruitment of other cells at this later time point (Geissmann et al., 2003).

In comparison, in embryonic skin, CX3CL1 was relatively highly expressed between E15 and E18.5 (Figure 3-11). In humans, CX3CL1 is expressed by keratinocytes (Sugaya et al., 2003), which appear in the mouse embryo from E9 (Jacob et al., 2023). Keratinocytes may be the source CX3CL1 expression in the embryonic skin, where it could act to coordinate the migration of Langerhans cell precursors, which express CX3CR1 and enter the epidermis between E16.5 and E18.5 (Chorro et al., 2009).

Some, although few, CXCR1 and CXCR2 ligands, which act as potent neutrophil chemoattractants, were detected in embryonic tissues between E15 and E18.5. Potential roles for neutrophils in embryonic development have been outlined, for example in a study that demonstrated that Ly6B.2⁺ (Ly6G⁻) neutrophils localise between arteries and veins in the skin at E14.5 where they contribute to the correct alignment of blood vessels in the skin by secreting matrix metalloproteinase-9 (MMP-9) (Kidoya et al., 2015). MMP-9 degrades basement membrane type IV collagen, remodelling the extracellular matrix and facilitating the repositioning of veins into the correct spatial position within the tissue (Kidoya et al., 2015). This study highlights a key homeostatic role for neutrophils in embryonic skin development. The precise localisation of neutrophils in this process implies a regulated recruitment mechanism, likely mediated by chemokines. The neutrophil chemoattractant CXCL1 was detected at the protein level in embryonic skin at E15, E17.5 and E18.5 and another neutrophil chemoattractant, CXCL6, was also present at detectable RNA levels (Figure 3-11 and Figure 3-12). This suggests that these chemokines may regulate neutrophil positioning within the developing skin and that this may contribute to correct development.

Evidence from the embryonic lung further supports the idea that chemokines may regulate neutrophil positioning during late embryonic development in mice. One of the most profound changes in gene expression observed during this analysis was the CXCL15 in the embryonic lung between E15 and E17.5 (Figure 3-7). CXCL15 was previously thought to

be expressed exclusively in the lung (Rossi et al., 1999), but is now recognised to also be expressed in multiple endocrine and mucosal tissues, including the gastrointestinal tract and adrenal glands (Schmitz et al., 2007). Functionally, CXCL15 is involved in neutrophil recruitment in response to infection and may also influence the proliferation of hematopoietic progenitor cells (Broxmeyer et al., 2021). Although CXCL15 does not have a direct human homolog, it is functionally analogous to human CXCL8 and likely signals through CXCR1 and CXCR2, which are predominantly expressed by neutrophils. By birth, neutrophils are the most abundant immune cells in the murine lung (Loffredo et al., 2020), suggesting that this upregulation may act as a developmental cue for neutrophil recruitment or positioning in the embryonic lung.

Beyond its role in neutrophil recruitment, CXCR2 has been shown to regulate chemokine induced angiogenesis (Addison et al., 2000) and adipocyte development (Dyer et al., 2019b). Therefore, CXCR2 ligand expression across embryonic tissues between E15-E18.5 may contribute to vascularisation or stromal cell differentiation during development.

In the embryonic heart and liver, CC-chemokines were detected at low transcript levels with considerable variability between samples (Figure 3-1 and Figure 3-13). Therefore, inconsistent high signals in individual samples may reflect residual DNA contamination rather than true transcriptional activity. In the liver, the lack of convincing iCCR ligand expression at E15, E17.5 and E18.5 correlates with very low expression of cognate iCCRs in macrophage populations within the tissue at these time points (Figure 3-24). In contrast, the lung and skin exhibit higher and more robust RNA expression of specific CC-chemokines (Figure 3-5 and Figure 3-9), suggesting that CC-chemokine signalling may regulate cell migration in these tissues during this developmental window. Among the chemokines expressed, inflammatory iCCR ligands were consistently more abundant at transcript level than non-iCCR ligands. This corresponds with higher expression of iCCRs across myeloid cells in these tissues in comparison with the liver (Figure 3-19, Figure 3-22 and Figure 3-24). CCR2 was most highly expressed by Ly6C^{high} monocytes, while CCR5 was most abundant on F4/80^{high} macrophages, though also present on F4/80^{low} macrophages. Furthermore, consistent with previous studies that suggest that F4/80^{high} CD11b^{low} macrophages in the embryo are derived from yolk sac progenitors, CD11b^{low} macrophages in embryonic, skin, liver and spleen were CCR2^{low} or CCR2⁻ and Ly6C⁻, suggesting that these cells are of yolk sac origin.

Between E12.5 and E15, the immune cell population in the embryonic dorsal skin consists exclusively of myeloid cells, including immature macrophages (CCR2⁺), dermal dendritic

cells (CCR7⁺), and mast cells (Jacob et al., 2023). By E17.5, we observed F4/80^{high} macrophages in the skin that were primarily CCR5⁺, with approximately 20 % expressing CCR1 (Figure 3-22). These are likely mature macrophages and may contribute to the increased CCL6 gene expression levels observed in embryonic skin at E18.5 (Figure 3-9). CCL6 transcript expression also increased significantly in the embryonic lung between E17.5 and E18.5 (Figure 3-5). Studies have demonstrated that CCL6 is upregulated during tissue remodelling following inflammation, for example in instances of skin wound healing (Kaesler et al., 2002) and lung fibrosis (Belperio et al., 2002). These findings suggest that CCL6 expression in these tissues during late development may contribute to tissue modelling by macrophages.

The higher surface expression of CCR5 on F4/80^{high} macrophages compared to F4/80^{low} macrophages (Figure 3-22B and Figure 3-22C) could indicate that CCR5 is upregulated during differentiation and maturation. High CCR5 surface expression by F4/80^{high} macrophages was also observed in the E17.5 mouse lung. Trajectory analysis from a study using single cell RNA-seq data of human foetal lung tissue spanning 5-and-22 weeks post conception (pcw) (equivalent to approximately mouse E10.5- P0) showed that genes associated with myeloid differentiation are increased across pseudo time and notably, both CCR2 and CCR5 were included in the top 100 most differentially expressed genes across pseudo time in the myeloid populations analysed (Barnes et al., 2023).

In addition, among iCCR ligands expressed in the embryonic lung, CCL11 was particularly notable at E17.5, with expression detected at both RNA and protein levels. CCL11 is a high affinity ligand for the inflammatory receptor CCR3 (Kitauro et al., 1996), which is predominantly expressed by eosinophils (Humbles et al., 2002, Bachelierie et al., 2014, Combadiere et al., 1996). CCR3 is also expressed by Th2 cells, as well as mast cells and bronchial fibroblasts (Puxeddu et al., 2006, Humbles et al., 2002, Sallusto et al., 1997). CCL11 plays a role in both regulating the migration of mast cell progenitors and as well as modulating fibroblast activity in the lung via CCR3 signalling (Puxeddu et al., 2006). Eosinophils and T cells only begin to colonise the murine lung postnatally, during early alveolarization (postnatal days P3-P10) (Loffredo et al., 2020). The high levels of CCL11 observed at E17.5 are therefore unlikely to be involved in recruiting these cell types at this stage. Furthermore, previous studies have reported that CCL11 expression peaks between E12 and E18 but then decreases and remains low during the P0-P10 period when eosinophil and T cell infiltration occurs (Loffredo et al., 2020). These observations suggest that embryonic CCL11 may instead be acting on mast cells, fibroblasts, or other CCR3-

expressing cell types in the lung. However, as CCR3 was only detected at very low transcript levels in the embryonic lung (Figure 3-20) it is possible that CCL11 is signalling via CCR2 or CCR5, as CCL11 also binds these receptors, but with lower affinity (Ogilvie et al., 2001, Bachelierie et al., 2014). CCR2 was expressed at transcript level in the lung and across monocytes and macrophages at E17.5 (Figure 3-19) and CCR5 was present on the surface of F4/80^{high} macrophages (Figure 3-19C.i) and detected at the transcript level (Figure 3-20). Therefore, CCL11 could be recruiting CCR2⁺ monocytes or specifically localising CCR2⁺CCR5⁺ macrophages within the tissue, as macrophages also peak in the developing mouse lung at E18-P3 (Loffredo et al., 2020).

Recent investigation using human foetal lung tissue from between 5 and 22-weeks post conception (pcw) (mouse E10.5 - P0) has underscored the significance of immune cell signalling in directing epithelial cell fate during lung development. Immunohistochemistry revealed that CD206⁺ macrophages and a population of dendritic cells establish direct contact with epithelial tip progenitors. This study demonstrated that IL-1 β released by myeloid cells promotes epithelial to basal cell differentiation within foetal lung organoids (Barnes et al., 2023). This is further supported by the findings that the upregulation of IL-1 β in transgenic mice leads to abnormal mouse lung development (Barnes et al., 2023, Bry et al., 2007). Furthermore, neutrophils and monocytes were also observed in the developing human lung epithelium (Barnes et al., 2023). This highlights that throughout lung development, myeloid cells are both strategically positioned and functionally active, supporting the likelihood that inflammatory chemokines, detected across multiple tissues during late gestation in mice, may contribute to tissue patterning and morphogenesis.

4 ACKR2 Deficiency is Associated with Altered Peritoneal Macrophages

4.1 Introduction

ACKR2 regulates macrophage numbers in multiple contexts including in the embryonic skin, with reduced numbers of F4/80^{int} macrophages observed in the skin of E14.5 ACKR2-deficient embryos (Lee et al., 2019), and during pubertal mammary gland branching morphogenesis (Wilson et al., 2020). This led to interest in whether altered macrophage numbers occur in naïve adult ACKR2-deficient mice at rest. The role of ACKR2 within the peritoneal cavity is currently not well established, although B1b cells in the peritoneal cavity are known to express ACKR2 (Hansell et al., 2011). This chapter will define inflammatory chemokine dynamics in the peritoneum and explore the functional consequence of the decreased numbers of a specific subset of macrophage in the peritoneal cavity of ACKR2-deficient mice.

The peritoneum is a membrane that lines the abdominal cavity and separates the abdominal organs from the pleural cavity, which contains the lungs and heart. The peritoneum consists of two main layers: the visceral layer, which covers the abdominal organs, and the parietal layer, which surrounds the abdominal wall and diaphragm. The peritoneal cavity is filled with fluid that reduces friction between organs, preventing fibrosis, and providing a niche for leukocytes, including macrophages, natural killer cells, and B-1b cells (Liu et al., 2021b). This niche enables rapid immune infiltration to nearby tissues following infection or damage (Capobianco et al., 2017, Young et al., 2013). The cavity houses two distinct macrophage populations: large peritoneal macrophages (LPMs) and small peritoneal macrophages (SPMs) (Ghosn et al., 2010). These macrophages regulate B-1b cells and provide immune surveillance for the peritoneal cavity as well as proximal tissues (Louwe et al., 2021).

LPMs are resident macrophages initially derived during embryonic development and maintained through self-renewal under homeostatic conditions (Hoeffel et al., 2015, Sheng et al., 2015). They are characterised by high F4/80 expression and GATA6 transcription factor expression, which is essential for their function (Ghosn et al., 2010, Rosas et al., 2014) (Figure 4-1). GATA6 expression by LPMs is driven by stromal cells within the

omentum which produce retinoic acid (Okabe and Medzhitov, 2014). LPMs play an integral role in tissue repair, immune regulation, and maintaining peritoneal homeostasis by producing anti-inflammatory cytokines such as IL-10 and TGF- β (Wang and Kubes, 2016, Okabe and Medzhitov, 2014). In contrast, SPMs are derived from CCR2⁺ peripheral monocytes (Bain et al., 2016). SPMs are characterised by low F4/80 expression and MHCII expression (Ghosn et al., 2010). SPMs express the transcription factor IRF4 and their differentiation from monocytes is dependent on IRF4 (Kim et al., 2016) and also often express CD226/DMAM-1 (Figure 4-1) (Takenaka et al., 2018). In the peritoneal cavity at rest, LPMs are the dominant macrophage population, comprising approximately 90% of macrophages, while SPMs are fewer in number, approximately 10% of macrophages in the peritoneum and have a shorter lifespan (Ghosn et al., 2010). Furthermore, SPMs can act as precursors for LPMs, and this happens constitutively, with male mice showing faster transition of SPMs to LPMs compared to female mice (Bain et al., 2016, Louwe et al., 2021). SPM derived LPMs can be distinguished from embryonically seeded LPMs by Tim-4 expression which is retained only by the latter (Bain et al., 2016, Wong et al., 2010).

Both LPMs and SPMs have phagocytic capacity (Ghosn et al., 2010, Cassado Ados et al., 2011) and SPMs, but not LPMs, can present antigen to naïve CD4⁺ T cells on MHCII with CD226 acting as a costimulatory molecule for this interaction (Takenaka et al., 2018). During infection, LPMs disappear from the cavity in a phenomenon referred to as the macrophage disappearance/adherence response (Barth et al., 1995). In response to fungal cell wall derived zymosan or *E. coli* infection, LPMs migrate to mesothelial cells in the peritoneal wall, where adhesion molecules are upregulated, enabling access to lymphatic vessels (Vega-Perez et al., 2021). The peritoneum contains stomata, pores that facilitate communication between the peritoneal cavity and lymphatic vessels, ultimately facilitating the draining of leukocytes into local lymph nodes (Liu et al., 2021b). Furthermore, following *E. coli* infection, LPMs from the peritoneal cavity form aggregates on the mesothelium of the peritoneal wall, which further recruit monocyte derived cells, neutrophils and B-1b cells. These aggregates are dependent on LPMs and fibrin, but do not require neutrophils or B cells to form (Vega-Perez et al., 2021).

We hypothesised that the absence of ACKR2 would result in elevated levels of its chemokine ligands, leading to altered macrophage recruitment within the adult tissues including the peritoneal cavity and wall and that this would result in functional implications.

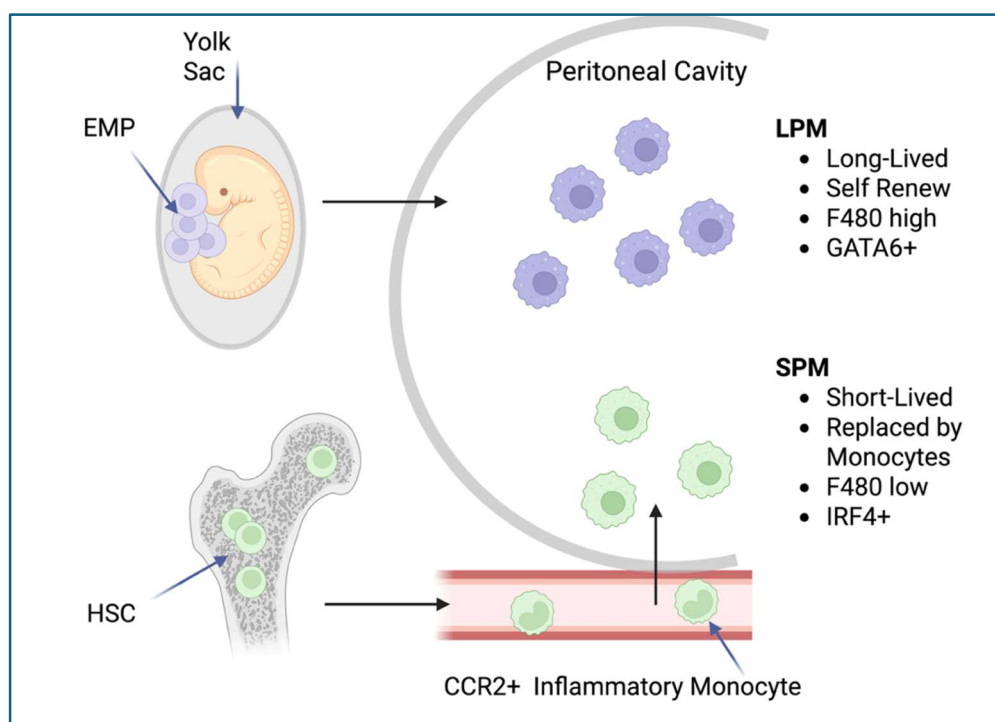


Figure 4-1 Schematic Diagram Summarising LPM and SPM Origins and Characteristics

LPMs (purple) are embryo derived, whilst SPMs (green) emerge from HSCs in the bone marrow, differentiating from classical inflammatory CCR2⁺ monocytes that travel through the circulation and infiltrate tissues. The key characteristics of these distinct populations are listed. Made using BioRender.com, adapted from figure by Mass et al., (Mass et al., 2023).

4.2 Results

4.2.1 SPM Numbers are Reduced in the Peritoneal Cavity and Peritoneal Wall of Naïve ACKR2-Deficient Mice

The SPM and LPM populations in WT and ACKR2-deficient mouse peritoneal fluid were examined by flow cytometric analysis according to the gating strategy outlined in Figure 4-2. This revealed a significant reduction in the number of SPMs within the peritoneal cavity of ACKR2-deficient mice under resting conditions (Figure 4-3A). SPMs decreased from a mean of 172.5 SPMs/ml in WT mice to 98.07 SPMs/ml in ACKR2-deficient mice ($p = 0.0152$) (Figure 4-3A). In contrast, the number of LPMs remained unchanged between WT and ACKR2-deficient mice in the peritoneal cavity at rest ($p > 0.05$) (Figure 4-3B).

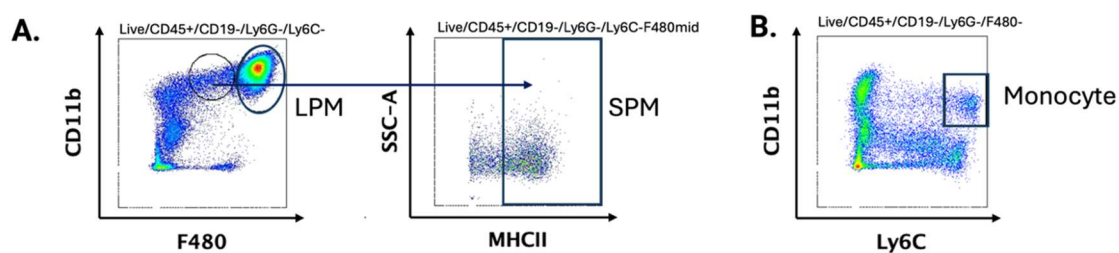


Figure 4-2 Gating Strategy to Define Cell Populations in the Peritoneal Cavity

A) Representative gating strategy for LPMs and SPMs in the peritoneal cavity. LPMs were gated as single cells, live cells, CD45⁺, CD19⁻, Ly6G⁻, Ly6C⁻, CD11b⁺, F4/80^{high}. SPMs were gated as single cells, live cells, CD45⁺, CD19⁻, Ly6G⁻, Ly6C⁻, CD11b⁺, F4/80^{mid}. **B)** Representative gating strategy for live CD45⁺, CD19⁻, Ly6G⁻, F4/80⁻, Ly6C^{high} monocytes in the peritoneal cavity.

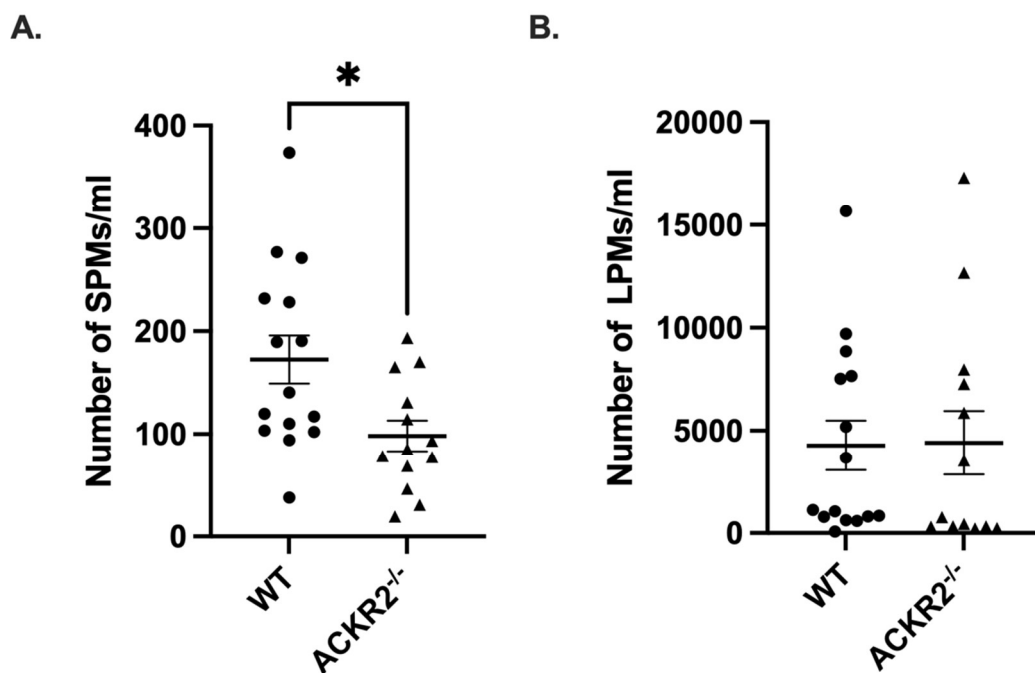


Figure 4-3 The Number of Small Peritoneal Macrophages in the Peritoneal Cavity of Wild Type and ACKR2-Deficient Mice at Rest

The number of SPMs (**A**) and LPMs (**B**) in the peritoneal cavity of naïve WT ($n = 15$) and ACKR2^{-/-} (ACKR2-deficient) ($n = 15$) 8-week-old female mice were determined by flow cytometry. Error bars represent SEM. Outliers were identified using the Grubbs test (Prism) and removed before pairwise statistical analysis. Statistical significance was determined by unpaired t-test or Mann Whitney ($p = < 0.05$) depending on normality of the data and is indicated as $p < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****).

To further investigate whether the reduction of SPMs in the peritoneal cavity might reflect altered migratory behaviour or tissue redistribution of these macrophages, we next assessed the numbers of SPMs (F4/80^{mid} MHCII⁺) macrophages in tissues proximal to the peritoneal cavity, including the peritoneal wall, omentum, spleen, and mesenteric lymph node. LPM or F4/80^{high} macrophage numbers were also measured in the peritoneal wall and omentum. The SPM population in the peritoneal wall of WT and ACKR2-deficient mice was examined by flow cytometry according to the gating strategy outlined in Figure 4-4. In comparison with the peritoneal cavity, the F4/80^{mid} macrophage gate for SPMs is less well defined in the peritoneal wall. CD226 is only expressed on SPMs and not LPMs and was therefore used to validate the accuracy of the SPM gating strategy in this tissue. Figure 4-4B highlights that the F4/80^{mid} MHCII⁺ macrophage population identified was also CD226⁺, confirming that the gate correctly captured the SPM population in the peritoneal wall.

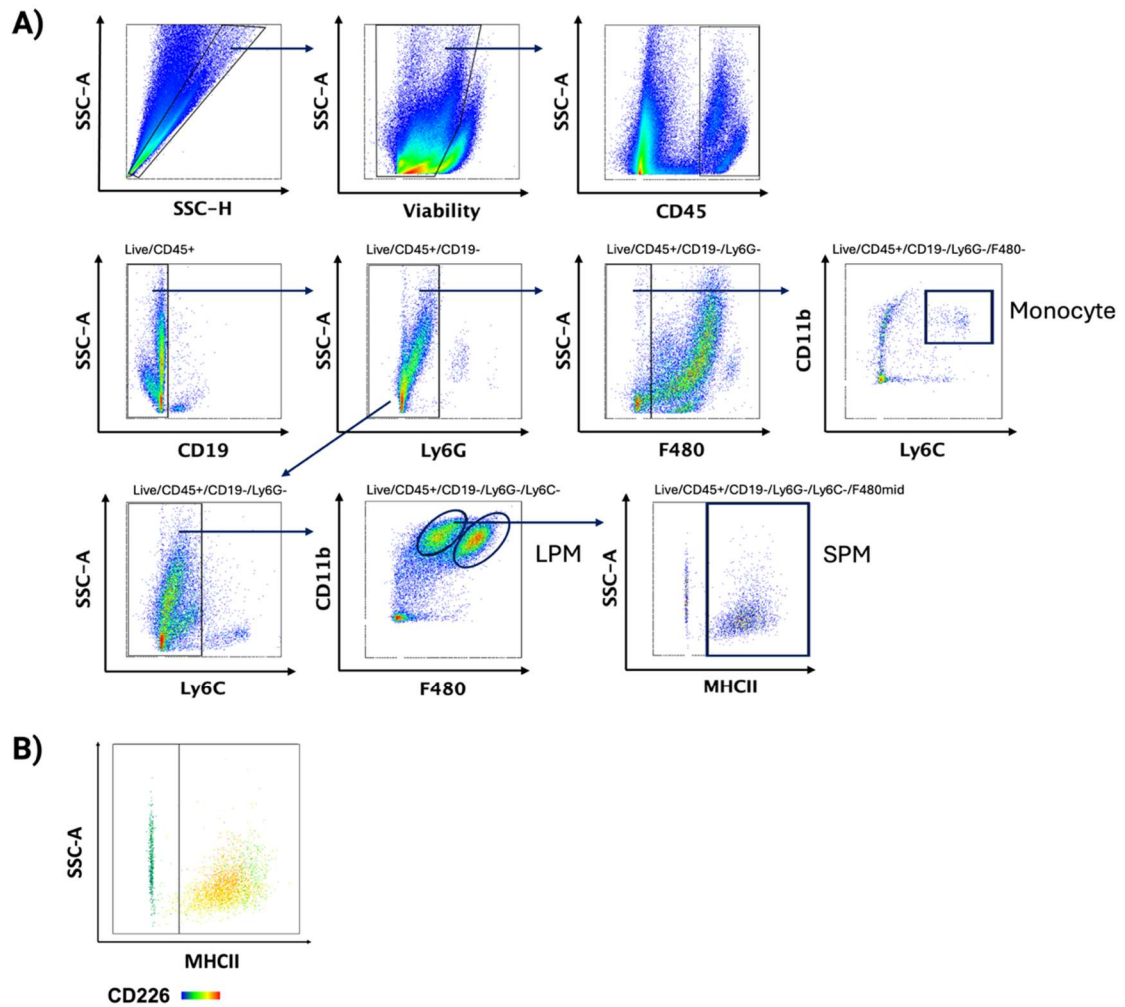


Figure 4-4 Gating Strategy to Define Cell Populations in the Peritoneal Wall

A) Representative gating strategy for LPMs, SPMs and monocytes (labelled) in the mouse peritoneal wall. **B)** Representative image of the single cells, live cells, CD45⁺, CD19⁻, Ly6G⁻, Ly6C⁻, CD11b⁺, F4/80^{mid} gate for SPMs, the MHCII⁺ gate overlaps with high CD226/DMAM-1 expression.

Figure 4-5A.i indicates that SPMs were also reduced in numbers in the peritoneal wall of naïve ACKR2-deficient mice in comparison with the WT. The mean number of SPMs in the peritoneal wall of WT mice was 10,768, compared to 5,800 in ACKR2-deficient mice ($p = 0.0057$) (Figure 4-5A.i). In contrast, the number of LPMs remained unchanged between WT and ACKR2-deficient mice the peritoneal wall at rest ($p > 0.05$) (Figure 4-5A.ii). These data suggest that SPMs from the peritoneal cavity of ACKR2-deficient mice do not accumulate at the peritoneal wall at rest.

To test whether SPMs relocate to other adjacent tissues, we next quantified the number of SPMs (F4/80^{mid} MHCII⁺) in the omentum of WT and ACKR2-deficient mice at rest. No difference was observed in either the number of SPMs (Figure 4-5B.i) or LPMs (F4/80^{high}) (Figure 4-5B.ii) between WT and ACKR2-deficient mice at rest ($p > 0.05$).). Similarly, no difference was observed in the number of MHCII⁺ macrophages measured in the spleen (Figure 4-5C) or mesenteric lymph node (Figure 4-5D) of WT or ACKR2-deficient mice ($p > 0.05$). Therefore, we found no evidence of compensatory SPM accumulation in tissues surrounding the peritoneal cavity. However, cell tracking methods would be required to clarify these dynamics due to low numbers of SPMs/ml in the peritoneal cavity, as it is possible that the markers F4/80 and MHCII may be downregulated by SPMs leaving the peritoneal cavity.

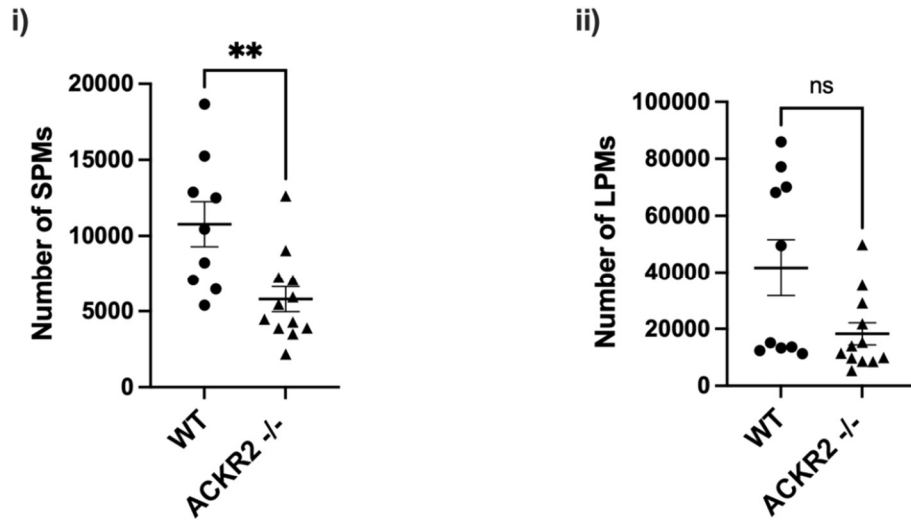
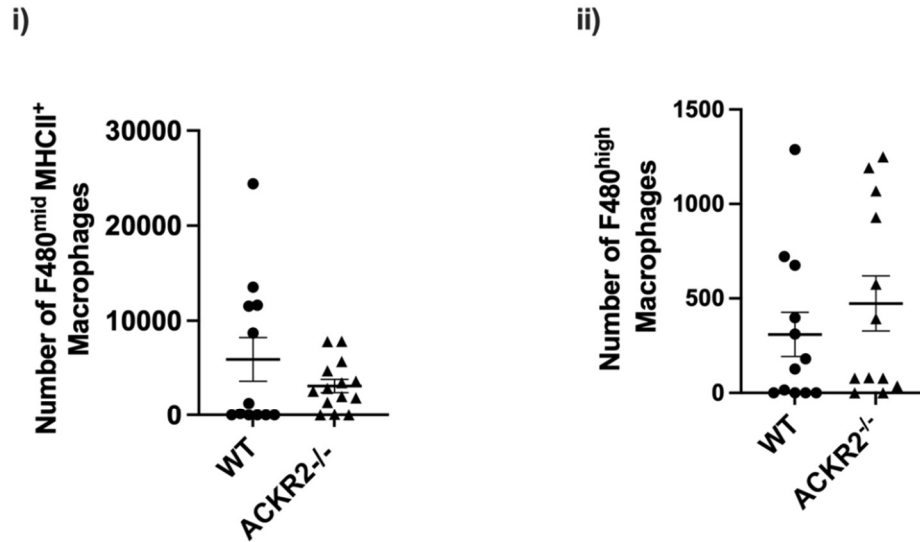
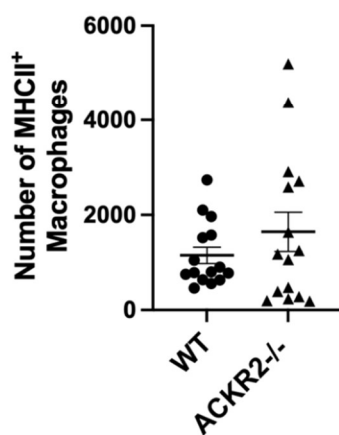
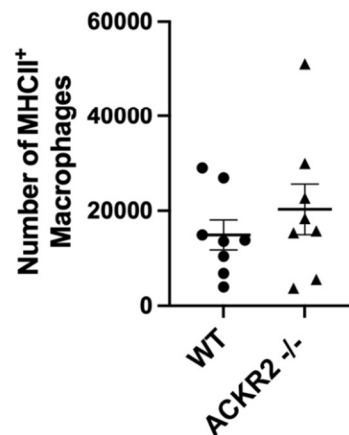
A. Peritoneal Wall**B. Omentum****C. Spleen****D. Mesenteric Lymph Node**

Figure 4-5 The Numbers of Specific Macrophages in the Peritoneal Wall, Omentum, Spleen and Mesenteric Lymph Node of Wild Type and ACKR2-Deficient Mice at Rest

A) The number of SPMs (i) and LPMs (ii) in the peritoneal wall of naïve WT ($n = 10$) and ACKR2-deficient (ACKR2^{-/-}) mice ($n = 12$) was determined by flow cytometry. **B)** The number of

F4/80^{mid} MHCII⁺ macrophages **(i)** and F4/80^{high} macrophages **(ii)** per total omentum of naïve WT ($n = 12$) and ACKR2^{-/-} ($n = 14$) was determined by flow cytometry. **C)** The number of MHCII⁺ macrophages per total spleen of naïve WT ($n = 17$) and ACKR2^{-/-} mice ($n = 17$) was determined by flow cytometry. **D)** The number of MHCII⁺ macrophages per total mesenteric lymph node of naïve WT ($n = 8$) and ACKR2^{-/-} ($n = 8$) mice was determined by flow cytometry. All mice used were 8-week-old females. Error bars represent SEM. Outliers were identified using the Grubbs test (Prism) and removed before statistical analysis. Statistical significance was determined by unpaired t-test or Mann Whitney ($p < 0.05$) depending on normality of the data and is indicated as $p < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****). Data are combined from 2 experiments for all tissues excluding the mesenteric lymph node.

4.2.2 Monocytes are Reduced in Numbers in the Peritoneal Wall of Naïve ACKR2-Deficient Mice

As recruited monocytes are the precursors of SPMs, their numbers were measured in WT and ACKR2-deficient mice to determine whether monocyte recruitment to peritoneal tissues was altered by ACKR2 deficiency. Classical inflammatory monocytes (Ly6C^{high}) in both the peritoneal cavity and the peritoneal wall were identified using the gating strategies defined in Figure 4-2B and Figure 4-4A respectively.

In the peritoneal cavity, monocyte levels remained unchanged at rest between WT and ACKR2-deficient mice ($p > 0.05$) (Figure 4-6A). In contrast, monocyte numbers were significantly reduced in the peritoneal wall of ACKR2-deficient mice, decreasing from a mean of 520 cells in WT mice to 357.8 cells in ACKR2-deficient ($p = 0.0374$) (Figure 4-6B). To rule out systemic effects on monocyte production or circulation that may have led to the low numbers observed in the peritoneal wall, we next measured monocyte numbers in peripheral blood and bone marrow. There was no difference between monocyte numbers in the peripheral blood or the bone marrow of WT and ACKR2-deficient mice ($p > 0.05$) (Figure 4-6C and Figure 4-6D). Together, these findings suggest that monocyte recruitment to the peritoneal wall in ACKR2-deficient mice is impaired in a specific manner that is not due to a general defect in monocyte production or migration to the peritoneal cavity. Furthermore, as both SPMs and monocytes were reduced in the peritoneal wall of ACKR2-deficient mice, SPMs do not appear to be being retained in the peritoneal wall or redirected from the peritoneal cavity to the wall in mice lacking ACKR2.

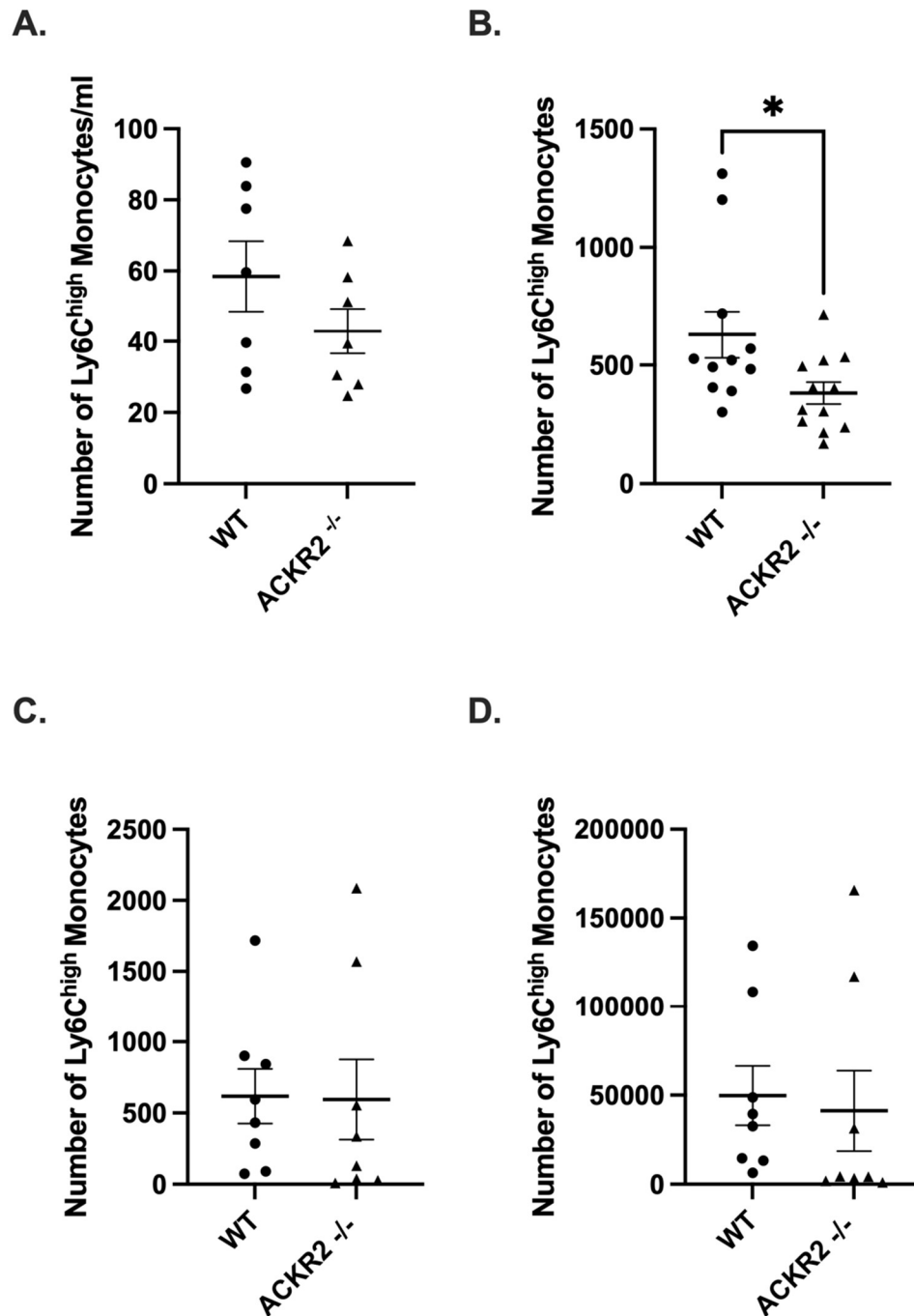


Figure 4-6 Monocyte Numbers in the Peritoneal Cavity, Peritoneal Wall, Blood and Bone Marrow of Wild Type and ACKR2-Deficient Mice at Rest

A) Flow cytometry was used to measure the number of Ly6C^{high} monocytes in the peritoneal wall of wild type (WT) ($n = 11$) and ACKR2-deficient mice (ACKR2^{-/-}) ($n = 12$). **B)** Flow cytometry was used to measure the number of Ly6C^{high} monocytes in the peritoneal cavity of WT ($n = 7$) and ACKR2^{-/-} mice ($n = 7$). Flow cytometry was used to measure the number of Ly6C^{high} monocytes in the peripheral blood (**C**) and bone marrow (**D**) of WT ($n = 8$) and ACKR2^{-/-} mice ($n = 8$). Error bars represent SEM. Outliers were identified using the Grubbs test (Prism) and removed before pairwise statistical analysis. Statistical significance was determined by unpaired t-test or Mann Whitney ($p < 0.05$) and is indicated as $p < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****).

4.2.3 ACKR2 RNA is Expressed across Lymphatic Vessels in the Peritoneal Wall

The peritoneal wall was proposed as a possible source of ACKR2 in the peritoneum that may contribute to the reduced macrophage numbers observed in the peritoneal cavity and wall of ACKR2-deficient mice. We investigated ACKR2 RNA expression in the peritoneal wall using RNAscope *in situ* hybridization (Figure 4-7). ACKR2 expression by lymphatic endothelial cells (LECs) in various other tissues is well characterised (Nibbs et al., 2001, McKimmie et al., 2013, Lee et al., 2014) and therefore, we co-stained the tissue with a FITC-conjugated Lyve-1 antibody, as Lyve-1 is a well-established marker of LECs, to assess whether ACKR2 is localised to these cells in the peritoneal wall.

ACKR2 RNA was colocalized with Lyve-1 in the peritoneal wall of naïve WT mice (Figure 4-7: WT Female + ACKR2). No ACKR2 signal was observed in the peritoneal wall of ACKR2-deficient mice, which served as a negative control. This identified a source of ACKR2 in the peritoneal wall that may be driving the observed changes in macrophage numbers within the tissue.

Blue: DAPI Green: Lyve-1 Red: ACKR2

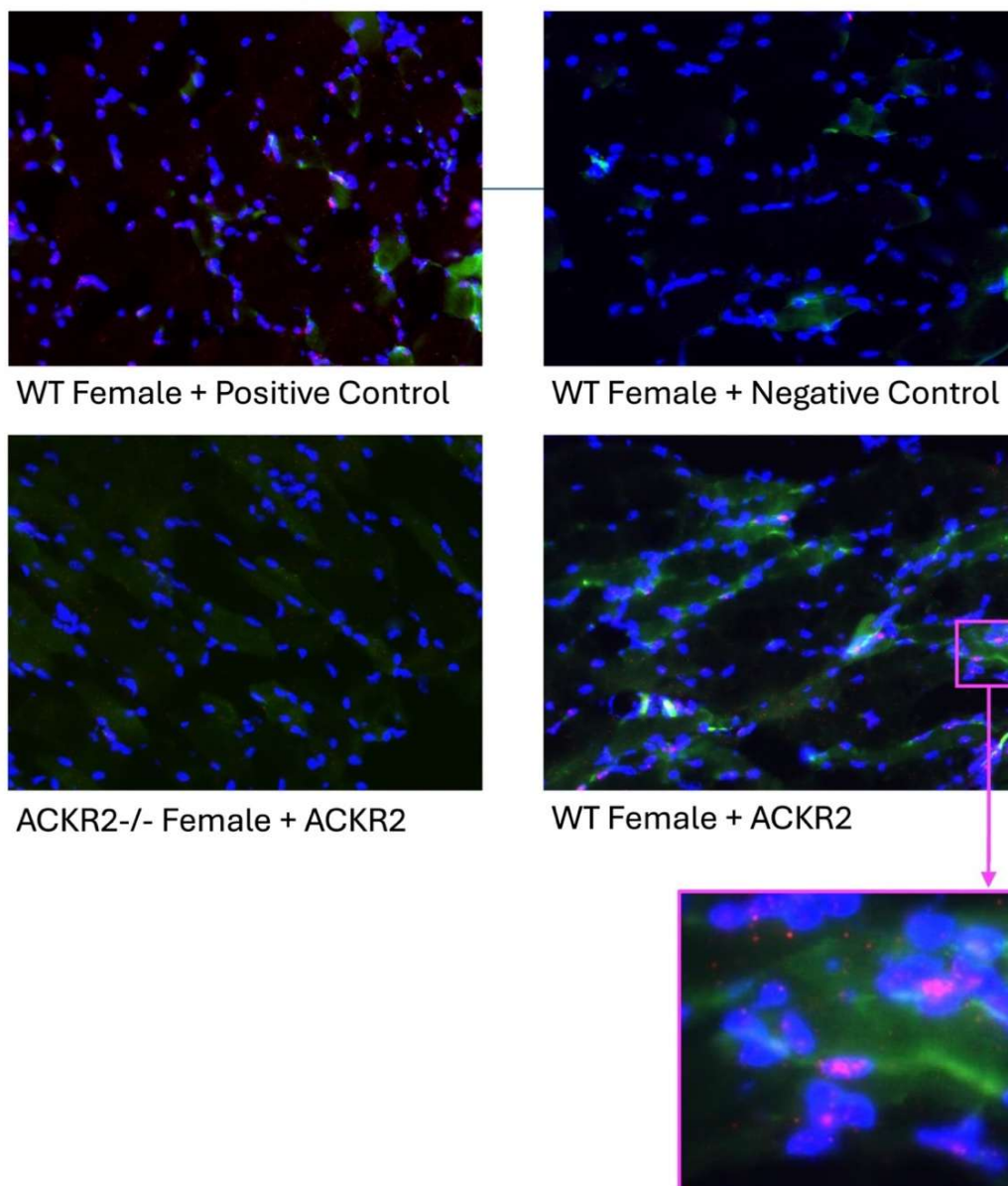


Figure 4-7 ACKR2 RNA Expression in the Peritoneal Wall at Rest

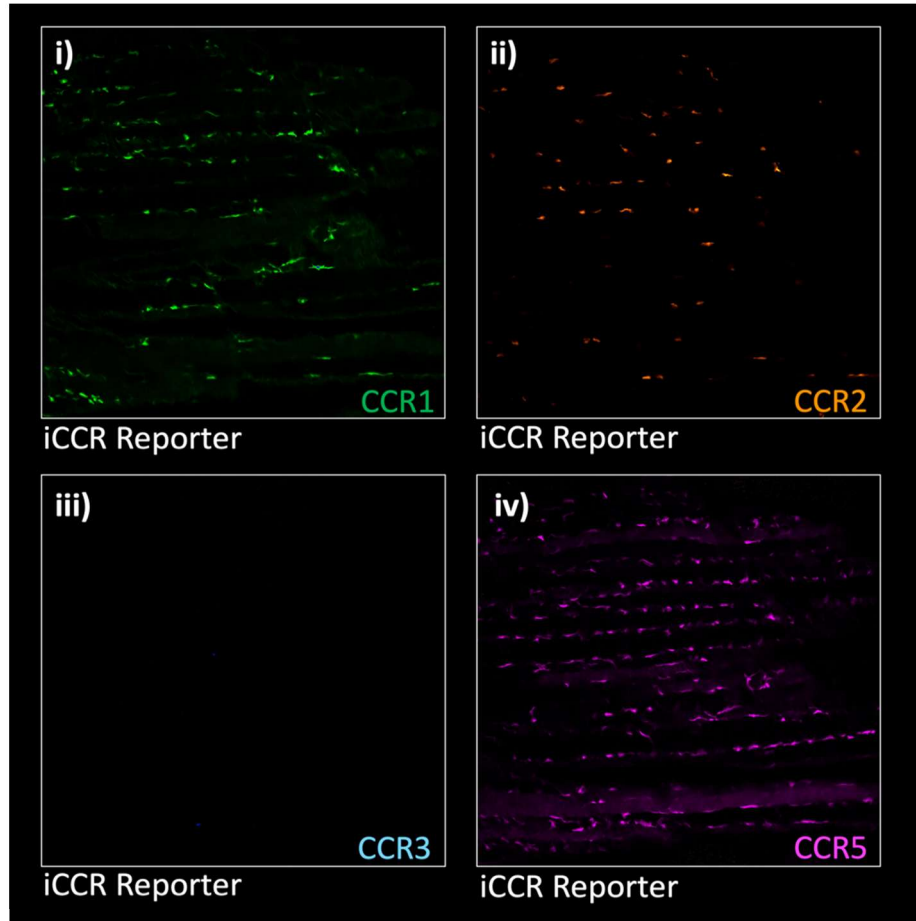
Immunofluorescent RNAscope *in situ* hybridisation of ACKR2 (red), DAPI staining (blue) and Lyve-1 antibody staining (green) in the peritoneal wall, imaged at 20X magnification. Dissection, tissue preparation and tissue sectioning by Lily Koumbas Foley, staining and imaging by Elise Pitmon and Lauren Fernandez.

4.2.4 Inflammatory Chemokine Receptors (iCCRs) are Expressed in the Peritoneal Wall

Following the identification of LECs as a source of ACKR2 in the peritoneal wall, we next investigated the expression of CCR1, CCR2, CCR3, and CCR5 in this tissue. As both ACKR2 and iCCRs share chemokine ligands (Chapter 1, Figure 1-3), confocal microscopy was used to image the peritoneal wall of iREP fluorescent reporter mice for the chemokine receptors CCR1, CCR2, CCR3, and CCR5 (iCCRs) (Medina-Ruiz et al., 2022) at rest.

Expression of CCR1, CCR2, and CCR5 was detectable in the naïve peritoneal wall by confocal microscopy of iCCR reporter mice (Figure 4-8). Notably, co-expression of iCCRs was also evident in the tissue. Co-localisation of CCR2 and CCR5 (Figure 4-8B.iii(a)), CCR1 and CCR5 (Figure 4-8B.iii(b)) and CCR2 and CCR5 expression (Figure 4-8B.iii (c)) are highlighted by white boxes (Figure 4-8). In contrast, CCR3 was not detected in the peritoneal wall at rest (Figure 4-8). Many of the CCR1/2/5-positive cells displayed an unusual, elongated morphology. These elongated cells may represent leukocytes situated between mesothelial layers or within lymphatic vessels throughout the peritoneal wall. However, the organised pattern in which the cells were positioned within the peritoneal wall may indicate smooth muscle (Figure 4-8). Ultimately, iCCR⁺ cells were identified in the peritoneal wall, suggesting that leukocyte migration in this tissue may be affected by altered ligand scavenging in the absence of ACKR2, which shares ligands with iCCRs.

A.



B.

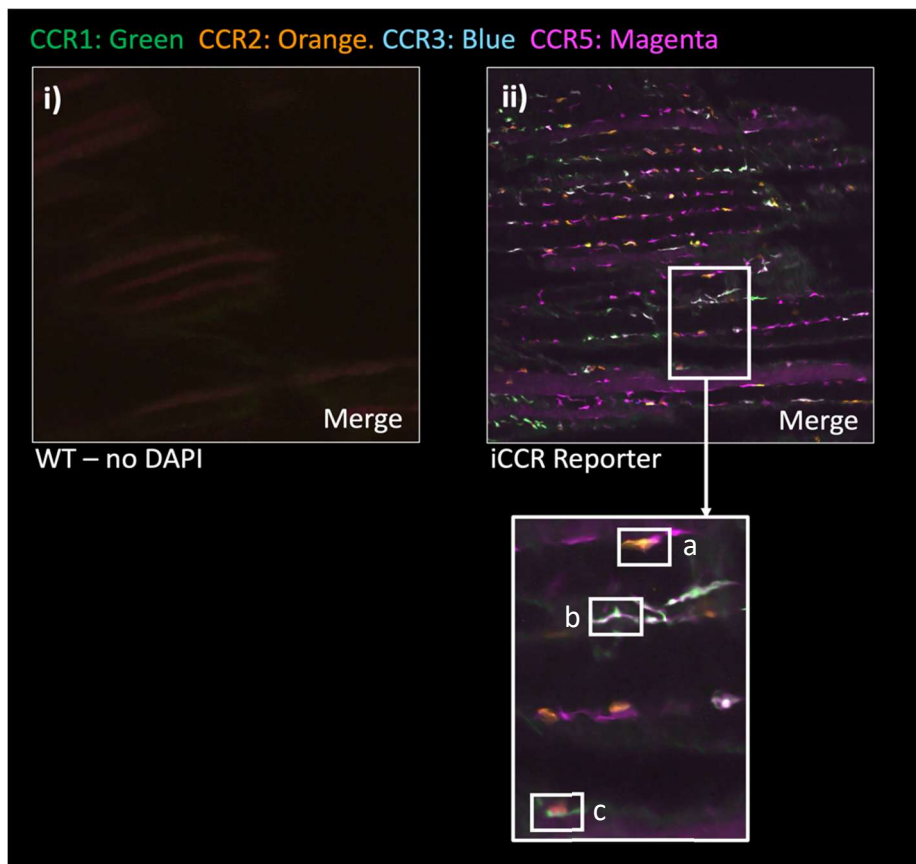


Figure 4-8 Inflammatory Chemokine Receptor Expression in the Peritoneal Wall at Rest

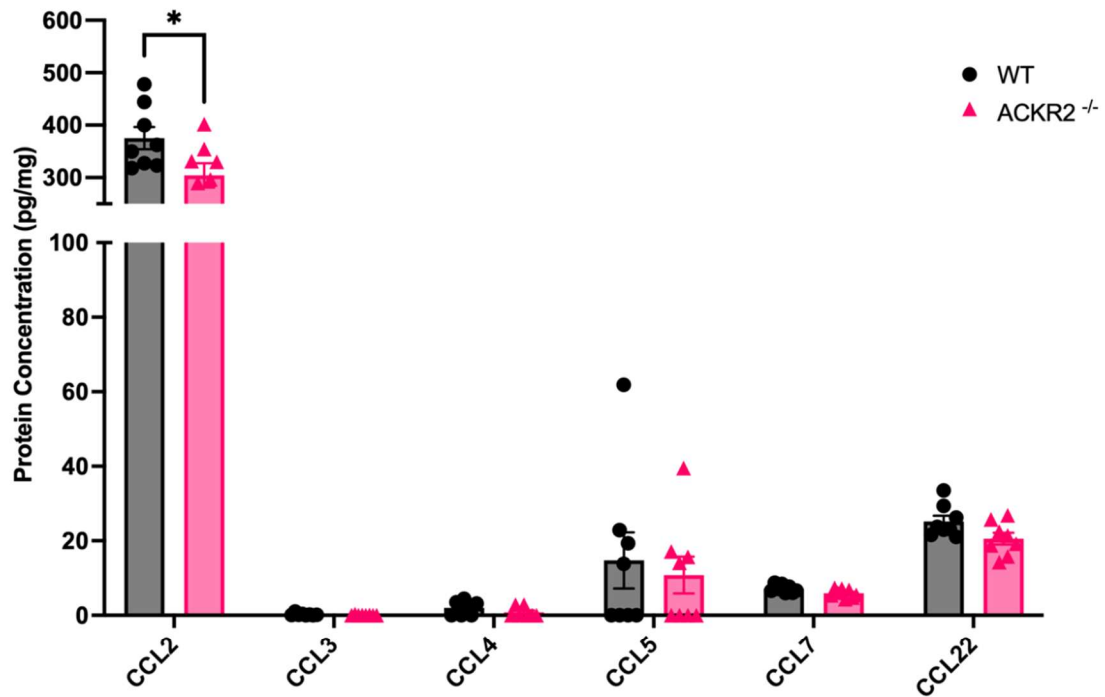
Whole-mount confocal imaging of the naïve peritoneal wall from transgenic reporter (REP) mice expressing CCR1-Clover (green), CCR2-mRuby2 (orange), CCR3- mTagBFP2 (blue), and CCR5-

iRFP682 (magenta), at 20X magnification. **A)** Single-channel images from the iREP peritoneal wall for each reporter: CCR1 **(i)**, CCR2 **(ii)**, CCR3 **(iii)**, and CCR5 **(iv)**. **B)** Merged images: **(i)** wild type peritoneal wall without DAPI, **(ii)** iCCR reporter peritoneal wall with zoomed in view highlighting receptor co-expression, shown by white boxes: **(a)** CCR2 and CCR5, **(b)** CCR2 and CCR5 **(c)** CCR1 and CCR2.

4.2.5 ACKR2 Does Not Scavenge Chemokines in the Peritoneal Cavity or the Peritoneal Wall at Rest

To examine whether ACKR2 modulation of chemokine levels may contribute to the reduced numbers of macrophages observed, we next analysed the concentrations of ACKR2 ligands; CCL2, 3, 4, 5, 7, 11, 12 and 22, in the peritoneal cavity fluid (Figure 4-9A), cavity cell lysate (Figure 4-9B) and peritoneal wall (Figure 4-10). In the peritoneal cavity fluid CCL2, CCL5, CCL7 and CCL22 were detected (Figure 4-9A), whilst CCL4 and CCL12 were not detected (Wilson et al., 2024). CCL2 decreased in the peritoneal lavage fluid of ACKR2-deficient mice from 296.5 pg/ml in the WT to 375.3 pg/ml ($p = 0.0396$) (Figure 4-9A). In contrast, in the peritoneal cavity cell lysate, CCL5 and CCL11 were detected and CCL5 concentration was lower in the cell lysate of ACKR2-deficient mice (70.7 pg/ml) compared to the WT cell lysate (177.1 pg/ml) ($p = 0.0413$) (Wilson et al., 2024). It should be noted that in the absence of ACKR2 chemokine ligands are expected to increase, not decrease, as the molecule is no longer being scavenged by the atypical receptor; however in both the lavage fluid and the cavity cell lysate, ligands were decreased in ACKR2-deficient mice (Figure 4-9A and Figure 4-9B). Notably, whilst CCL3 is known to be produced by SPMs in response to LPS stimulation *in vitro* (Takenaka et al., 2018), CCL3 was not detected in the peritoneal cavity at rest. Taken together, ACKR2 does not appear to be actively scavenging ligands in the peritoneal cavity at rest.

A.



B.

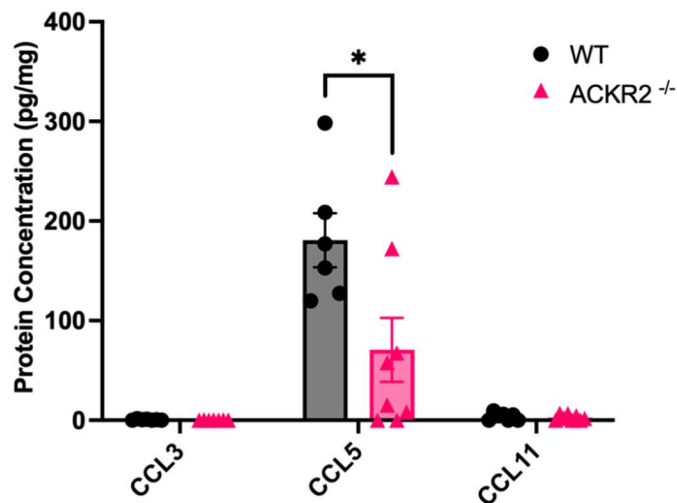


Figure 4-9 ACKR2 Ligand Protein Expression in the Peritoneal Cavity at Rest

Chemokine protein concentration in wild type (WT) and ACKR2-deficient (ACKR2^{-/-}) peritoneal cavity fluid (A) and peritoneal cavity cell lysate (B) ($n = 8$ per group) was determined by Luminex assay and normalised to total protein concentration (mg) measured by BCA assay. Statistical significance was determined by unpaired t-test or Mann Whitney ($p < 0.05$) depending on normality of the data and is indicated as $p < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****).

In the peritoneal wall, CCL2 was the most highly expressed ACKR2 ligand detected at rest (Figure 4-10). In contrast, CCL22 was present at concentrations below 10 pg/mg and CCL3, CCL5, CCL7 and CCL12 were not detected in the peritoneal wall by Luminex assay (Figure 4-10). Furthermore, none of the ACKR2 ligands were significantly increased in the peritoneal wall of ACKR2-deficient mice ($p > 0.05$), indicating that ACKR2 protein scavenging does not mediate macrophage distribution in the peritoneal wall at rest (Figure 4-10). Other selected chemokines in the peritoneal wall were also measured, including CCL19, CCL20, CXCL13 and CXCL16 (Figure 4-11).

CCL20 and CXCL13 were the most highly detected chemokines in the peritoneal wall (Figure 4-11). High CXCL13 may reflect the fact that peritoneal B1b cells, which comprise up to 80% of B cells in the cavity (Ghosn et al., 2010) are homed by CXCL13 (Ansel et al., 2002). In contrast, CCL19 and CXCL16 were not detected in the peritoneal wall by Luminex assay, indicating that they are not involved in leukocyte migration in the wall at rest (Figure 4-11). Ultimately, ACKR2 did not appear to scavenge chemokines in either the peritoneal cavity or wall at rest (Figure 4-9 and Figure 4-10). This suggests that the reduced numbers of macrophages observed in these tissues is not caused by impaired migration regulated by ACKR2.

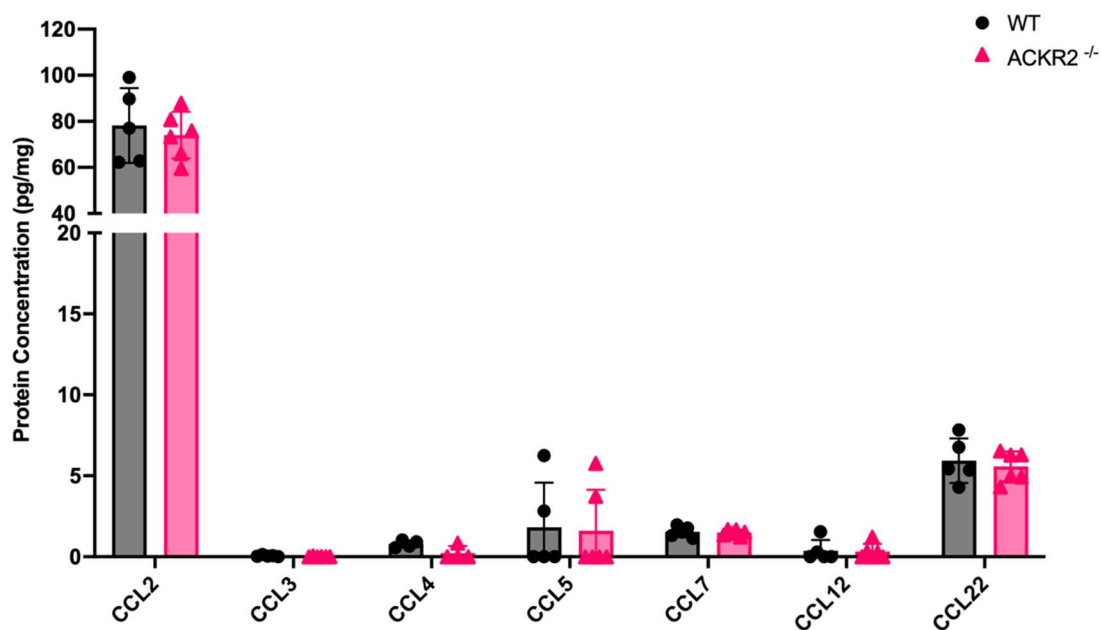


Figure 4-10 ACKR2 Ligand Expression in the Peritoneal Wall at Rest

Chemokine protein concentration in WT ($n = 5$) and ACKR2^{-/-} (ACKR2-deficient) ($n = 6$ per group) 8-week-old female mice was determined by Luminex assay and normalised to total protein concentration (mg) measured by BCA assay. Error bars represent SEM. Statistical significance was determined by unpaired t-test or Mann Whitney ($p < 0.05$) depending on normality of the data and is indicated as $p < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****).

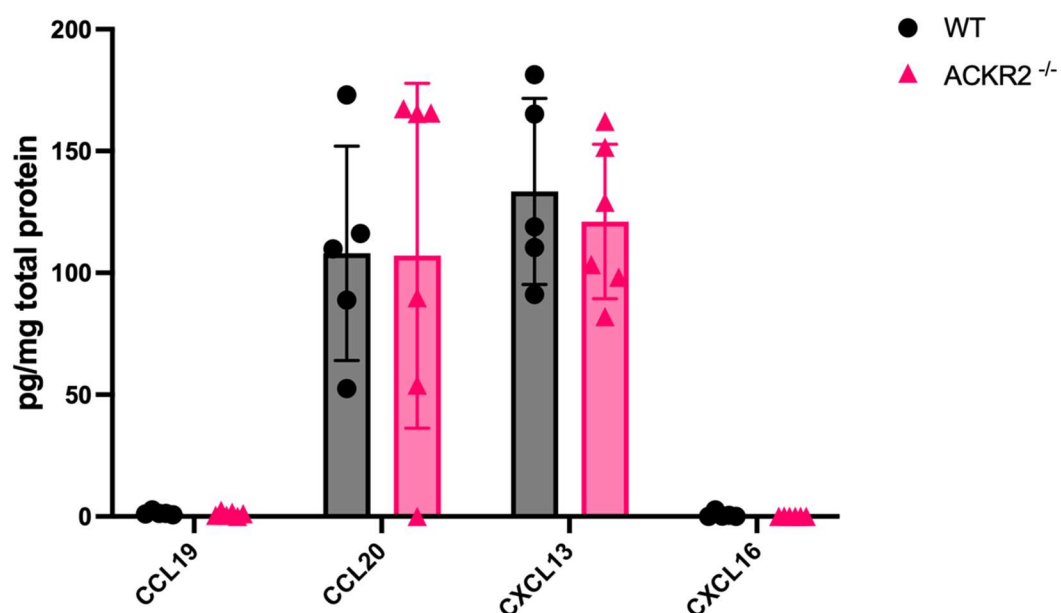


Figure 4-11 Non-ACKR2- Ligand Chemokine Protein Expression in the Peritoneal Wall at Rest

Chemokine protein concentration in WT ($n = 5$) and ACKR2^{-/-} ($n = 6$ per group) 8-week-old female mice was determined by Luminex assay and normalised to total protein concentration (mg) measured by BCA assay. Error bars represent SEM. Statistical significance was determined by unpaired t-test or Mann Whitney ($p < 0.05$) depending on normality of the data and is indicated as $p < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****).

4.2.6 Bacterial Clearance is Defective in ACKR2-Deficient Mice

To investigate the functional impact of reduced monocyte and macrophage numbers in the peritoneal cavity and wall of ACKR2-deficient mice, WT and ACKR2-deficient were intraperitoneally injected with *E. coli*, and bacterial clearance and leukocyte dynamics in response to infection were evaluated. Colony forming units (CFU) were counted in WT and ACKR2-deficient mice 18 h post intraperitoneal challenge. CFU count/ml in the peritoneal cavity of ACKR2-deficient mice was significantly higher (CFU/ml = 5214) than in WT mice (CFU/ml = 63) ($p = 0.0033$) (Figure 4-12A). Similarly, bacterial load was increased in the spleen, which is a site of bacterial clearance, of ACKR2-deficient mice (CFU/ml = 21000), in comparison with WT controls (CFU/ml = 4163) ($p = 0.0214$) (Figure 4-12B). Bacterial clearance was not changed in the liver or peritoneal wall ($p > 0.05$) (Figure 4-12C and Figure 4-12D). No CFU were detected in the PBS treated mice indicating that results were not the result of contamination (Figure 4-12). CFU was also not detected in any blood samples highlighting that the infection did not become systemic (data not shown). Thus, bacterial elimination was defective in ACKR2-deficient mice.

The number of neutrophils and monocytes in the peritoneal cavity, as well as the number of SPMs and LPMs in both the peritoneal cavity and peritoneal wall of WT and ACKR2-deficient mice were analysed 18 h following *E. coli* infection using flow cytometry. Gating strategies are shown in Figure 4-2 and Figure 4-4. This analysis was performed to determine whether the impaired bacterial clearance observed in the peritoneal cavity of ACKR2-deficient mice was associated with altered leukocyte dynamics in peritoneal tissues. The number of neutrophils observed in the peritoneal cavity of ACKR2-deficient mice following *E. coli* infection was higher (mean = 11,178.2 neutrophils/ml) than in the peritoneal cavity of WT mice (mean = 3,387.4 neutrophils/ml) ($p = 0.0069$) (Figure 4-13A.i). Similarly, a greater number of monocytes were also observed in the peritoneal cavity of ACKR2-deficient mice following bacterial infection (mean = 680.5 monocytes) compared to the WT (mean = 200.7) ($p = 0.0052$) (Figure 4-13A.ii). These data suggest that ACKR2-deficient mice were less effective at resolving bacterial infection within 18 hours compared to WT mice, consistent with the higher CFU counts observed in the peritoneal cavity of ACKR2-deficient mice (Figure 4-12A).

In addition, both SPMs and LPMs were reduced in the peritoneal cavity following *E. coli* infection, with lower cell numbers observed after PBS injection than after *E. coli* injection in both WT and ACKR2-deficient mice ($p < 0.05$) (Figure 4-13B.i and Figure 4-13B.ii).

This likely reflects the macrophage disappearance reaction, in which, during infection of the peritoneal cavity, macrophages, in particular LPMs, disappear from peritoneal fluid and adhere to the peritoneal wall, as well as migrate to other not yet well-established sites (Barth et al., 1995). Notably, in ACKR2-deficient mice, both SPMs and LPMs did not decrease to the same extent following infection, suggesting that the disappearance process was disrupted. The number of SPMs found in ACKR2-deficient mice was higher (18.4 SPMs/ml) than in WT mice (mean = 7.0 SPMs/ml) ($p = 0.0047$) (Figure 4-13B.i). This was also observed for LPMs. A mean number of 62.0 LPMs/ml was observed in the WT peritoneal cavity following *E. coli* infection whilst a mean of 382.8 LPMs/ml was observed in the cavity of ACKR2-deficient mice ($p = 0.0005$).

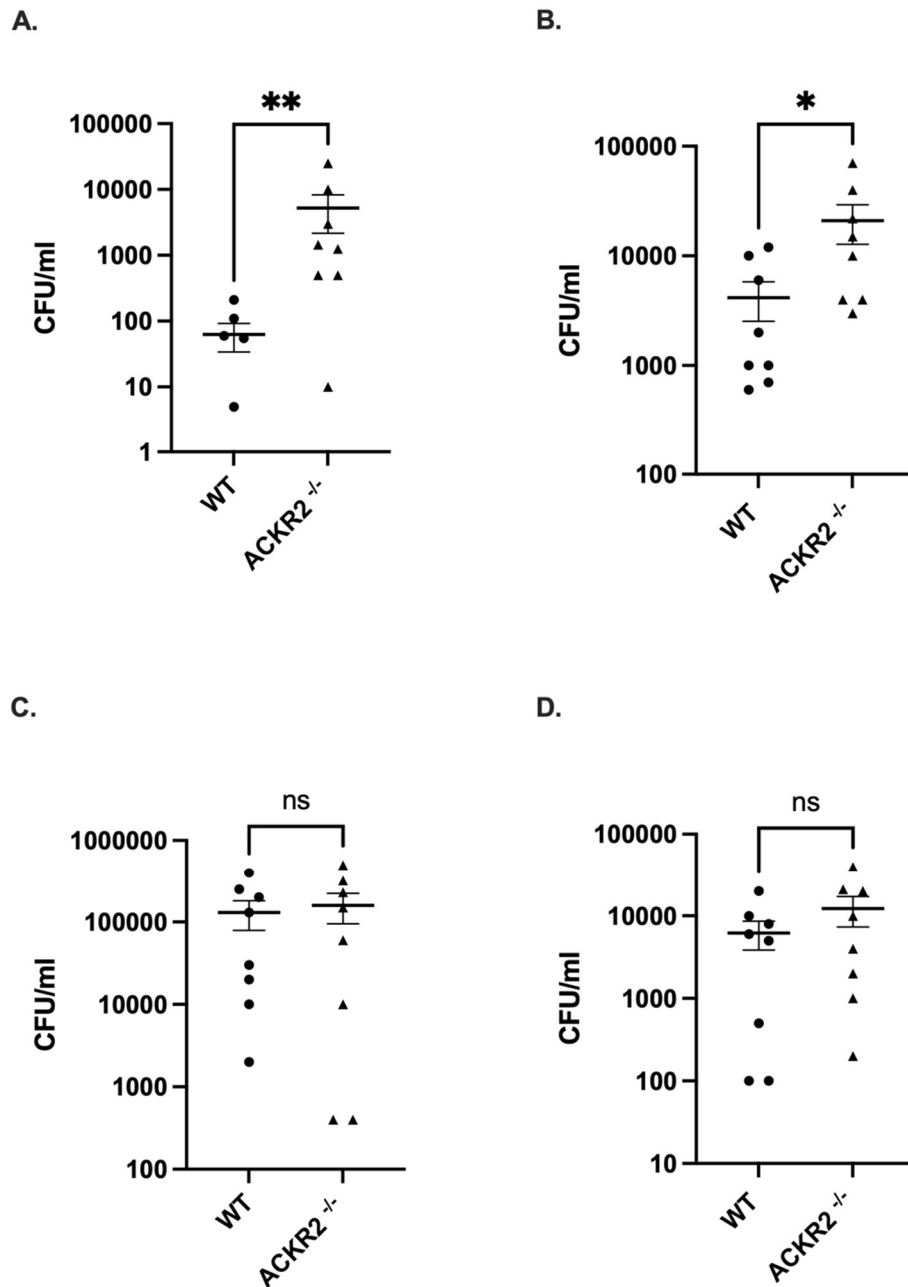
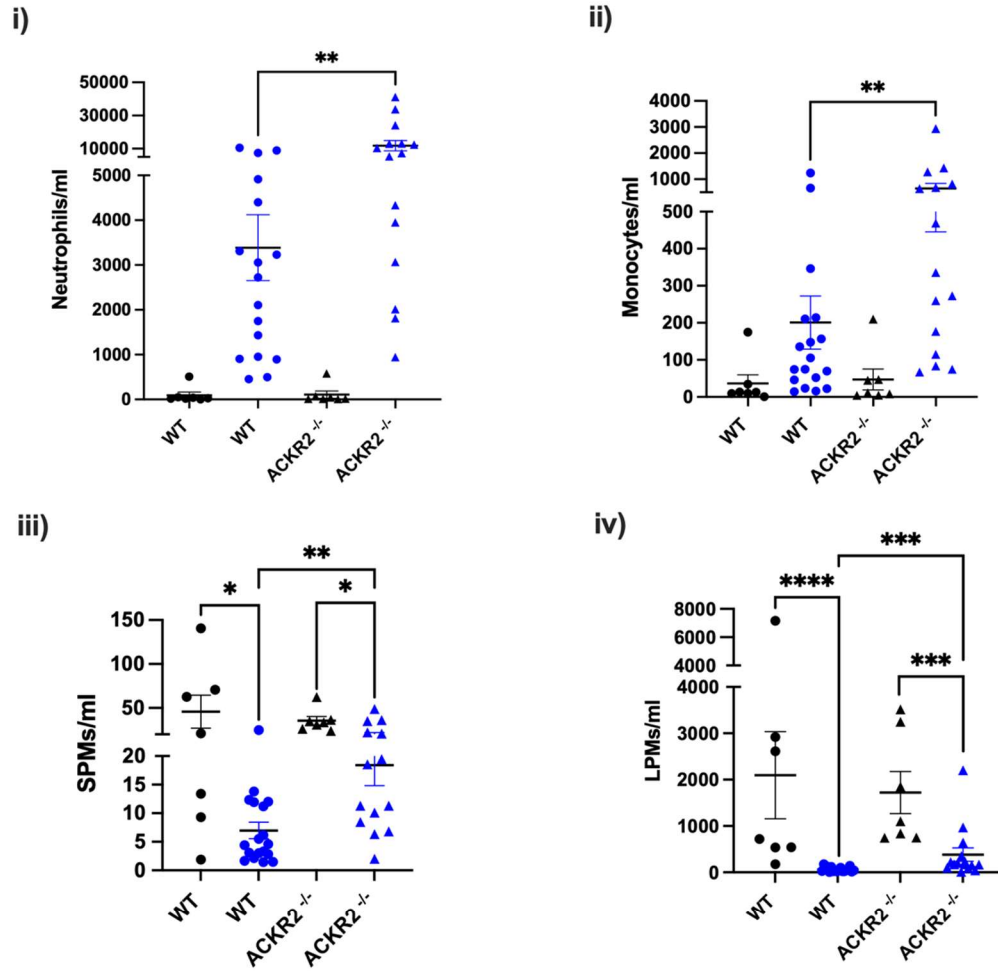


Figure 4-12 CFU counts in Wild Type and ACKR2-Deficient Tissues Following *E. coli* Infection

Bacterial colony forming units (CFU) / ml were counted from **A)** peritoneal cavity, **B)** spleen, **C)** peritoneal wall, and **D)** liver ($n = 8$ per group). Error bars represent SEM. Statistical significance was determined by unpaired t-test or Mann Whitney ($p = < 0.05$) depending on normality of the data and is indicated as $p < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****). Administration of *E. coli* and CFU count data by Gill Wilson.

A. Peritoneal Cavity

● WT PBS ● WT *E. coli* ▲ ACKR2^{-/-} PBS ▲ ACKR2^{-/-} *E. coli*



B. Peritoneal Wall

● WT PBS ● WT *E. coli* ▲ ACKR2^{-/-} PBS ▲ ACKR2^{-/-} *E. coli*

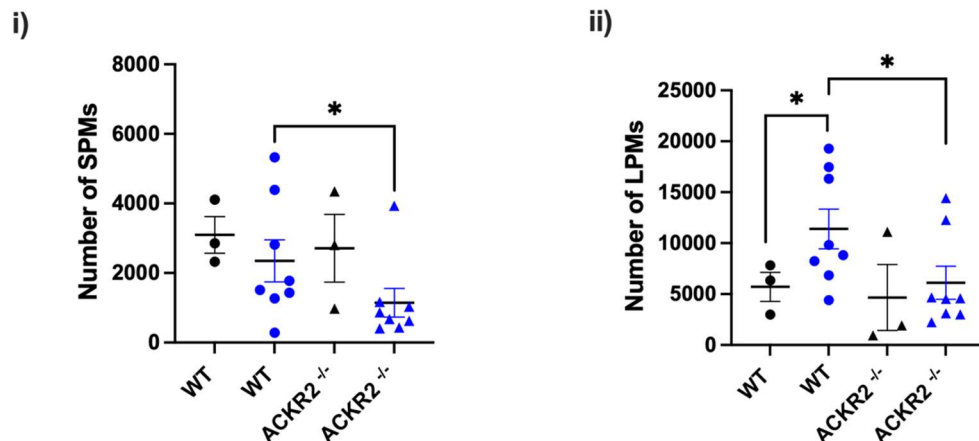


Figure 4-13 Leukocyte Numbers in the Peritoneal Cavity and Peritoneal Wall of Wild Type and ACKR2-Deficient Mice Following Intraperitoneal *E. coli* Infection

A) Flow cytometry of the peritoneal cavity was used to quantify neutrophils (i), monocytes (ii), SPMs (iii) and LPMs (iv) in WT and ACKR2^{-/-} (ACKR2-deficient) mice following injection with either PBS ($n = 7$ per group) or *E. coli* (WT $n = 18$, ACKR2^{-/-} $n = 17$). Peritoneal cavity data were

provided by Dr. Gillian Wilson (Wilson et al., 2024). **(B)** Flow cytometry of the peritoneal wall was used to determine the number of SPMs **(i)** and LPMs **(ii)** in WT and ACKR2^{-/-} mice following injection with either PBS ($n = 3$ per group) or E. coli ($n = 8$ per group). Error bars represent SEM. Outliers were identified using the Grubbs test (Prism) and removed before statistical analysis. Statical significance was determined by unpaired t-test or Mann Whitney ($p = < 0.05$) depending on normality of the data and is indicated as $p < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****).

Additionally, although bacterial clearance in the peritoneal wall was not affected by ACKR2, differences in the number of SPMs and LPMs in the peritoneal wall of ACKR2-deficient mice compared to WT mice were observed 18 h post *E. coli* infection (Figure 4-13A and Figure 4-13B). Fewer SPMs were measured in the peritoneal wall of ACKR2-deficient mice following *E. coli* infection (mean = 744.4) (Figure 4-13A) in comparison with the WT (mean = 1643) ($p = 0.0499$) (Figure 4-13B). However, no significant difference was observed in the number of SPMs in PBS treated WT and ACKR2-deficient mice. This may seem to contradict earlier results (Figure 4-5A.i); however, the sample size of the PBS group was very small (Figure 4-13), and we have also observed that PBS injection alone can alter leukocyte dynamics within the peritoneum, likely in response to the injection induced injury, as the procedure involves directly piercing the peritoneal wall (data not shown).

In addition, in the WT peritoneal wall, LPM numbers rose from a mean of 4715.5 LPMs/wall following PBS injection to 11400.0 LPMs/wall in response to bacterial challenge ($p = 0.0456$) (Figure 4-13B). This increase in LPMs during infection has previously been reported, e.g. following treatment with *E. coli*, LPS or zymosan, LPMs migrate from the peritoneal cavity to the peritoneal mesothelium where they adhere to the wall and form aggregates that provide a scaffold for neutrophil and SPM attachment to resolve inflammation (Vega-Perez et al., 2021). In contrast, no significant difference was observed between the number of LPMs in the peritoneal wall of ACKR2-deficient mice injected with PBS or *E. coli* ($p > 0.05$) (Figure 4-13B). In addition, Figure 4-13B demonstrates that LPMs reached higher numbers in the peritoneal wall of WT mice (mean = 11400.9 LPMs/wall) compared to ACKR2-deficient mice (mean = 6108.2 LPMs/wall) following *E. coli* challenge ($p = 0.0499$). It is possible that the lower number of LPMs observed in ACKR2-deficient mice after bacterial infection was due to LPMs becoming less adherent to the wall in the absence of ACKR2. This may explain why there was no observable difference in the number of LPMs at rest (Figure 4-5A.ii) as cells measured in naïve mice may be mostly localised within the wall, as opposed to adhered to the mesothelial surface.

4.2.7 Examination of Protein Levels of Other Potential Mediators.

Given that we observed that ACKR2 was expressed by LECs but did not actively scavenge chemokines in the peritoneal wall, we conducted a broader Luminex analysis that included selected cytokines, growth factors and matrix metalloproteinases to identify other factors potentially contributing to reduced macrophage numbers in the peritoneum.

Limited but specific changes in the protein expression of selected cytokines, MMPs, growth factors and other mediators were observed in the peritoneal wall of naïve ACKR2-deficient mice compared with WT mice, as measured by Luminex assay (Figure 4-14). Of the cytokines analysed, IL-6 and BAFF were detected at the highest levels, IL-10 was also detected at lower levels, whilst TNF- α was not detected (Figure 4-14A). Figure 4-14A demonstrates that there was no difference in protein expression of the detected cytokines between WT and ACKR2-deficient mice ($p > 0.05$), indicating that the release of these cytokines is not affected by ACKR2. Both MMP-2 (WT mean = 979 pg/mg) and MMP-9 (WT mean = 47.84 pg/mg) were detected in the peritoneal wall; however, MMP-2 was detected at substantially higher levels (Figure 4-14B). Furthermore, MMP-2 was detected at a significantly higher level (mean = 1422 pg/mg) in the ACKR2-deficient peritoneal wall than in the WT peritoneal wall (mean = 979.5 pg/mg) ($p = 0.0103$) (Figure 4-14B). MMP-2 is known to contribute to mesothelial adhesion by degrading the extracellular matrix and modifying adhesion molecules in the context of tumour cell-mesothelial adhesion (Carey et al., 2021). Therefore, it is possible that the increase in MMP-2 observed in ACKR2-deficient mice may be related to changes in adhesion of LPMs and SPMs to the peritoneal wall. However, in contrast to tumour models where increased MMP-2 is associated with enhanced tumour cell adhesion to the peritoneal surface (Ramos et al., 2024), the upregulation of MMP-2 in ACKR2-deficient mice is associated with reduced numbers of SPMs in the peritoneal wall. This suggests that elevated MMP-2 in this context may not promote, and could potentially inhibit, macrophage adhesion. These findings highlight a potentially distinct, context-dependent role for MMP-2 in regulating interactions between peritoneal macrophages and the mesothelial surface of the peritoneum.

A variety of analytes were also measured. Chil-1 and CD105 (Endoglin) were detected at the highest levels (Figure 4-14C). The growth factors IGF-1, EGF, FGF-1 and were also detected, but at much lower levels. M-CSF (macrophage colony stimulating factor-1) was not detected at all in the peritoneal wall (Figure 4-14C) despite its key role in monocyte to

macrophage differentiation (Ushach and Zlotnik, 2016). This raises questions about how macrophages are maintained in this tissue. Monocytes are present at rest (Figure 4-6B), suggesting ongoing recruitment, but their differentiation may rely on alternative CSF1R ligands such as IL-34 or TGF- β . Therefore, it is possible that the peritoneum utilises an M-CSF-independent pathway for macrophage differentiation, potentially shaping a unique subset of macrophages with specialised functional properties. Furthermore, IL-10, detected at rest in the peritoneal wall (Figure 4-14A), may contribute to macrophage differentiation as it does following infection (Nguyen et al., 2012). Although M-CSF is essential for LPM proliferation at rest (Davies et al., 2013), its absence in the wall suggests it may instead be produced in the peritoneal cavity.

Finally, levels of Chil-1 were reduced in the peritoneal wall of ACKR2-deficient mice from a mean of 123.8 pg/mg in the WT to 61.49 pg/mg ($p = 0.0192$) (Figure 4-14C).

Downregulation of *CHIL1* in ACKR2-deficient mice has also been observed in RNA sequencing of SPMs (Wilson et al., 2024). Finally, levels of Endoglin (CD105) protein were significantly higher in the ACKR2-deficient peritoneal wall at rest (Figure 4-14C). A mean of 378.5 pg/mg of Endoglin was detected in peritoneal wall of ACKR2-deficient mice, compared to 231.0 pg/mg in the peritoneal wall of WT mice ($p = 0.0173$) (Figure 4-14C).

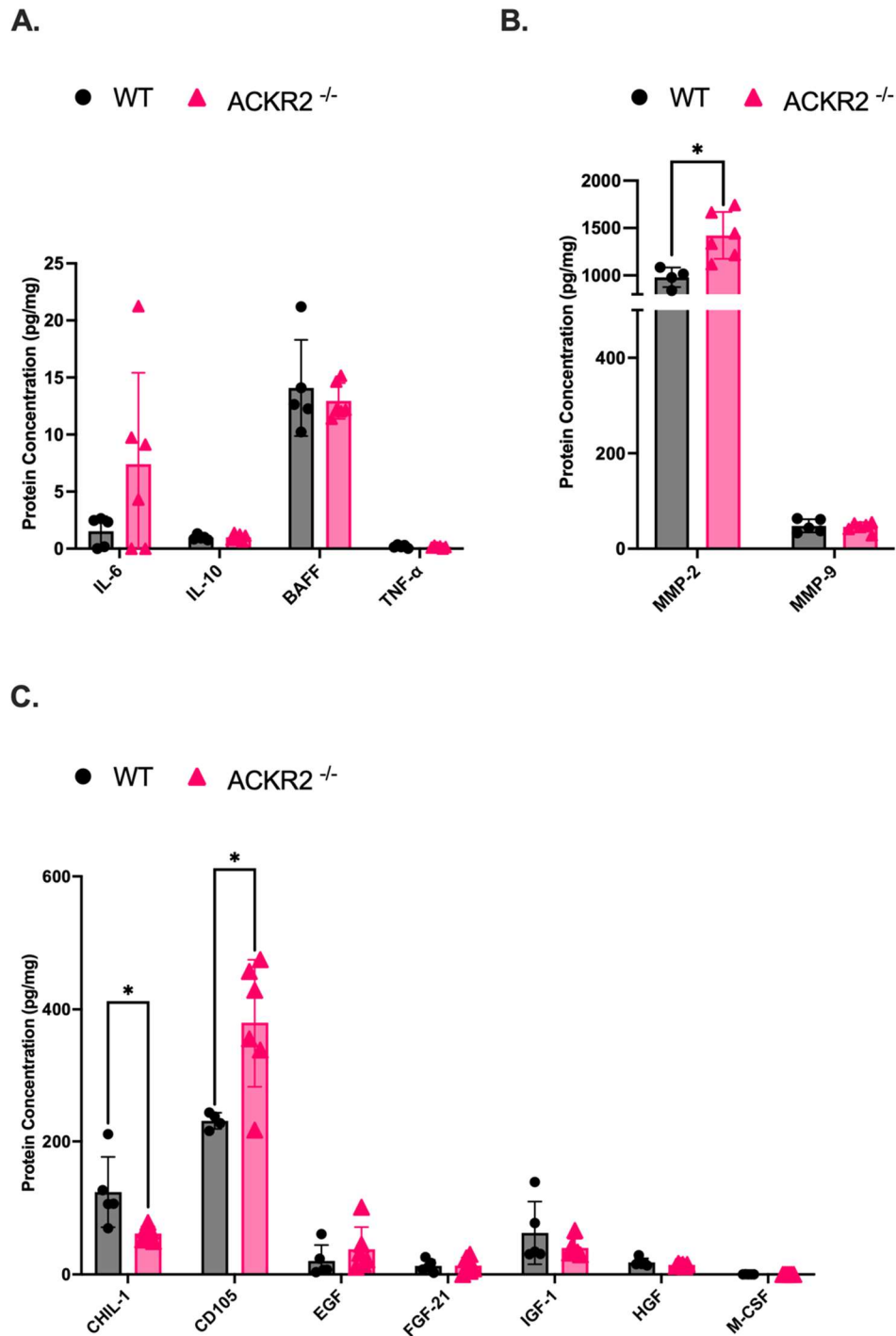


Figure 4-14 Protein Concentration of Other Potential Mediators in the Peritoneal Wall at Rest

Cytokine (A) MMP (B) and other mediator (C) protein concentration in wild type (WT) ($n = 5$) and ACKR2-deficient (ACKR2^{-/-}) ($n = 6$ per group) 8-week-old female mice was determined by Luminex assay and normalised to total protein concentration (mg) measured by BCA assay. Error bars represent SEM. Statistical significance was determined by unpaired t-test or Mann Whitney ($p = < 0.05$) depending on normality of the data and is indicated as $p < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****).

4.2.8 ACKR2 is Associated with Reduced Numbers in a Specific Lung Macrophage Population

Finally, to assess whether ACKR2 deficiency disrupts macrophage numbers in other tissues, MHCII⁺ and MHCII⁻ macrophages were quantified across multiple tissues, including sites comparable to the peritoneal cavity (pleural cavity) as well as sites distal to the peritoneum (lung, skin, inguinal lymph node and bone marrow). Additionally, LPMs were also analysed in the pleural cavity where they are conventionally found. ACKR2 deficiency did not alter the number of SPMs in the pleural cavity (Figure 4-15A.i) ($p > 0.05$). Similarly, the number of pleural LPMs were also unaffected by ACKR2 ($p > 0.05$) (Figure 4-15A.ii). This indicates that the mechanism underlying the reduction of SPMs in the peritoneal cavity is not common to all similar fluid compartments.

Next, MHCII⁻ and MHCII⁺ macrophage populations in distal tissues were investigated. Although ACKR2 deficiency did not affect the number of interstitial MHCII⁺ macrophages in the lung (Figure 4-15B.i) ($p > 0.05$), a significant reduction in interstitial MHCII⁻ macrophages was observed in the lung of ACKR2-deficient mice (mean = 4466) compared to WT controls (mean = 6980) ($p = 0.0018$) (Figure 4-15B.ii). Neither MHCII⁺ nor MHCII⁻ macrophage populations were changed in numbers in the skin (Figure 4-15C.i and Figure 4-15C.ii), inguinal lymph node (Figure 4-15D.i and Figure 4-15D.ii), or bone marrow (Figure 4-15E.i and Figure 4-15E.ii) ($p > 0.05$). These findings suggest that although ACKR2 does not globally alter macrophage numbers, it reduces specific macrophage populations in the lung, as well as in the peritoneal cavity and peritoneal wall (Figure 4-15B.ii, Figure 4-3A and Figure 4-5A.i). Notably, the lung macrophage population reduced in ACKR2-deficient mice was MHCII⁻, whereas SPMs are MHCII⁺, indicating that these populations are likely to be highly distinct. It is therefore important to determine whether the association between ACKR2 and the reduction in their numbers is driven by a shared mechanism. If so, identifying common features between these macrophage populations may elucidate why these macrophages are selectively affected.

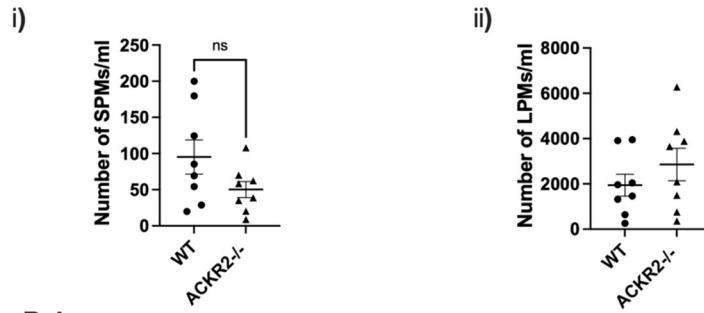
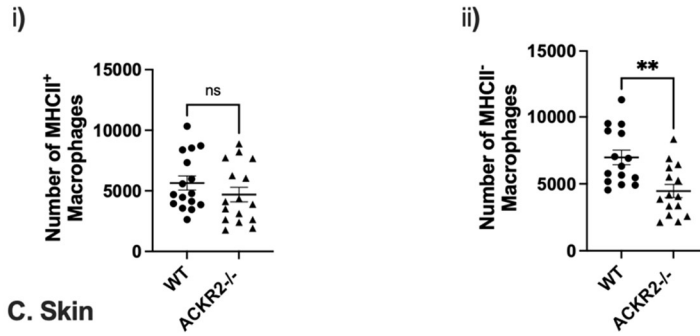
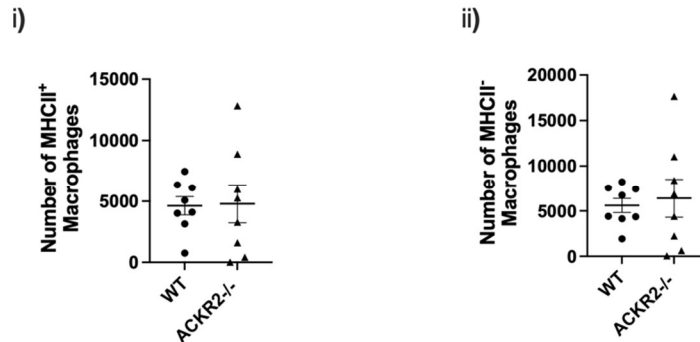
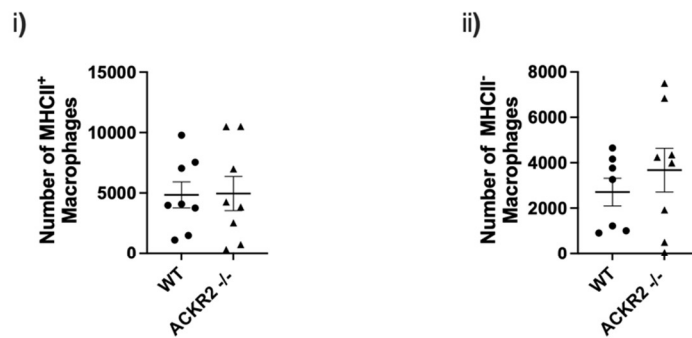
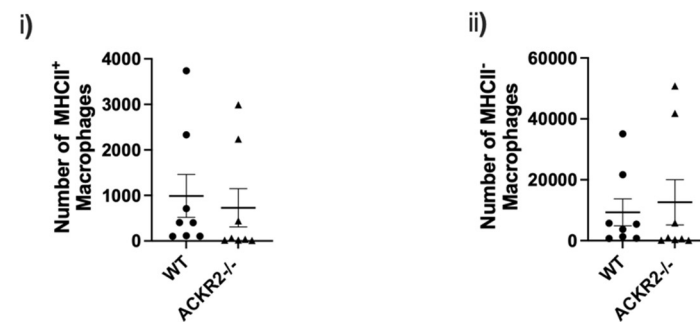
A. Pleural Cavity**B. Lung****C. Skin****D. Inguinal Lymph Node****E. Bone Marrow**

Figure 4-15 Numbers of Macrophages Across Multiple Tissues in Wild Type and ACKR2-Deficient Mice at Rest

Flow cytometric analysis was used to determine the number of macrophages within different subsets in naïve wild type (black circles) and ACKR2^{-/-} (black triangles) 8-week-old female mice. All macrophages were gated as CD45⁺/CD19⁻/Ly6G⁻/Ly6C⁻. **A)** Pleural Cavity: **(i)** SPMs/ml **(ii)** LPMs/ml of pleural lavage fluid ($n = 8$ per group). CD11b⁺F480⁺ macrophages were divided into MHCII⁺ and MHCII⁻ populations. **B)** Lung: **(i)** F480^{mid} MHCII⁺ macrophages **(ii)** F480^{mid} MHCII⁻ macrophages ($n = 16$ per group). **C)** Skin: **(i)** F480^{mid} MHCII⁺ macrophages **(ii)** F480^{mid} MHCII⁻ macrophages ($n = 8$ per group). **D)** Inguinal Lymph Node: **(i)** F480^{mid} MHCII⁺ macrophages **(ii)** F480^{mid} MHCII⁻ macrophages ($n = 8$ per group). **E)** Bone Marrow: **(i)** F480^{mid} MHCII⁺ macrophages **(ii)** F480^{mid} MHCII⁻ macrophages ($n = 8$ per group). Error bars represent SEM. Outliers were identified using the Grubbs test (Prism) and removed before pairwise statistical analysis. Statistical significance was determined by unpaired t-test or Mann Whitney ($p = < 0.05$) depending on normality of the data and is indicated as $p < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****). Data are representative of repeats.

4.3 Discussion

Following the finding that SPMs were reduced in the peritoneal cavity and wall of ACKR2-deficient mice, this chapter aimed to investigate the role of ACKR2 in regulating the number of macrophages within adult tissues. Whilst CCR1, CCR2 and CCR5 expression was observed in the peritoneal wall (Figure 4-8), and a source of ACKR2 RNA expression was identified on lymphatic endothelial cells in the peritoneal wall (Figure 4-7), ACKR2 chemokine ligands were not elevated in the peritoneal wall of ACKR2-deficient mice, highlighting that despite presence of ACKR2 in this tissue ACKR2 does not scavenge chemokines in the peritoneal cavity at rest (Figure 4-9 and Figure 4-10). This suggests that ACKR2 does not mediate the migration of SPMs in the peritoneum and that the observed phenotype is not the result of dysregulated monocyte or macrophage recruitment. This was further supported by the fact that SPMs did not appear to be accumulating in proximal tissues such as the omentum, spleen, or mesenteric lymph node (Figure 4-5B.i, Figure 4-5C and Figure 4-5D). Peritoneal and pleural macrophages, collectively referred to as serous macrophages, occupy a similar anatomical niche and both tissues contain a resident LPM population and a monocyte derived SPM population (Bain and Jenkins, 2018). It was therefore hypothesised that ACKR2 deficiency would also be associated with a reduction in the number pleural SPMs; however, no difference was observed in the number of pleural SPMs in ACKR2-deficient mice (Figure 4-15). This suggests that the mechanism responsible for the reduction of SPMs in the peritoneal cavity is not shared across comparable fluid tissue compartments and is not specific to serosal macrophages, further supported by the observed reduction in a lung macrophage population (Figure 4-15A.i).

The reduced levels of SPMs in the peritoneal cavity, as well as monocytes and SPMs in the peritoneal wall, were associated with impaired bacterial clearance in ACKR2-deficient mice 18 h after *E. coli* infection (Figure 4-12). The formation of cellular aggregates at the peritoneal wall, in which LPMs adhere to the mesothelium and scaffold adhesion of further leukocytes such as neutrophils, is crucial for the destruction of bacteria during peritoneal infection (Vega-Perez et al., 2021). In this chapter, we show that LPM numbers are reduced in the ACKR2-deficient peritoneal wall compared to the WT following *E. coli* infection (Figure 4-13) and therefore this may explain the higher CFU counts observed in the peritoneal cavity of infected ACKR2-deficient mice. Despite these observations, the cellular dynamics shown in Figure 4-13 are associations and do not establish a causal

relationship with the increased bacterial burden in ACKR2-deficient mice. In addition, investigation carried out by Wilson et al., demonstrated that SPMs in the peritoneal cavity of ACKR2-deficient mice do not have normal phagocytic capacity, therefore it is unclear whether impaired clearance of *E. coli* was due to the reduced numbers of SPMs, reduced LPMs in the peritoneal wall, altered phagocytic capacity or a combination of all of these factors (Wilson et al., 2024). However, altered tissue cell migration driven by dysregulated chemokine scavenging is unlikely to account for the defect as Wilson et al., measured the protein level of ACKR2 ligands CCL2, CCL3, CCL4, CCL5, CCL12 and CCL22 in the peritoneal fluid 18 h after *E. coli* infection in ACKR2-deficient and WT mice. All of the chemokines measured were detected after infection; however, no differences in ACKR2 ligand concentrations between WT mice and ACKR2-deficient mice were observed, highlighting that ACKR2 does not scavenge these ligands in the peritoneum during infection (Wilson et al., 2024)

Furthermore, LPM numbers in the peritoneal wall were unchanged at rest but significantly reduced in ACKR2-deficient mice after *E. coli* infection, suggesting that ACKR2 may regulate LPM adhesion to the mesothelium during infection, rather than their recruitment under resting conditions. To test this, an adhesion assay or microscopy-based quantification of LPMs at the mesothelium could be performed in WT and ACKR2-deficient mice. LPM adhesion to the peritoneal surface plays a key role in tissue repair (Ardavin et al., 2023, Zindel et al., 2021, Vega-Perez et al., 2021). However, uncontrolled inflammation at the peritoneum can lead to fibrin deposition that leads to fibrosis, and this scarring of the peritoneal wall is associated with severe morbidities (Zindel et al., 2021, Ardavin et al., 2023). Although the data in this chapter showed reduced bacterial clearance in the peritoneal cavity of ACKR2-deficient mice, if the mice eventually resolve the infection, they may exhibit reduced or enhanced fibrosis. Therefore, histology of the peritoneum, for example by hematoxylin (stains nuclei) and eosin (stains extracellular matrix and cytoplasm) staining, after recovery from *E. coli* infection should be analysed in WT and ACKR2-deficient mice. LPMs have been shown to help prevent post-operative adhesions (Ito et al., 2021). Exploring whether ACKR2 plays a role in regulating LPMs could have important implications for ameliorating post-operative complications like adhesions.

Specific changes in protein levels in response to ACKR2 deficiency were identified. For example, Chil-1 protein (human homolog encoded by *CHI3L1*) was downregulated in the ACKR2-deficient peritoneal wall (Figure 4-14). Our group has also shown that *CHIL1* is a

differentially expressed gene between SPMs isolated from WT and ACKR2-deficient mice (Wilson et al., 2024). This reduction is observed in both peritoneal tissues, possibly due to lower SPM numbers. However, since protein was isolated from the entire peritoneal wall, it is unclear which specific cells contributed to the observed changes.

Despite this, the corresponding reduction in the *CHIL1* transcript in peritoneal cavity SPMs suggests that SPMs may be the source of Chil-1 in the peritoneal wall. Chil-1 can bind chitin, which is a polysaccharide component of the cell wall of pathogenic fungi and some bacteria and protists, heparin, and collagen (Kognole and Payne, 2017); however, its ligand remains unknown (Yu et al., 2024). Chil-1 is highly expressed by macrophages in the context of various inflammatory diseases, including liver fibrosis and Multiple Sclerosis (Kumagai et al., 2016, Quintana et al., 2018). Activation of Chil-1 has numerous downstream implications on macrophage activity, including migration and proliferation, and it has been associated with alternative immune activation and M2 macrophage polarisation (Yu et al., 2024). Chil-1 is also able to activate TGF- β signalling pathways through interaction with its receptor, promoting inflammation (He et al., 2013, Yu et al., 2024). The role of Chil-1 in the peritoneal cavity and wall remains unclear.

In addition, CD105 (Endoglin) levels were elevated in the ACKR2 deficient peritoneal wall (Figure 4-14). Endoglin is a coreceptor for TGF- β and exists as two isoforms, L-Endoglin and S-Endoglin. Endoglin is expressed on endothelial cells and is upregulated during endothelial cell proliferation and plays established roles in angiogenesis (Liu et al., 2020). Imaging of the vasculature and lymphatic network throughout the peritoneal wall using immunohistochemistry or immunofluorescence would determine any changes in vessel structure related to altered Endoglin levels associated with ACKR2 deletion. Furthermore, Endoglin is also expressed on macrophages, including peritoneal macrophages and is increased throughout monocyte to macrophage differentiation both *in vitro* and *in vivo* (peritoneal cavity) (Ojeda-Fernandez et al., 2016). In a zymosan-induced peritonitis model, Endoglin expression was downregulated to 0% expression in SPMs until day 3 post infection. Additionally, peritoneal macrophages from Endoglin-deficient mice exhibited reduced phagocytic capacity and altered expression of TGF- β -regulated genes (Ojeda-Fernandez et al., 2016). Both Endoglin and Chil-1 can activate TGF- β signalling pathways through different means. Pathway analysis of bulk RNA-seq data of bone marrow monocytes revealed significant changes in the NF- κ B1 pathway in ACKR2-deficient mice. Notably, TGF- β 1 levels were reduced, which is critical for alternative macrophage activation (Wilson et al., 2024). It is important to highlight that we cannot determine the source of Endoglin in the WT peritoneal wall from the data obtained.

However, given that both Chil-1 and Endoglin contribute to monocyte-to-macrophage differentiation (Yu et al., 2024, Ojeda-Fernandez et al., 2016), further investigation is needed to determine whether ACKR2 leads to broader dysregulation of macrophage development via disrupted TGF- β signalling.

Overall, the data so far demonstrate that ACKR2-deficient mice had reduced numbers of specific macrophage populations in the peritoneal cavity, peritoneal wall and lung. This had functional consequences but was not associated with impaired chemokine scavenging by ACKR2. Although levels of MMP-2, Chil-1 and Endoglin were altered in the ACKR2-deficient peritoneum, a specific mechanism underlying the reduction in macrophages has not yet been established. The selective impact of ACKR2 on peritoneal SPMs and not pleural SPMs also remains unclear. However, as monocyte recruitment to the peritoneal cavity was affected by the absence of ACKR2, it is possible that macrophage differentiation, rather than recruitment, is altered in ACKR2-deficient mice.

5 Transcriptomic Analysis of Lung Macrophage Populations

5.1 Introduction

In the previous chapter, ACKR2 was shown to be associated with a reduced number of macrophages in the peritoneal cavity, peritoneal wall and lung. In the lung, this reduction specifically affected a population of MHCII⁺ macrophages, which are likely to represent an interstitial lung macrophage population, (Chapter 4, Figure 4-3, Figure 4-5 and Figure 4-15B.ii). Notably, the number of MHCII⁺ interstitial macrophages was not affected by ACKR2 deficiency (Chapter 4, Figure 4-15B.i).

At rest, the mouse lung contains two main types of macrophages, resident alveolar macrophages and monocyte derived interstitial macrophages. Alveolar macrophages, the most abundant lung macrophage population, express SiglecF, originate from foetal liver monocyte precursors and can self-renew (Guilliams et al., 2013). Although they are long-lived, alveolar macrophages can be replenished by circulating monocytes following infection or injury, or over the course of ageing as their numbers decline (Machiels et al., 2018, Liu et al., 2019, Byrne et al., 2020). These cells reside within the alveolar spaces, the primary site of gas exchange, and play essential roles in maintaining pulmonary homeostasis, for example by regulating pulmonary surfactant (Bain and MacDonald, 2022). Interstitial macrophages are derived from embryonic progenitors or are bone-marrow derived and can be broadly categorised into two main subpopulations (Schyns et al., 2019, Chakarov et al., 2019, Gibbings et al., 2017). These subpopulations have recently been categorised as CD206⁻ or CD206⁺ (Schyns et al., 2019), and overlap with populations previously defined as Lyve-1^{low} MHCII^{high} or Lyve-1^{high} MHCII^{low} respectively (Gibbings et al., 2017, Chakarov et al., 2019, Li et al., 2024). These subpopulations are spatially segregated, with Lyve-1^{low} MHCII^{high} predominantly localised to nerves (Ural et al., 2020) and Lyve-1^{high} MHCII^{low} localised to blood vessels (Chakarov et al., 2019). Both subsets of interstitial macrophages are phagocytic and constitutively release the immunoregulatory cytokine IL-10 (Bain and MacDonald, 2022, Bedoret et al., 2009, Kawano et al., 2016). In general, Lyve-1^{low} MHCII^{high} are considered more inflammatory and release proinflammatory cytokines whilst Lyve-1^{high} MHCII^{low} macrophages have established roles in wound healing and immunoregulation. For example, in models of bleomycin induced lung fibrosis, depletion of Lyve-1^{high} MHCII^{low} macrophages is associated with increased

damage, underlining a function for these macrophages in regulating inflammation (Mass et al., 2023, Chakarov et al., 2019). Further heterogeneity within interstitial macrophages have been described based on expression of TIM4, Lyve-1, FOLR2, CCR2 and MHCII (Mass et al., 2023, Chakarov et al., 2019, Gibbings et al., 2017, Ural et al., 2020). This heterogeneity reflects both the diverse activation states of these macrophages and the potential for their replacement by monocytes (Li et al., 2024). This heterogeneity is further exemplified by a recent study which identified ten distinct interstitial macrophage subsets based on chemokine expression patterns that correspond with specialised function (Li et al., 2024).

We previously observed a reduction in the numbers of MHCII⁻ interstitial macrophages in the lungs of naïve ACKR2-deficient mice (Chapter 4, Figure 4-15B.ii). In the lung, ACKR2 is expressed by lymphatic endothelial cells, alveolar blood endothelial cells and a subpopulation of CD140a⁺ Sca-1^{high} fibroblasts. In contrast, CD45⁺ immune cells in the lung do not express detectable ACKR2 protein (Hansell et al., 2020), suggesting ACKR2 expression within this tissue is not immune cell derived.

To investigate whether ACKR2 influences the transcripts of lung macrophages, rather than just the abundance of these macrophages, RNA-sequencing was performed on both MHCII⁻ and MHCII⁺ lung macrophage populations. This aimed to uncover transcriptomic changes associated with ACKR2 deficiency. Additionally, we compared the transcriptomes of MHCII⁻ and MHCII⁺ macrophages to explore the intrinsic differences between these two populations, which may explain why only the MHCII⁻, and not MHCII⁺ subset, was affected by the absence of ACKR2.

5.2 Results

5.2.1 The Impact of ACKR2 on the Number of Alveolar Macrophages and Monocytes in the Naïve Lung

As MHCII⁻ interstitial macrophages were reduced in numbers in ACKR2-deficient mice, alveolar macrophages in the resting lung were quantified by flow cytometry using the gating strategy shown in Figure 5-1. This aimed to determine whether the impact of ACKR2 deficiency in the lung is specific to particular macrophage populations, or more broadly impacts multiple macrophage populations. Figure 5-2A highlights that there was no significant difference observed in the number of alveolar macrophages in the lungs of ACKR2-deficient mice compared to WT mice at rest ($p = > 0.05$). This indicates that the reduction of MHCII⁻ interstitial macrophages is specific to this population (as MHCII⁺ macrophages were also not affected (Chapter 4, Figure 4-15B.ii), and that ACKR2 is unlikely to interrupt the seeding or turnover of resident alveolar macrophages. This is similar to the observation in peritoneal tissues, as monocyte derived SPMs were reduced in numbers in the absence of ACKR2, but resident LPMs were unaffected (Chapter 4, Figure 4-3 and Figure 4-5).

Following this, Ly6C^{high} monocytes in the lung were quantified by flow cytometry using the gating strategy presented by Figure 5-1, to determine whether the reduction of lung monocyte derived MHCII⁻ macrophages in ACKR2-deficient mice was associated with impaired recruitment of monocytes. The number of monocytes measured in the resting lungs of ACKR2-deficient mice were comparable to that of WT mice ($p = > 0.05$) (Figure 5-2B). This indicates that the observed reduction in MHCII⁻ macrophages in ACKR2-deficient mice was not the result of impaired migration of monocytes to the site.

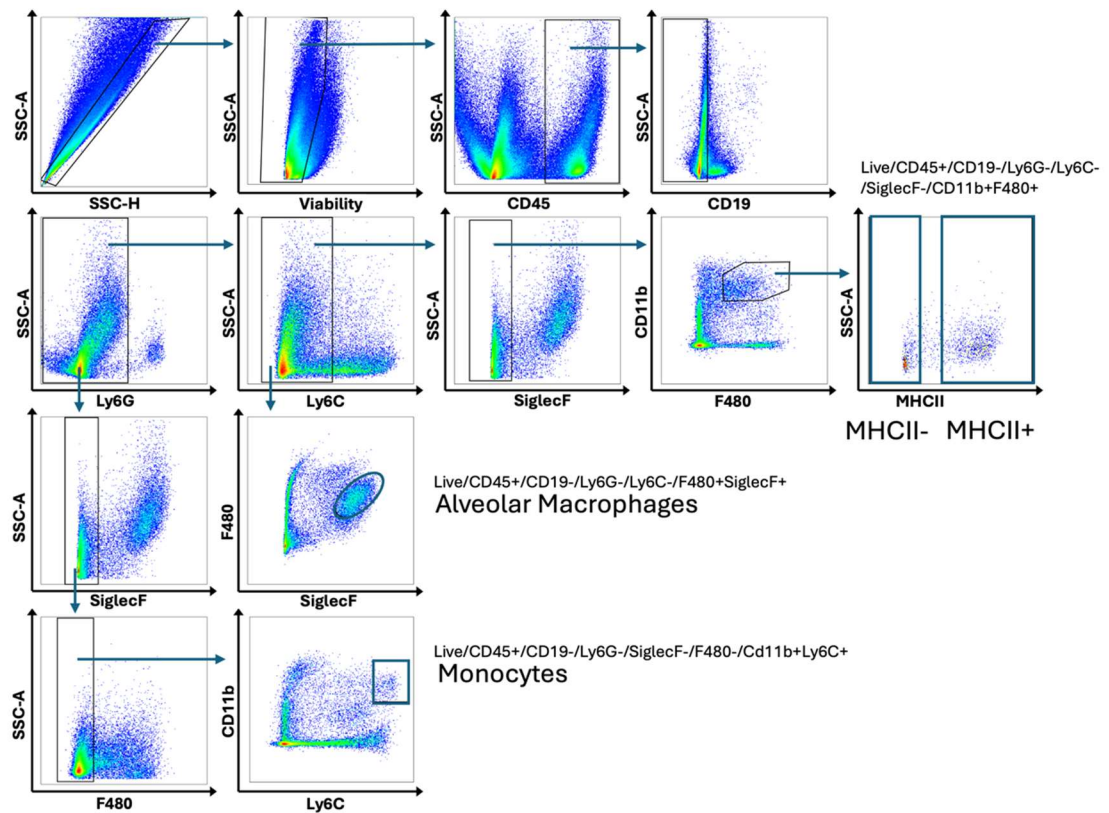


Figure 5-1 Gating Strategy Used to Define Cell Types in the Lung

Flow cytometry was carried out to identify cell types in the lung. All cell types analysed were defined as single cells, live cells, CD45⁺, CD19⁻. MHCII⁻ and MHCII⁺ interstitial macrophages were defined as Ly6G⁻, Ly6C⁻, SiglecF⁻, CD11b⁺, F4/80⁺ and further divided into MHCII⁺ or MHCII⁻ populations. Classical monocytes were defined as Ly6G⁻, SiglecF⁻, F4/80⁻, Ly6C^{high}. Alveolar macrophages were defined as Ly6G⁻, Ly6C⁻, F4/80⁺, SiglecF⁺.

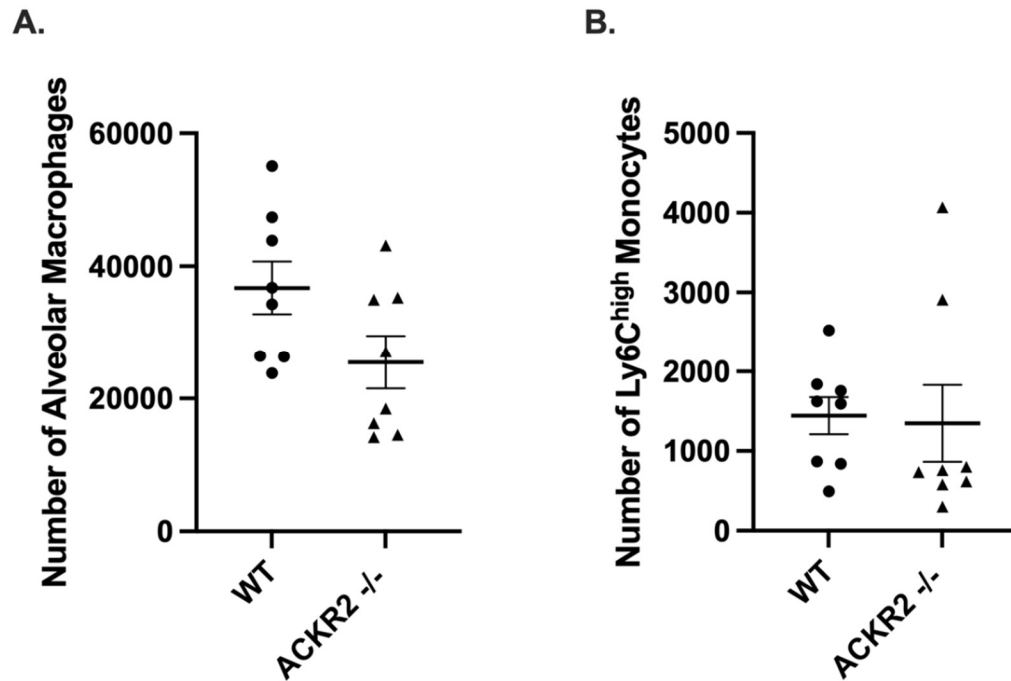
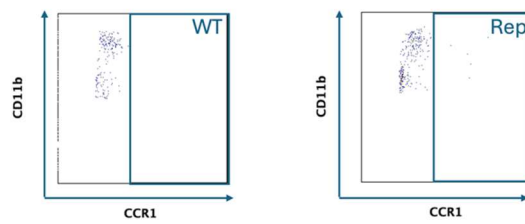
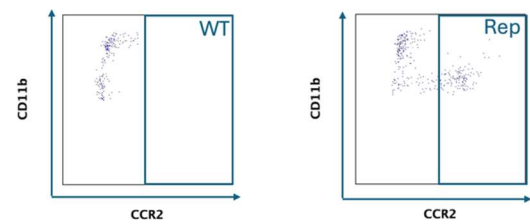
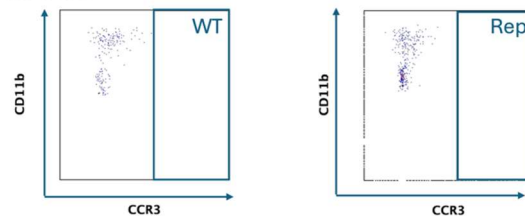
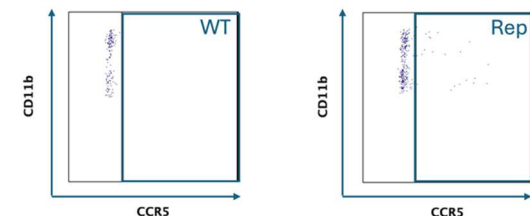
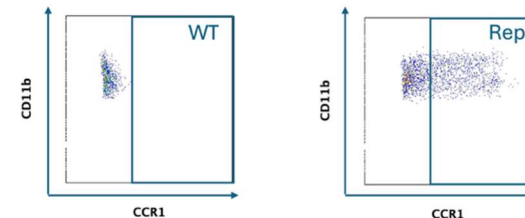
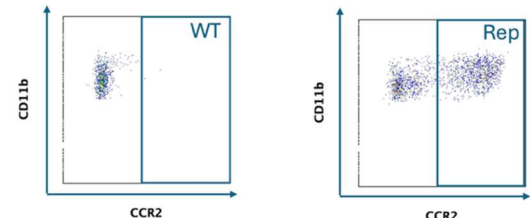
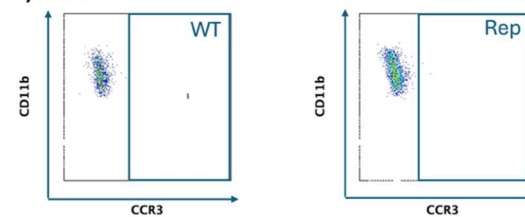
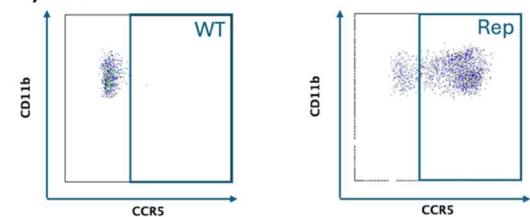


Figure 5-2 The Number of Alveolar Macrophages and Monocytes in the Lungs of Wild Type and ACKR2-Deficient Mice at Rest

Flow cytometry was used to measure the number of alveolar macrophages and monocytes in the total lung of wild type (WT) and ACKR2-deficient mice (ACKR2^{-/-}) ($n = 8$ per group). Alveolar macrophages (**A**) were gated as single cells, live cells, CD45⁺, CD19⁻, Ly6G⁻, Ly6C⁻, F4/80⁺, SiglecF⁺. Ly6C^{high} monocytes (**B**) were gated as single cells, live cells, CD45⁺, CD19⁻, Ly6G⁻, SiglecF⁻, F4/80⁻, CD11b⁺, Ly6C^{high}. Error bars represent SEM. Outliers were identified using the Grubbs test (Prism) and removed before statistical analysis. Statistical significance was determined by unpaired t-test or Mann Whitney ($p < 0.05$) depending on normality of the data and is indicated as $p < 0.05$ (*), < 0.01 (**), < 0.001 (***), < 0.0001 (****). Data are representative of repeats.

5.2.2 iCCR Expression by MHCII⁻ and MHCII⁺ Lung Macrophages

ACKR2 acts via interaction with ligands that signal through CCR1, CCR2, CCR3 and CCR5. To investigate whether recruitment of MHCII⁻ macrophages to the lung is regulated by these inflammatory chemokine receptors, we assessed surface expression of CCR1, CCR2, CCR3, and CCR5 on both MHCII⁻ and MHCII⁺ macrophages isolated from the lungs of iCCR fluorescent reporter mice using flow cytometry. The MHCII⁺ and MHCII⁻ macrophage populations were identified according to the gating strategy outlined in Figure 5-1 and iCCR expression was determined using the gating approach depicted by Figure 5-3. The results revealed that a greater proportion of MHCII⁺ macrophages express surface CCR1, CCR2, and CCR5 compared to MHCII⁻ macrophages. Specifically, 6.7 % of MHCII⁻ macrophages expressed CCR1, compared to 55.9 % of MHCII⁺ macrophages; 19.98 % of MHCII⁻ macrophages expressed CCR2, compared to 65.65 % of MHCII⁺ macrophages; and 16.7 % of MHCII⁻ macrophages expressed CCR5, compared to 90.13 % of MHCII⁺ macrophages (Figure 5-4). Neither MHCII⁻ nor MHCII⁺ lung macrophages expressed CCR3 (Figure 5-4), which aligns with literature that highlights that CCR3 is primarily expressed on eosinophils, and is unlikely to influence macrophage recruitment (Medina-Ruiz et al., 2022, Dyer et al., 2019a).

A. MHCII⁻ Lung Macrophages**i) CCR1****ii) CCR2****iii) CCR3****iv) CCR5****B. MHCII⁺ Lung Macrophages****i) CCR1****ii) CCR2****iii) CCR3****iv) CCR5****Figure 5-3 iCCR Reporter Gating Strategy in the Lung**

Chemokine receptor expression in **A) MHCII⁻ lung macrophages** and **B) MHCII⁺ lung macrophages** was determined for CCR1 (**i**), CCR2 (**ii**), CCR3 (**iii**) and CCR5 (**iv**) using transgenic reporter mice (REP). iCCR is on the x-axis of representative flow cytometry plots and CD11b was selected on the y-axis for clear visualisation. Wild type (WT) mice were used as negative controls. MHCII⁻ and MHCII⁺ macrophages were gated using the strategy outlined by Figure 5-1 and were defined as single cells, live cells, CD45⁺, CD19⁻, Ly6G⁻, Ly6C⁻, SiglecF⁻, CD11b⁺, F4/80⁺.

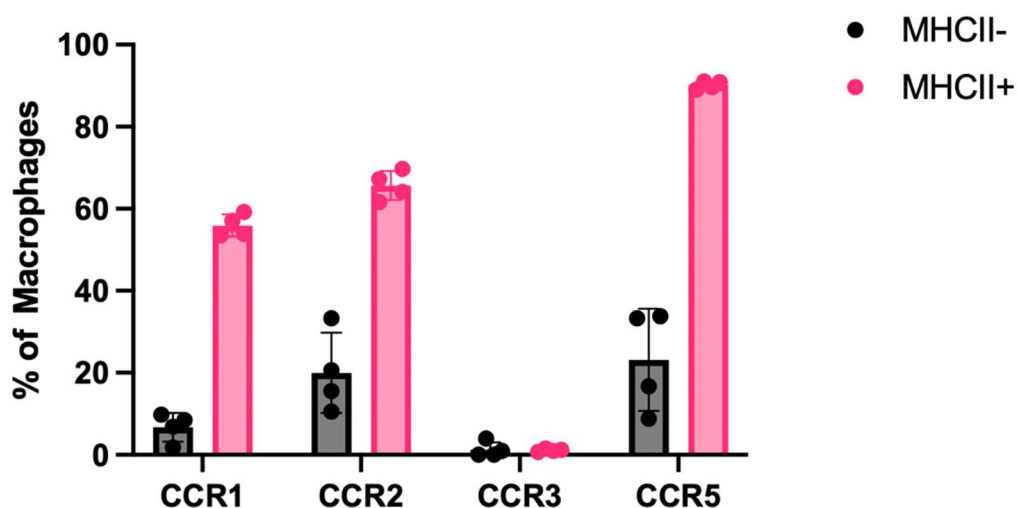


Figure 5-4 iCCR Expression Across Macrophage Subpopulations in the Lung at Rest

Flow cytometry was used to measure the percentage of MHCII⁺ and MHCII⁻ macrophages expressing CCR1, CCR2, CCR3 and CCR5 using iREP fluorescent reporter mice. MHCII⁺/MHCII⁻ macrophages were defined as single cells, live cells, CD45⁺, CD19⁻, Ly6G⁻, Ly6C⁻, CD11b⁺, F4/80⁺. Wild type mice were used as negative controls for reporter signals ($n = 4$ per group).

5.2.3 Transcriptomic Analysis of MHCII⁻ and MHCII⁺ Macrophage Populations

Figure 5-4 highlights that the impact of ACKR2 on MHCII⁻ macrophage abundance in the lung was unlikely to be caused by altered levels of iCCR ligands, as a low percentage of this population expressed iCCRs. Therefore, focus shifted to whether ACKR2 influences the transcriptomic profile of these macrophages, as gene expression changes could account for their lower numbers or indicate how ACKR2 deficiency may impact their function in the lung. To investigate this, bulk-RNA sequencing was performed on MHCII⁻ and MHCII⁺ macrophages from the resting lungs of WT and ACKR2-deficient mice.

MHCII⁺ and MHCII⁻ macrophages were sorted by fluorescence-activated cell sorting (FACS) according to the gating strategy previously illustrated in Figure 5-1. Sorted macrophages were gated as single, live, CD45⁺, SiglecF⁻, Ly6G⁻, Ly6C⁻, CD11b⁺, F4/80⁺, and either MHCII⁺ or MHCII⁻ (Figure 5-1). Each sample represents pooled cells from four mouse lungs to obtain enough cells for RNA extraction. RNA from each sample was subsequently sequenced by Novogene using the Illumina NovaSeq X Plus platform. RNA quality was assessed and found to be sufficient for downstream analysis, which was carried out using Searchlight 2 (version 2.0.3) (Cole et al., 2021). The RNA quality across samples is demonstrated by the density plots shown in Figure 5-5. These plots visualise the overall distribution of log transformed gene expression values across samples, with each plot representing one sample. A high degree of similarity in expression distribution across samples is expected and reflects high quality RNA and technical consistency. While most samples showed comparable expression profiles, two outliers (in groups WT MHCII⁻ and ACKR2^{-/-} MHCII⁻) showed slightly atypical gene expression distribution, which may reflect insufficient RNA quantity during library preparation (Cole et al., 2021).

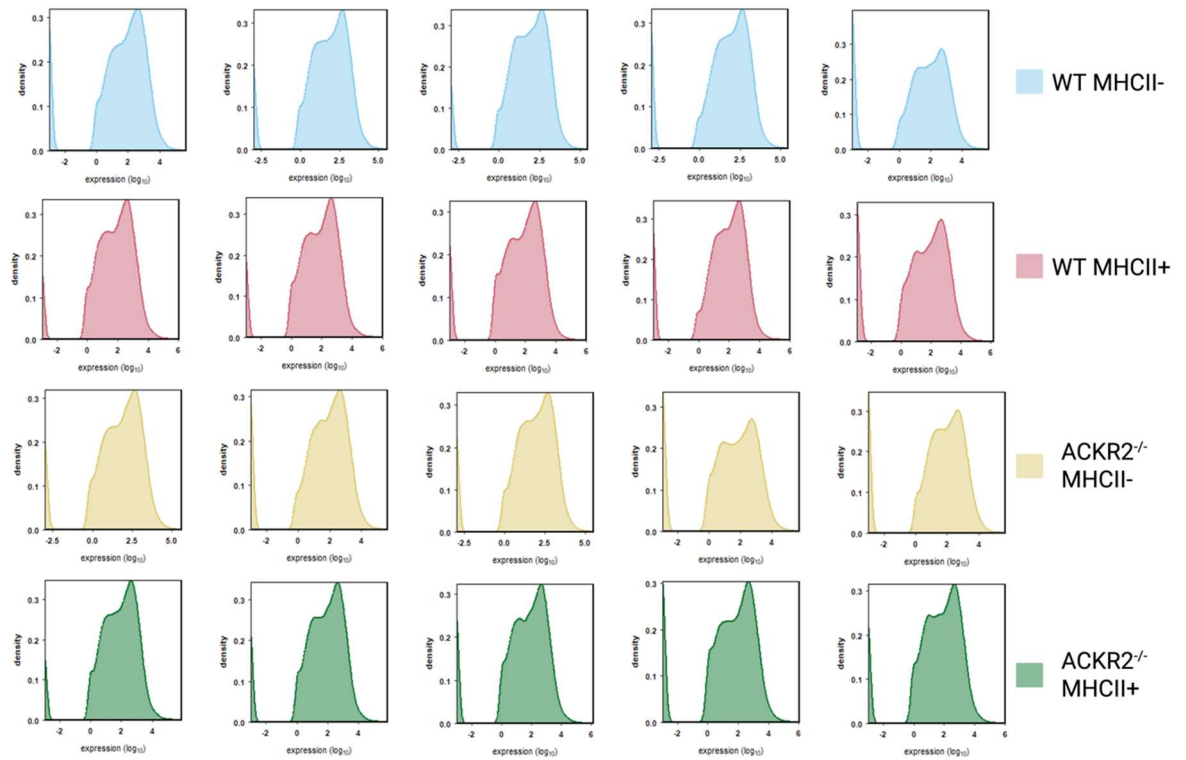


Figure 5-5 Overall Distribution of Gene Expression Across Samples Used for Transcriptomic Analysis

Density plots showing the overall distribution of expression values for each sample of sorted wild type (WT) and ACKR2-deficient (ACKR2^{-/-}) MHCII⁻ and MHCII⁺ lung macrophages. Expression values are given on x-axis and are on a log₁₀ scale. The gene density is given on the y-axis.

Figure 5-6A illustrates the extent to which the 20 highest sources of variation between the four groups analysed (WT MHCII⁻, WT MHCII⁺, ACKR2^{-/-} MHCII⁻, and ACKR2^{-/-} MHCII⁺ macrophages) contribute to the overall variation across samples. These sources of variation are referred to as principal components. Figure 5-6A highlights that four principal components account for the majority of the variation observed between the macrophage populations. Figure 5-6B plots the position of each sample along Principal Component 1 (PC1) and Principal Component 2 (PC2) and reveals that MHCII⁺ and MHCII⁻ lung macrophages form distinct clusters along PC1 in both WT and ACKR2-deficient mice. This suggests that PC1 primarily reflects differences based on MHCII expression. In contrast, PC2 appears to capture other sources of biological variability between individual mice. Notably, within each MHCII subset, WT and ACKR2-deficient macrophages do not form separate clusters, indicating a largely overlapping transcriptional profile between genotypes. Additionally, PC3 and PC4 also capture other sources of variability that do not include ACKR2 expression and none of the four groups cluster separately along these components (Figure 5-6C).

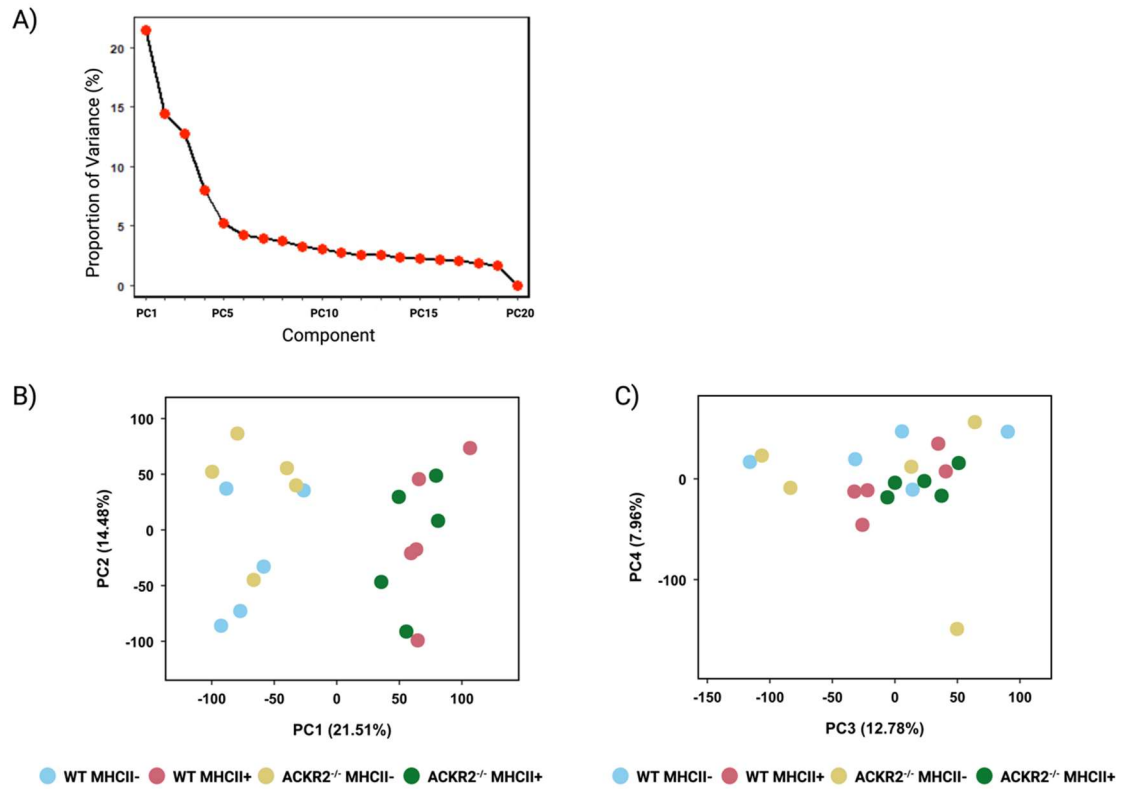


Figure 5-6 Principal Component Analysis of Gene Expression Across Samples Used for Transcriptomic Analysis

A) Waterfall plot showing the proportion of variance explained by the top 10 principal components (PCs) derived from the gene expression dataset. Components are ranked in descending order, with PC1 accounting for the greatest variability and PC10 the least. **B)** PCA scatter plot illustrating sample clustering based on PC1 (x-axis) and PC2 (y-axis), which represent the two most variable components. Each point corresponds to an individual sample. WT MHCII⁻ samples = blue, WT MHCII⁺ = pink, ACKR2^{-/-} MHCII⁻ = yellow, and ACKR2^{-/-} MHCII⁺ = green ($n = 5$ per group). **C)** PCA scatter plot illustrating sample clustering based on PC3 (x-axis) and PC4 (y-axis), which represent the third and fourth most variable components. Each point corresponds to an individual sample. WT MHCII⁻ samples = blue, WT MHCII⁺ = pink, ACKR2^{-/-} MHCII⁻ = yellow, and ACKR2^{-/-} MHCII⁺ = green ($n = 5$ per group).

Comparisons between macrophages from WT and ACKR2-deficient mice revealed few transcriptional differences (Figure 5-7). MHCII⁻ macrophages in ACKR2-deficient mice downregulated expression of 6 genes and upregulated expression of 5 genes. MHCII⁺ macrophages from ACKR2-deficient mice downregulated expression of 5 genes and upregulated expression of 2 genes (Figure 5-7). Interestingly, both MHCII⁺ and MHCII⁻ macrophages display a similar number of differentially expressed genes between genotypes, despite only the MHCII⁻ population being reduced in numbers in ACKR2-deficient lungs (Chapter 4, Figure 4-15B). In comparison, MHCII⁺ and MHCII⁻ macrophages in WT lungs exhibit many differentially expressed genes, with 2,394 downregulated and 1,926 upregulated.

A very limited number of differentially expressed genes were identified when comparing MHCII⁺ and MHCII⁻ macrophage populations between WT and ACKR2-deficient mice (Figure 5-7, Figure 5-8 and Figure 5-9). This reflects the overlapping PCA points shown in Figure 5-6B. The differentially expressed genes between WT MHCII⁻ macrophages and ACKR2-deficient MHCII⁻ macrophages ($p_{\text{adj}} < 0.05$, absolute $\log_2\text{FC} > 0.5$) are outlined by the heatmap in Figure 5-8 and corresponding $\log_2$ fold change in expression is also noted. In MHCII⁻ macrophages, the genes ACKR2, CRPPA, ST18, GM39469, CCR5, ENSMU S00000120808 were downregulated in ACKR2-deficient mice ($p_{\text{adj}} < 0.05$, absolute $\log_2\text{FC} > 0.5$) compared to the WT. The most downregulated gene transcript was ST18, which showed a $\log_2$ fold change of -5.37 in the MHCII⁻ lung macrophages of ACKR2-deficient mice. In contrast, the gene expression of SH2D2A, AW112010, CENPL, TRAV7D-4, WDFY-1 were upregulated in MHCII⁻ macrophages in ACKR2-deficient mice compared with the WT in the lung at rest ($p_{\text{adj}} < 0.05$, absolute $\log_2\text{FC} > 0.5$). The most highly upregulated gene transcript observed was TRAV7-D4, which increased by a $\log_2\text{FC}$ of 6.51 in the instance of ACKR2 deficiency.

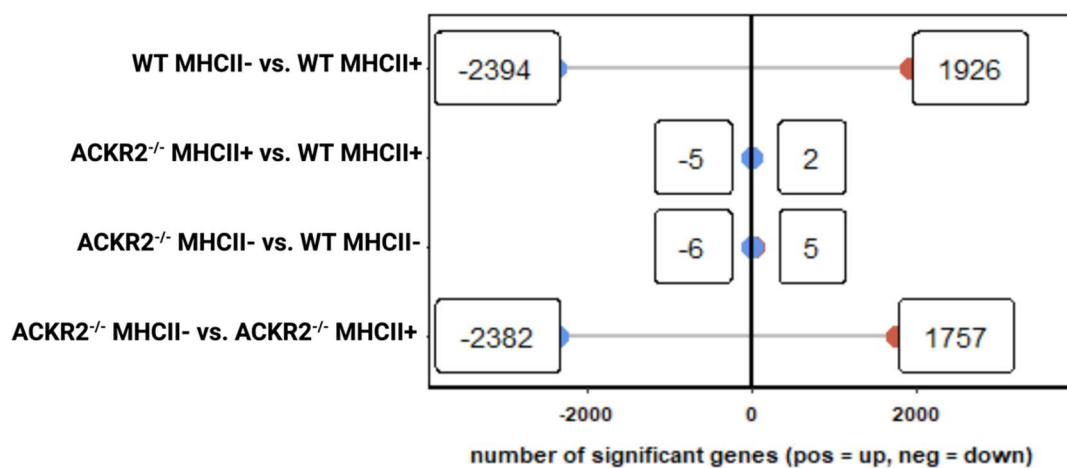


Figure 5-7 The Number of Differentially Expressed Genes Between Wild Type and ACKR2-Deficient MHCII⁻ and MHCII⁺ Lung Macrophages at Rest

Lollipop plot displays the number of genes which were significantly downregulated and upregulated ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5) in expression for each comparison ($n = 5$ per group).

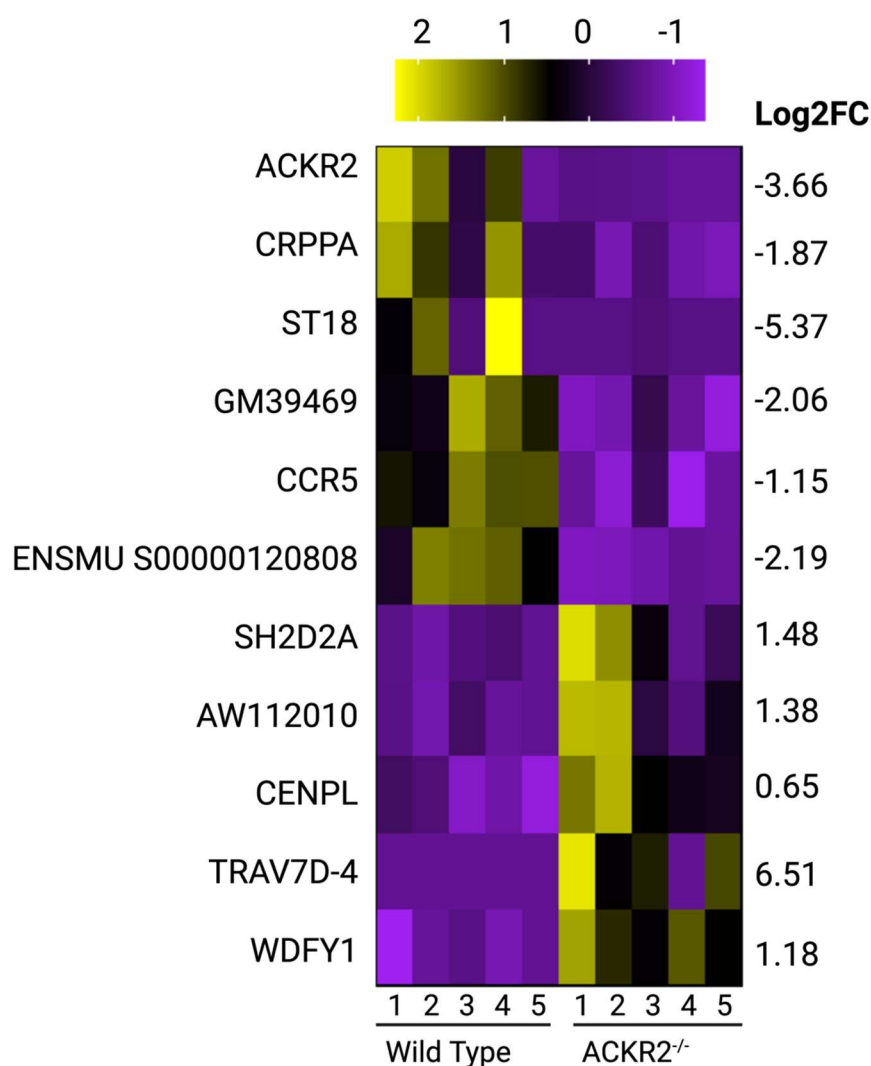


Figure 5-8 Transcriptional Differences Between Wild Type and ACKR2-Deficient MHCII⁻ Lung Macrophages at Rest

Heatmap of the significantly differentially expressed genes ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5) for wild type MHCII⁻ and ACKR2-deficient (ACKR2^{-/-}) MHCII⁻ macrophages. The genes have been hierarchically clustered based on similarity in expression profile using Spearman distances with UPMGA agglomeration and mean reordering (Cole et al., 2021). Sample number is shown on the x-axis and gene names are shown on the y-axis. Colour intensity represents expression level, with purple representing low expression, and yellow representing high expression. Expression levels have been row scaled into z-scores.

In comparison, in MHCII⁺ lung macrophages, the gene expression of *ENSMU* *S00000120808*, *CYP8B1*, *ACKR2*, *GM39469*, and *CCR5* were downregulated in ACKR2-deficient mice ($p.\text{adj} < 0.05$, absolute $\log_2\text{FC} > 0.5$) compared to the WT (Figure 5-9). The most downregulated transcript in MHCII⁺ ACKR2-deficient lung macrophages was *CYP8B1* ($\log_2\text{FC} = -6.17$). However, this particular gene is located proximal to *ACKR2* on chromosome 9 and passenger mutations for this mouse model have previously been reported (Hayes et al., 2024). Interestingly, the next most downregulated gene transcript was *ACKR2* ($\log_2\text{FC} = -3.79$) (Figure 5-9). A similar reduction was also observed in MHCII⁻ macrophages, where *ACKR2* expression showed a $\log_2$ fold change of -3.66. Although this aligns with the genotype of the mice used, *ACKR2* has not been reported to be expressed by macrophages and was previously undetectable on the surface of lung macrophages as measured by a functional fluorescent CCL22 uptake assay (Hansell et al., 2020). However, interestingly, this transcriptomic data shows that *ACKR2* is expressed at transcript level by lung macrophages.

In contrast, Figure 5-9 outlines that the genes *IRF2BP2* and *WDFY1* were upregulated in MHCII⁺ macrophages in ACKR2-deficient mice compared with the WT in the lung at rest ($p.\text{adj} < 0.05$, absolute $\log_2\text{FC} > 0.5$). The expression of *WDFY1* was most highly upregulated in MHCII⁺ macrophages of ACKR2-deficient mice with a $\log_2\text{FC}$ value of 1.27 (Figure 5-9). Notably, the transcript expression of this gene was also upregulated in MHCII⁻ macrophages from ACKR2-deficient mice, indicating that this gene may be generally associated with *ACKR2* activity.

Overall, the $\log_2$ fold change across the differentially expressed genes was mostly comparatively lower than those observed between wild type MHCII⁻ and MHCII⁺ macrophages, indicating that even amongst the few transcriptomic differences identified, the magnitude of change in expression was also relatively smaller. Furthermore, too few differential genes were observed in either the comparison of MHCII⁻ or MHCII⁺ macrophages in WT and ACKR2-deficient mice to carry out pathway analysis. Therefore, it is only possible to speculate the significance and potential roles of the limited differentially expressed genes identified.

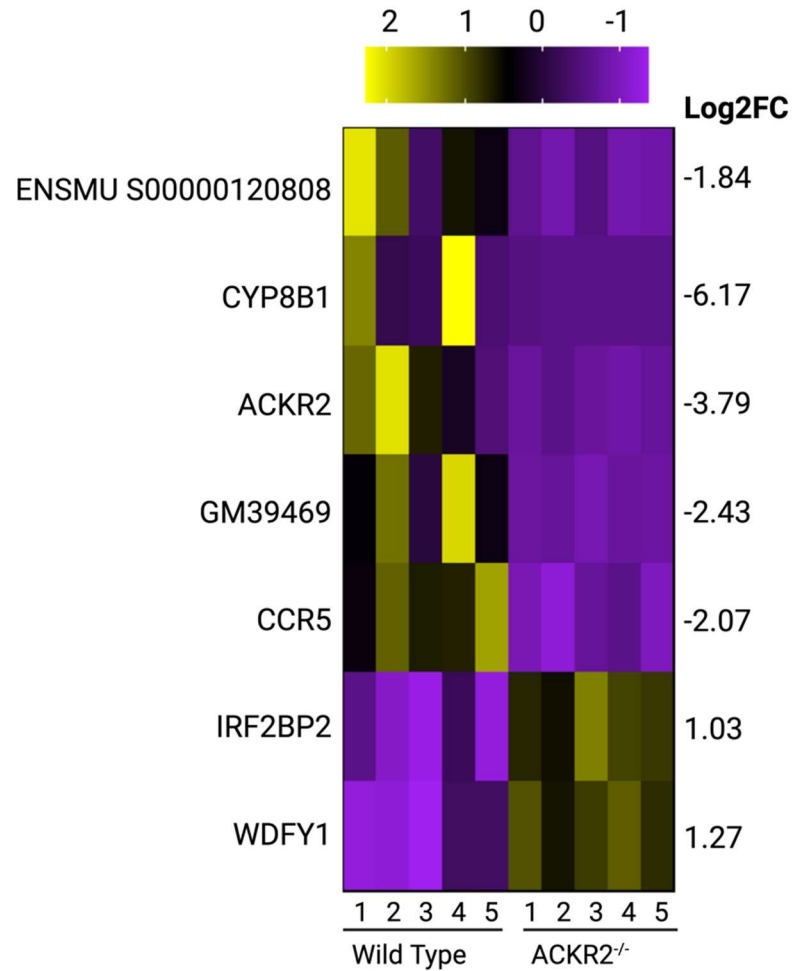


Figure 5-9 Transcriptional Differences Between Wild Type and ACKR2-Deficient MHCII⁺ Lung Macrophages at Rest

Heatmap of the significantly differentially expressed genes ($p_{\text{adj}} < 0.05$, absolute $\log_2\text{FC} > 0.5$) for wild type MHCII⁺ and ACKR2-deficient (ACKR2^{-/-}) MHCII⁺ macrophages. The genes have been hierarchically clustered based on similarity in expression profile using Spearman distances with UPMGA agglomeration and mean reordering (Cole et al., 2021). Sample number is shown on the x-axis and gene names are shown on the y-axis. Colour intensity represents expression level, with purple representing low expression, and yellow representing high expression. Expression levels have been row scaled into z-scores.

In comparison, substantial transcriptional differences were observed between MHCII⁻ and MHCII⁺ macrophages in WT mice (Figure 5-10). The most significantly upregulated genes between WT MHCII⁻ and MHCII⁺ macrophages, ranked by adjusted *p*-value (*p*.adj), are *ITGAL* and *MDM1* (Figure 5-10). Conversely, Figure 5-10 labels the most significantly downregulated genes ranked by *p*.adj, which include *H2-AA*, *TSPAN33*, *SWAP70*, *H2-DMA*, *CD74*, *CCR2*, *6330537M06RIK*, and *H2-DMB1*. The observed upregulation of *CCR2* RNA in MHCII⁺ macrophages relative to MHCII⁻ lung macrophages is consistent with flow cytometric analysis of MHCII⁺ and MHCII⁻ macrophages which shows that a greater percentage of MHCII⁺ lung macrophages express this receptor than MHCII⁻ macrophages (Figure 5-4).

Furthermore, the MA plot (Figure 5-10), which shows the log₂ fold change in gene expression against average expression level, demonstrates a well-balanced distribution of differentially expressed genes across a broad range of expression values. This plot measures the extent to which the data are impacted by the inherent bias which arises in bulk RNA-sequencing, where genes expressed at low levels tend to have fewer reads, and therefore these genes are less likely to be identified as significantly differentially expressed compared to highly expressed genes (Cole et al., 2021). The presence of significant changes not only among highly expressed genes but also in those with moderate and lower expression levels (Figure 5-10) suggests that the experiment was sufficiently powered to detect differential expression across the transcriptome, despite some samples demonstrating an atypical distribution of gene expression values (Figure 5-5).

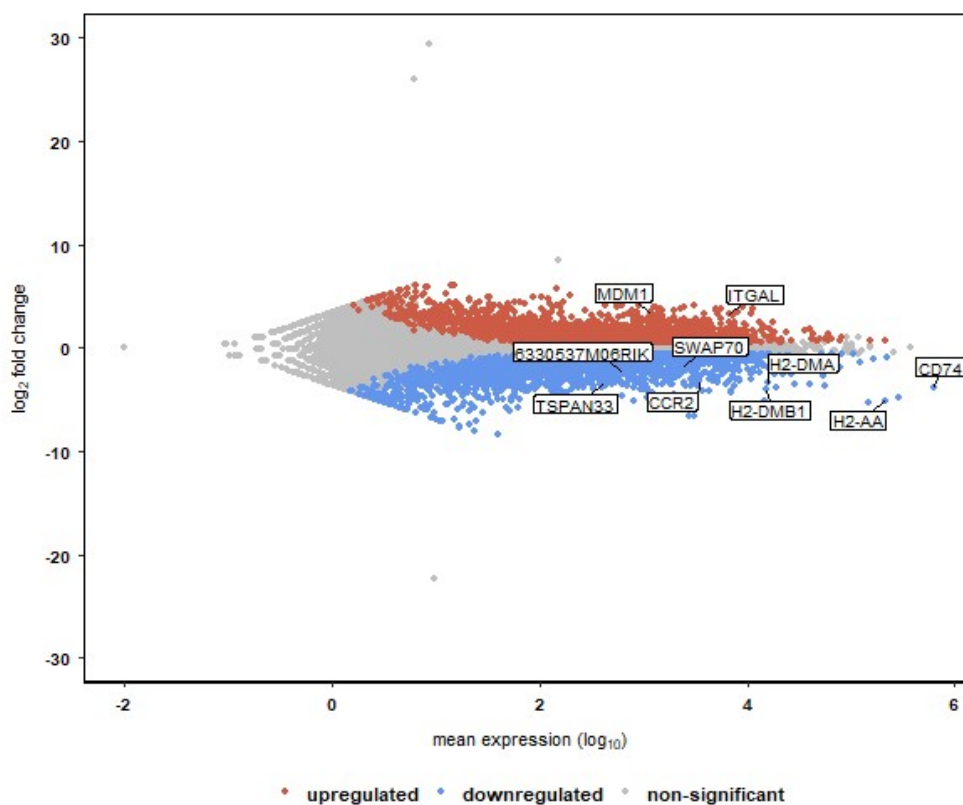


Figure 5-10 Mean Expression and Log2Fold Change of Genes in Naïve Wild Type MHCII⁻ Versus MHCII⁺ Lung Macrophages

MA plot displaying mean gene expression on the x-axis and log₂ fold change in gene expression between wild type MHCII⁻ versus MHCII⁺ lung macrophages on the y-axis. Each dot represents a gene. Blue signifies downregulated genes, red indicates upregulated genes, and grey indicates non-significant genes. Labelled points are the most statically significant differentially expressed genes ($p_{\text{adj}} < 0.05$, absolute log₂ fold > 0.5). A positive fold change indicates higher expression in WT MHCII⁻ than in WT MHCII⁺ cells ($n = 5$ per group).

Additionally, genes ranked by log2FC, the most strongly downregulated transcripts (lower in MHCII⁺ than MHCII⁻ lung macrophages) included *IBSP*, *BTNL1*, *MUCL1*, *TLR11*, *KCNJ9*, *LRRC3*, *GM8221*, *H2-EA*, *F730038M01RIK*, and *OR5V1* (Table 5-1). Among these, all except *IBSP* were significantly differentially expressed ($p.\text{adj} < 0.05$). In contrast, the genes most upregulated by log2FC (higher in MHCII⁻ lung macrophages) were: *ENSMUSG00000120173*, *SLITRK4*, *CAR3*, *GM7784*, *GM31774*, *HSD17B2*, *GLT1D1*, *PCDH11X*, *SLC5A5*, and *ENSMUSG00000121360*, of which *GM7784*, *GM31774*, *HSD17B2*, *GLT1D1*, *PCDH11X*, *SLC5A5*, and *ENSMUSG00000121360* were statistically significant ($p.\text{adj} < 0.05$) (Table 5-2). Although broadly, the differentially expressed genes mentioned are associated with functions such as immune regulation (i.e. *H2-AA*, *H2-DMA*, *H2-DMB1*, *H2-EA*, *CD74*, *CCR2*, *TSPAN33*, *TLR11*, *BTNL1*) and cell adhesion (i.e. *ITGAL*, *PCDH11X*), further analysis is required to make meaningful conclusions about the biological significance of the transcriptional differences observed between the two macrophage populations.

Gene	log2FC	p.adj	Significant
<i>IBSP</i>	-22.36	1	NO
<i>BTNL1</i>	-8.29	5.25435236038305e-15	YES
<i>MUCL1</i>	-8.11	1.8588127104533e-13	YES
<i>TLR11</i>	-7.63	1.91087029209361e-10	YES
<i>KCNJ9</i>	-7.61	1.53309309749362e-09	YES
<i>LRRC3</i>	-7.34	2.81437823185089e-09	YES
<i>GM8221</i>	-7.07	4.38688996472355e-08	YES
<i>H2-EA</i>	-7.01	1.59651886855749e-07	YES
<i>F730048M01RIK</i>	-7.0	3.58624575749003e-06	YES
<i>OR5V1</i>	-6.88	3.33806913048714e-08	YES

Table 5-1 Most Downregulated (Log₂ Fold) Genes in Naïve Wild Type MHCII⁻ Versus MHCII⁺ Interstitial Lung Macrophages

Downregulated genes are more highly expressed in WT MHCII⁺ than in WT MHCII⁻ cells. A significant *p*.adj value was <0.05. Significant *p*.dj values are indicated as 'YES'; non-significant results are marked 'NO'.

Gene	log2FC	p.adj	Significant
<i>ENSMUSG00000120173</i>	29.43	1	NO
<i>SLITRK4</i>	26.1	1	NO
<i>CAR3</i>	8.44	1	NO
<i>GM7784</i>	6.14	0.00062857333417646	YES
<i>GM31774</i>	6.12	0.00981265744715146	YES
<i>HSD17B2</i>	6.05	3.10144759313868e-08	YES
<i>GLT1D1</i>	6.04	6.26735758759749e-06	YES
<i>PCDH11X</i>	6.03	0.348521935916348	YES
<i>SLC5A5</i>	5.89	0.00669289744658528	YES
<i>ENSMUSG00000121360</i>	5.84	1.87602383731374e-05	YES

Table 5-2 Most Upregulated (Log₂ Fold) Genes in Naïve Wild Type MHCII⁻ Versus MHCII⁺ Interstitial Lung Macrophages

Upregulated genes are more highly expressed in WT MHCII⁻ than in WT MHCII⁺ lung macrophages. A significant *p*.adj value was < 0.05. Significant *p*.dj values are indicated as 'YES'; non-significant results are marked 'NO'.

To elucidate the meaning and biological context of the list of differentially expressed genes identified between MHCII⁻ and MHCII⁺ lung macrophages, pathway analysis was undertaken using Over-Representation Analysis (ORA). This method involved comparing the list of differentially expressed genes identified between WT MHCII⁻ and MHCII⁺ lung macrophages to curated databases (Gene Ontology (GO) or KEGG), in which genes are grouped into sets representing defined pathways or functions. Genes that were both differentially expressed in MHCII⁻ and MHCII⁺ macrophages and belonged to specific gene sets were considered enriched, and statistical analysis revealed whether particular gene sets were significantly enriched within the gene list. The most enriched gene sets identified were those with the highest statistical significance (lowest *p*-values). This allowed the identification of specific pathways likely to distinguish the two macrophage populations and removed the bias of focusing on individual genes in isolation, which ultimately overlooks broader biological patterns (Cole et al., 2021).

Figure 5-11A lists the ten most enriched gene sets amongst the significantly upregulated genes in MHCII⁻ lung macrophages compared to MHCII⁺ macrophages. These include sets labelled “abnormal IgG1 level, mixed, incl.mhc classes i/ii-like antigen recognition protein, and natural killer like, c-type lectin-like domain”, “abnormal nk cell physiology” and “incl. mhc classes i/ii antigen recognition protein and fceri mediated ca²⁺ mobilisation”. These are vague and unclear and closer analysis of the gene sets is required to gather meaning from this overall pathway analysis. Figure 5-11B displays the differential expression across the genes within the most enriched gene set for genes significantly upregulated in MHCII⁻ macrophages.

In contrast Figure 5-12A presents the ten most enriched gene sets among the significantly upregulated genes in MHCII⁺ lung macrophages compared with MHCII⁻ macrophages. Figure 5-12B shows the differential expression across the genes within this gene set. Notably, these genes are expressed at significantly higher levels by MHCII⁺ macrophages than MHCII⁻ lung macrophages. MHCII⁺ macrophages exhibit higher relative expression across a variety of inflammatory genes, including inflammatory chemokine ligands (i.e. *CCL2*, 3, 4, 7, 9, *CXCL1*, 2, 10) and receptors (i.e. *CCR2*, *CCR5*) as well as TLRs (i.e. *TLR1*, *TLR2*, *TLR4*) for recognition of PAMPs and DAMPs and inflammatory cytokines (i.e. *IL-12B*, *IL-18*, *IL-1B*, *TNF*) (Figure 5-12B). In contrast, MHCII⁻ macrophages upregulated various histocompatibility (H2) genes as well as multiple natural killer c-lectin like receptors including *KLR1*, 3, 4, 7, 8, 9 and 10 (Figure 5-11B), indicating a signalling phenotype potentially involved in immune cell cross talk and innate immunity.

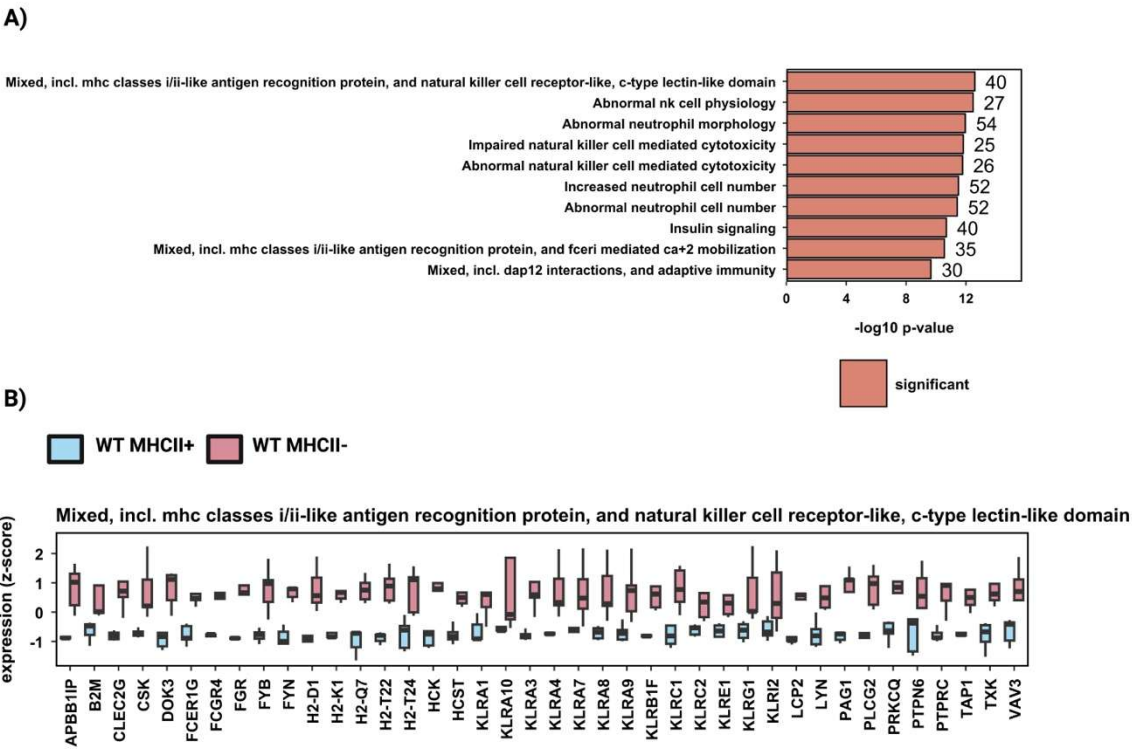


Figure 5-11 Pathway Analysis of Genes Upregulated in MHCII⁻ Lung Macrophages versus MHCII⁺ Lung Macrophages at Rest

A) Bar charts of the ten most enriched gene sets upregulated (red) ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5) in MHCII⁻ compared to MHCII⁺ lung macrophages (upregulated genes are higher expression in MHCII⁻ than MHCII⁺). The x-axis shows $-\log_{10}$ enrichment p -value and the y-axis shows the name of the gene sets. The number of significantly differential expressed genes within each gene set is shown at the end of each bar. Enrichment analysis was performed using Hypergeometric Gene Set Enrichment on the gene set database STRING 11.5. **B)** Box plots highlighting the differential expression of the genes within the most upregulated gene set in MHCII⁻ macrophages (red) compared with MHCII⁺ macrophages (blue). Names of the significantly differential genes within the gene set are shown on the x-axis and expression values scaled into per gene z-scores are shown on the y-axis. Enrichment analysis was performed using Hypergeometric Gene Set Enrichment on the gene set database STRING 11.5.

A) Bar charts of the ten most enriched gene sets upregulated (red) ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5) in MHCII⁺ compared to MHCII⁻ lung macrophages (upregulated genes are higher expression in MHCII⁺ than MHCII⁻). The x-axis shows $-\log_{10}$ enrichment p-value and the y-axis shows the name of the gene sets. The number of significantly differential expressed genes within each gene set is shown at the end of each bar. Enrichment analysis was performed using Hypergeometric Gene Set Enrichment on the gene set database STRING 11.5. **B)** Box plots highlighting the differential expression of the genes within the most upregulated gene set in MHCII⁺ macrophages (blue) compared with MHCII⁻ macrophages (red). Names of the significantly differential genes within the gene set are shown on the x-axis and expression values scaled into per gene z-scores are shown on the y-axis. Enrichment analysis was performed using Hypergeometric Gene Set Enrichment on the gene set database STRING 11.5.

5.3 Discussion

This chapter aimed to explore the effect of ACKR2 on the transcriptomic profile of a specific lung macrophage population, MHCII⁻ macrophages, that was reduced in numbers in naïve ACKR2-deficient mice. Previously, our group demonstrated that macrophage numbers in embryonic skin are impacted by ACKR2 (Lee et al., 2019), and Chapter 4 extended this observation to adult tissues, as SPMs and MHCII⁻ lung macrophages were also diminished in ACKR2-deficient mice at rest. In both tissues, the embryonically derived resident macrophage populations were not affected by ACKR2 (Chapter 4; Figure 4-3B and Figure 4-5B.ii and Chapter 5; Figure 5-2A). In the peritoneum, the reduction in SPMs was not driven by an excess of ACKR2 ligands in the absence of the receptor (Chapter 4; Figure 4-9 and Figure 4-10). Therefore, this chapter initially addressed whether the decrease in MHCII⁻ lung macrophages in ACKR2-deficient mice could be attributed to altered recruitment, or whether a shared mechanism may underlie the selective loss of specific macrophages across adult tissues. The data showed that monocytes were recruited to the lung as normal in ACKR2-deficient mice and that a low percentage of MHCII⁻ lung macrophages expressed iCCRs compared with MHCII⁺ macrophages. As MHCII⁺ macrophage numbers were not impacted by the absence of ACKR2, this suggests that impaired recruitment due to dysregulated chemokine signalling within the lung is unlikely to account for the reduction in MHCII⁻ lung macrophages. However, ELISA or Luminex analysis of ACKR2 ligands in resting WT and ACKR2-deficient lung tissue is needed to further investigate this. To gain understanding of the mechanisms driving the change in MHCII⁻ numbers and ascertain potential differences in the function of these macrophages compared to the wild type, RNA bulk sequencing of MHCII⁻ and MHCII⁺ ACKR2-deficient and WT lungs was undertaken.

In ACKR2-deficient mice, limited and highly specific changes were observed in both MHCII⁻ and MHCII⁺ lung macrophage populations compared to WT controls. No clear biological pathway or unifying functional signature could be identified among the differentially expressed genes and the number of significant hits was too low to support pathway analysis. There was also overlap in the differentially expressed genes across both MHCII⁻ and MHCII⁺ populations in ACKR2-deficient mice. Changes in the expression of these genes are likely to reflect off-target effects of the ACKR2 knockout, rather than biologically meaningful changes. This is exemplified as the genes *ENSMU S00000120808*, *CYP8B1*, *GM39469*, and *CCR5* are all located on mouse chromosome 9 proximal to

ACKR2 and passenger mutations for this mouse model have previously been reported (Hayes et al., 2024). Therefore, the differential expression of these genes should be interpreted with caution due to the likelihood of passenger effects.

It should also be noted that both MHCII⁻ and MHCII⁺ lung macrophages appear to express *ACKR2*, which has not previously been reported. Previous studies indirectly assessed *ACKR2* protein expression on lung CD45⁺ cells by measuring the binding of fluorescently labelled CCL22 using flow cytometry; however, *ACKR2* could not be detected on these cells (Hansell et al., 2020). Although *ACKR2* expression by interstitial macrophages has not been confirmed at the protein level, *ACKR2* has also been detected at the transcriptional level using RT-qPCR in bone marrow derived macrophages and SPMs (Wilson et al., 2024).

Furthermore, a similar number of differentially expressed genes was observed between MHCII⁻ and MHCII⁺ WT vs *ACKR2*-deficient macrophages. The genes downregulated in MHCII⁻ *ACKR2*-deficient lung macrophages (not located on chromosome 9) included *CRPPA* and *ST18*. The upregulated genes outside chromosome 9 included *SH2D2A*, *AW112010*, *CENPL*, *TRAV7D-4*, and *WDFY1*. Among the differentially expressed genes, some have known or suspected roles in macrophages. For example, mutation in the long non-coding RNA *AW112010* in macrophages leads to increased IL-10 expression and decreased IL-6 expression in response to LPS *in vitro* (Yang et al., 2020). In addition, *ST18* encodes a zinc finger protein expressed by macrophages that is involved in several signalling pathways, including the SP1 pathway, which regulates VEGF-A expression (Maruyama et al., 2020). Myeloid-specific *ST18*-deficient mice show elevated VEGF-A levels and increased susceptibility to various sepsis models, likely due to enhanced vascular permeability. Pharmacological inhibition of VEGF-A has been shown to rescue this phenotype (Maruyama et al., 2020). Finally, *WDFY1*, located on mouse chromosome 1, is significantly upregulated in both MHCII⁻ and MHCII⁺ lung macrophages in *ACKR2*-deficient mice. Furthermore, Wilson et al., also identified this gene as differentially expressed *ACKR2*-deficient bone marrow monocytes (Wilson et al., 2024). *WDFY1* plays a key role in TLR3 and TLR4 signalling, and its upregulation has been shown to enhance NF- κ B and interferon regulatory factor 3 signalling pathways, leading to increased production of proinflammatory type I interferons and cytokines (Hu et al., 2015). As *WDFY1* is differentially expressed across various *ACKR2*-deficient cell populations, it is possible that *ACKR2* somehow regulates this gene.

In contrast, the differentially expressed genes, *CENPL*, *SH2D2A*, *TRAV7D-4* have unclear or no established roles in macrophages. Although expression of *CRPPA*, which encodes C-reactive protein (CRP), a clinical marker of inflammation, in the lung is known, a role for CRP in macrophages is not yet established (Sproston and Ashworth, 2018). However, as ACKR2 has a role in inflammation, particularly in the resolution of inflammation (Jamieson et al., 2005, Martinez de la Torre et al., 2005), the observed change in *CRPPA* expression may reflect an increase in baseline inflammation within the resting lungs of ACKR2-deficient mice. Ultimately, MHCII⁻ macrophages in ACKR2-deficient mice demonstrate limited and specific transcriptional changes relative to the wild type. Although transcriptomic differences between MHCII⁻ and MHCII⁺ macrophages in ACKR2-deficient mice were subtle, investigation using proteomic and phospho-proteomic approaches may reveal additional changes at the protein level. Luminex analysis of the lung could also help determine whether there are shared changes in protein expression between the lung and peritoneal tissues in WT versus ACKR2-deficient mice at rest.

MHCII⁻ and MHCII⁺ WT macrophages show extremely distinct transcriptomes. Pathway analysis revealed that MHCII⁺ interstitial macrophages display a more inflammatory profile, with relative upregulation of multiple inflammatory cytokines such as *TNF* and chemokines including *CCL2*, *3*, *4*, *7*, *9*, *CXCL1* and *CXCL10*. In contrast, MHCII⁻ macrophages upregulated various histocompatibility (H2) genes as well as multiple natural killer c-lectin like receptors including: *KLRA1*, *3*, *4*, *7*, *8*, *9* and *10*, indicating a signalling phenotype involved in immune cell cross talk and innate immunity. In addition, both *CCR2* and *CCR5* were significantly downregulated in MHCII⁻ macrophages and are present in the most enriched pathway for downregulated genes (upregulated in MHCII⁺ macrophages), which correlates with the earlier finding that fewer MHCII⁻ macrophages express surface level iCCRs in comparison with MHCII⁺ macrophages (Figure 5-4).

Ultimately, the reduction in MHCII⁻ lung macrophages in ACKR2-deficient mice was associated with few transcriptomic changes, indicating that the reduced number of these cells is unlikely to result from altered gene expression. Although MHCII⁻ lung macrophages expressed low levels of surface iCCRs, it remains possible that their numbers are regulated by chemokines, which may be altered in the ACKR2-deficient lung. It is not yet clear whether the reduced number of macrophages observed in both the lung and peritoneal tissues is coordinated by a shared mechanism.

6 Final Discussion

In this thesis, a map of chemokine expression throughout embryonic mouse development during late gestation (E15-E18.5) has been generated. A transcriptomic atlas of the E14.5 mouse embryo using RNA-situ hybridisation has previously been presented and included 18,000 coding genes including many chemokine transcripts (Diez-Roux et al., 2011). Although this resource offers a visual indication of tissue specific chemokine expression across the embryo, it is limited to one time point and is not quantifiable. Furthermore, the resolution for chemokine expression on the currently available version of this data, the Genepaint database (<https://gp3.mpg.de>), is often very low.

Here we provide a comprehensive view of chemokine expression across multiple stages of late gestational development. E15 was selected as the earliest time point of interest due to a previous study that identified an F4/80^{int} macrophage population that is reduced in the E14.5 embryonic skin of ACKR2-deficient mice, which was associated with changes in inflammatory chemokine levels in embryo circulation (Lee et al., 2019). Furthermore, the embryo inflammatory response becomes functionally active from around E14 (Takaya et al., 2022). However, CCL2 has been detected in the mouse embryo as early as E12.5 (Lee et al., 2019), therefore future work could extend analysis of chemokine expression to preceding embryonic time points to investigate their potential roles during earlier stages of tissue morphogenesis.

The homeostatic chemokine CXCL12 was consistently and robustly expressed across all time points and organs analysed, underlining its critical role in organogenesis (Sierro et al., 2007, Belmadani et al., 2005, Gerrits et al., 2008). In addition, CXCL12 in the embryonic lung has been shown to regulate normal branching of the pulmonary arterial tree, with its deletion leading to reduced arterial and capillary density (Chandrasekaran et al., 2022). Other homeostatic CXC chemokines, such as CXCL14, also showed strong and sustained expression from E15-E18.5. Although the role of CXCL14 in the tissues analysed in this study remains elusive, CXCL14-deficient E14.5 embryos have been shown to have markedly reduced neurons in a specific brain region, which has been associated with CXCL14-mediated fatty acid and glycerophospholipid synthesis (Zhang et al., 2024). In addition, human transcriptome data indicates that CXCL14 may impact synaptic plasticity and transmission as well as neuron projection (Zhang et al., 2024). These findings

implicate CXCL14 in embryonic brain development and raise the possibility that CXCL14 may regulate metabolic pathways in other tissues between E14.5 and E18.5.

Furthermore, inflammatory iCCR chemokine ligands were detected from E15 to E18.5, with notably higher relative expression in the lung and skin compared to the heart and liver. This could reflect that the heart and liver become more functionally active in the embryo earlier in development compared with the lungs and skin, with alveolarization in mouse lungs not occurring until after birth (Ji et al., 2003, Mu et al., 2020). In addition, both the lung and skin are barrier tissues, and chemokine regulated recruitment of immune cells during late development may be important for priming these tissues for birth and subsequent exposure to pathogens.

A key observation made during the characterisation of chemokine expression across the embryo was a pronounced peak in CCL11 in the E17.5 lung, which was confirmed at both RNA and protein levels. CCL11 promotes the chemotaxis of mast cells, eosinophils, neutrophils, macrophages, Th2 cells, and basophils and is known to be associated with inflammatory diseases such as asthma in the adult lung (Wu et al., 2014). Whilst CCL11 expression was elevated in the E17.5 lung, its primary receptor, CCR3, was not detected, reflecting the fact that eosinophils are not located in the mouse lung at this developmental stage (Loffredo et al., 2020). This raises the possibility that CCL11 in the E17.5 lung may instead act through CCR2 or CCR5, which were expressed by monocytes (CCR2) and macrophages (CCR2 and CCR5) in the lung at this stage of development.

Notably, transcript levels of CCL6, CCL12 and CXCL4 increased in the embryonic lung between E17.5 and E18.5. Interstitial lung macrophages in the adult mouse constitutively express CXCL4 (Li et al., 2024) suggesting that the source of CXCL4 at E18.5 may be macrophages and indicating that macrophages may increase in the lung around this time point.

Given that the lung is a branching organ, and that macrophages, regulated by inflammatory chemokines and ACKR2, are known to coordinate branching morphogenesis in various contexts, such as in the pubertal mammary gland (Wilson et al., 2020), and during lymphangiogenesis in embryonic skin (Wilson et al., 2020, Lee et al., 2014), it is possible that iCCR⁺ macrophages play a similar role in the embryonic lung. Future study to determine the function of CCL11 in the developing lung and to identify the cellular targets and inflammatory chemokine receptors underlying elevated CCL11 at E17.5 would provide important insight. This could include analysis of tissue morphology and immune cell distribution in CCL11-deficient and receptor-deficient mice using flow cytometry and

histology. Additionally, in WT mice, RNAscope or immunofluorescence staining could be used to localise CCL11 within the tissue and correlate it with receptor activity using iCCR-reporter mice.

Further investigation is needed to understand the roles of inflammatory chemokine signalling during embryogenesis at a single cell level. The use of whole tissue lysates for both RNA and protein detection during this investigation limited resolution. To improve the resolution of the dataset, future studies could use single cell sequencing of embryonic tissues or spatial transcriptomics, such as the Visium spatial platform to precisely identify the cellular sources and potential targets of chemokine signalling during embryogenesis. Additionally, whole-mount imaging of the entire embryo at multiple developmental time points using iCCR reporter mice (iREP) would provide complementary insights. Whole embryo imaging analysis is currently being optimised (Figure 6-1). Once tissue processing and autofluorescence issues are addressed, this approach will allow precise visualisation of iCCR localisation across organs, and amongst tissues not included in this study, such as the kidney, which is also a branching organ, and the brain. 3D-imaging of iCCR reporters using tissue clearing techniques could also further enhance our understanding of chemokine receptor distribution and dynamics during embryogenesis. Although E15 was selected as a time point of interest for this thesis, earlier time points should also be investigated, as CCL2 has been detected in the yolk sac and embryonic skin as early as E12.5 (Lee et al., 2019).

At the protein level, the quantification of many target chemokines required extrapolation from MFI signals below their respective lower limits of detection. These extrapolated values are less reliable and not suitable for statistical analysis. To overcome the problem of extrapolated values, a more sensitive assay, such as specific ELISAs, or Meso Scale Discovery assays (MSDs) could be carried out for chemokine ligands of interest, which may enhance the accuracy of changes in quantification across time points or between tissues.



Figure 6-1 Tile Scan of the E17.5 Embryo at 20X Magnification

Exemplar tile scan image of full embryo section of E17.5 iCCR reporter taken at 20X using a Nikon inverted AX-R at the Glasgow Imaging Facility, within the Institute of Infection and Immunity, University of Glasgow.

A previous study has demonstrated that LPS injection of ACKR2 deficient pregnant female mice leads to increased foetal loss (Martinez de la Torre et al., 2007). However, this study focused on the placenta and did not explore how changes in inflammatory chemokines within embryonic tissues affected morphogenesis. Improving understanding of the role of inflammatory chemokine signalling in shaping the embryonic immune landscape and organ patterning could help to elucidate how maternal inflammation might influence embryonic development. For instance, maternal immune activation has been associated with elevated levels of the chemokine CCL2 in the cerebrospinal fluid (CSF) of E14.5 mouse embryos (Cui et al., 2020). This increase correlates with the accumulation of activated macrophages at the choroid plexus, which serves as the blood-CSF barrier and regulates the transport of nutrients and secretion of signalling molecules involved in brain development during embryogenesis (Cui et al., 2020). The CCL2 mediated recruitment of macrophages to the choroid plexus facilitates their entry into the CSF, triggering local inflammation with potential neurodevelopmental consequences (Cui et al., 2020). These findings align with growing epidemiological evidence linking maternal viral or bacterial infections during pregnancy with a significantly increased risk of neurodevelopmental disorders in children (Hall et al., 2023). This emphasises the need to further investigate the role of inflammatory chemokines during embryonic development and to explore whether their expression is modulated by maternal inflammation. One approach could include injection of individual ACKR2-ligands into trophoblast specific ACKR2-deficient pregnant female mice. This could determine how increased levels of specific ligands into embryo circulation impact development using flow cytometry, immunofluorescence staining and histological analysis of embryo tissues. These data would help to identify changes in immune cell distribution and tissue morphogenesis in response to elevated levels of specific maternal chemokines in the embryo.

Furthermore, in this study, macrophage numbers across adult tissues were explored in detail. This study has demonstrated that monocyte derived SPMs in both the peritoneal cavity and peritoneal wall were reduced in number in ACKR2-deficient mice. Although ACKR2 is expressed by lymphatic endothelial cells in the peritoneal wall and inflammatory chemokine receptors are present within the tissue, the reduction in SPMs was not attributable to a migratory defect caused by disrupted chemokine dynamics in the peritoneum. Monocytes were reduced in the peritoneal wall, but not the peritoneal cavity, lung, blood or bone marrow of ACKR2-deficient mice. This is surprising as the levels of CCR2 ligands, which regulate recruitment of CCR2⁺ bone marrow derived monocytes into tissues, were not altered in the peritoneal wall in the absence of ACKR2. This could

potentially suggest that the migration of monocytes to the peritoneal wall specifically is dependent on other chemokine signals that were not measured, such as CX3CL1, which despite not being an ACKR2 ligand could be indirectly affected downstream of dysregulation caused by the absence of ACKR2. This could be explored by analysing remaining monocytes in the peritoneal wall of *CCR2*^{-/-} mice using flow cytometry. Furthermore, ACKR2 ligand concentration was measured in tissue lysate, but it is possible that the absence of ACKR2 leads to localised changes in functional CCR2/ACKR2 ligand concentration gradients at specific sites within the peritoneal wall that were not identified. Chemokine localisation in WT and ACKR2-deficient mice could be explored by immunofluorescence staining to provide these insights. Increased MMP-2 in the peritoneal wall of ACKR2-deficient mice in comparison to the WT, could indicate that the extracellular matrix is being remodelled in the wall in such a way that monocytes are not retained as effectively in the tissue. Therefore, the level of other factors which regulate the ECM, such as other MMPs, could be investigated in WT versus ACKR2-deficient mice.

In addition, a key biological consequence of reduced SPM numbers in ACKR2-deficient peritoneal tissues was identified as bacterial clearance was impaired in ACKR2-deficient mice following intraperitoneal *E. coli* infection. This was accompanied by changes to macrophage dynamics within the peritoneum. Specifically, there were less LPMs in the peritoneal wall of ACKR2-deficient mice during infection. LPM numbers in the peritoneal cavity decrease markedly in WT mice following infection due to the macrophage adherence/disappearance reaction (Barth et al., 1995, Vega-Perez et al., 2021). Our findings indicate that this reaction is impaired in ACKR2^{-/-} mice, and less LPMs adhere to the wall during infection (Vega-Perez et al., 2021). However, the observed changes in SPM and LPM dynamics alone cannot causally explain the reduced bacterial clearance in the peritoneal cavity. Recent investigation has demonstrated that SPMs isolated from ACKR2-deficient mice exhibit altered clearance following phagocytosis, as shown by fluorescent *E. coli* PHRODO particle uptake assays (Wilson et al., 2024). Therefore, a combination of both altered macrophage numbers in the peritoneum during infection and defective bacterial clearance by SPMs is likely to contribute to impaired bacterial clearance in the peritoneal cavity of ACKR2-deficient mice. To further elucidate the role of LPM dynamics in the peritoneal immune response, future studies could employ whole-mount imaging of the peritoneal wall in ACKR2-deficient versus WT mice post bacterial infection. This could be complemented by flow cytometry and electron microscopy of isolated resident macrophage aggregates following *E. coli* infection, using a protocol for aggregate isolation (Ferriz et al., 2023). These approaches would help determine how

ACKR2 deficiency affects aggregate cellular composition, clustering patterns, size, and spatial distribution across the tissue surface during infection. Additionally, adhesion assays should be conducted to assess whether LPMs and SPMs from ACKR2-deficient mice can adhere to the mesothelium of the peritoneal wall with equal capacity to WT macrophages.

Future investigations could also investigate the impact of ACKR2 deficiency on other infection models in the peritoneum. For example, Chil-1 expression was downregulated in the ACKR2-deficient peritoneum. Chil-1 is a receptor which recognizes chitin, a component of the fungal cell wall and it would therefore be interesting to observe whether ACKR2-deficient mice display an impaired response to peritoneal fungal infection, such as *Candida albicans*, the most common fungus to cause peritonitis (Ashizawa et al., 2019). This could be determined by measuring both clinical signs of infection and fungal burden in the peritoneal cavity of ACKR2-deficient versus WT mice. These results would have clinical implications, as although fungal peritonitis is rare in the total population, it occurs with an incidence rate of between 1 % and 23 % amongst patients undergoing peritoneal dialysis and is associated with high mortality (Mayra et al., 2025). In addition, Chil-1-deficient mice demonstrate decreased survival following *Pseudomonas aeruginosa* infection (Marion et al., 2016). Therefore, ACKR2-deficient mice could be challenged with *Pseudomonas aeruginosa* to determine whether the associated reduction of Chil-1 in the peritoneum has pathological consequences.

A distinct MHCII⁻ lung macrophage population was also reduced in number in ACKR2-deficient mice. A low proportion of these cells expressed surface iCCRs in comparison with MHCII⁺ lung macrophages, which were not changed in numbers in ACKR2-deficient mice. This suggests that, similarly to the peritoneal cavity and wall, impaired iCCR chemokine mediated migration is unlikely to explain the reduced abundance of this macrophage population. In addition, transcriptional analysis uncovered very few differentially expressed genes between WT and ACKR2-deficient MHCII⁻ lung macrophages, indicating that ACKR2 has a limited effect on the transcriptome of these cells. Recent work has used single cell sequencing of enriched interstitial lung macrophages at rest and 24 hours post-LPS treatment to analyse heterogeneity across interstitial lung macrophages (Li et al., 2024). This work revealed 10 distinct IM subsets based on chemokine expression (Li et al., 2024). This could suggest that the reduction in MHCII⁻ lung macrophages observed in ACKR2-deficient mice may represent a more pronounced reduction in the number of a more specific subset that has been obscured by the resolution of our gating strategy. Furthermore, the fact that various macrophage subsets

could be classified based on chemokine expression could point towards the possibility that changes in the chemokine landscape within the lung in the absence of ACKR2 could be responsible for the reduction in a specific macrophage population. To investigate this, a Luminex assay of the lung is required to measure ACKR2 ligand concentration at rest and assess whether this is altered in the absence of ACKR2. This could be accompanied with spatial transcriptomics of both WT and ACKR2-deficient lung sections using the Visium platform to determine whether there are greater transcriptional changes in a more specific MHCII⁺ lung macrophage population, identified with higher resolution than MHCII surface expression alone, between the WT and ACKR2-deficient mice. These data could also assess whether any changes observed in particular MHCII⁺ macrophage populations are related to a specific chemokine transcript expression profile and finally whether any of these observations are related to changes in the position of MHCII⁺ macrophages within the lung in the absence of ACKR2.

The specific functional consequences of the reduced number of MHCII⁺ lung macrophages in ACKR2 deficiency have not yet been assessed. ACKR2 has associations with pathogenesis in the lung including worsened response to *Mycobacterium Tuberculosis* infection (Di Liberto et al., 2008), as well as in models of allergy, in which ACKR2-deficient mice show an increased inflammatory response, with more eosinophils found in both the lung parenchyma and the airways (Whitehead et al., 2007). The reduced number of MHCII⁺ macrophages in the absence of ACKR2 may contribute to this pathology. In addition, the depletion of particular chemokine expressing subsets of IMs was found to lead to changes in the recruitment of specific leukocytes to the tissue (Li et al., 2024). Therefore, it is possible that the reduction in MHCII⁺ macrophages in ACKR2-deficient mice could be responsible for impaired recruitment of other leukocytes during infection models.

The role of MHCII⁺ macrophages in infection could be measured directly by assessing the number and position of MHCII⁺ macrophages in response to lung infection. MHCII⁺ macrophages appear to be less inflammatory than MHCII⁺ macrophages, which were not decreased in the absence of ACKR2. Therefore, other lung infection models that are more associated with an M2 macrophage response, such as infection with the helminth *Nippostrongylus brasiliensis*, should also be analysed (Chen et al., 2012). Allergy also induces a type-2 immune response, therefore a lung allergy model using intranasal administration of house dust mites could uncover functional consequences of the reduced number of MHCII⁺ lung macrophages in ACKR2-deficient mice. In addition, as MHCII⁺

macrophages are associated with wound healing (Chakarov et al., 2019), scarring of lung tissue may be altered in ACKR2-deficient mice, where there are fewer of these macrophages. A model of fibrosis, such as bleomycin treatment in ACKR2-deficient versus WT lung, could therefore be conducted and scarring could be analysed using picrosirius red staining. Finally, changes in the function of MHCII⁺ lung macrophages in ACKR2-deficient mice could be explored for example by assessing phagocytic capacity using non-infectious fluorescent pHRODO *E. coli* particles.

The work presented in this thesis has contributed to the identification of a mechanism for the reduced numbers of macrophages observed in adult ACKR2-deficient mice (Wilson et al., 2024). Specifically, the decrease in the number of SPMs in the naïve ACKR2-deficient peritoneal cavity has been demonstrated to result from altered monocyte to macrophage differentiation in the ACKR2-deficient bone marrow (Wilson et al., 2024). Transcriptional changes were observed in ACKR2-deficient monocytes, shown to be driven by the loss of eosinophils from the bone marrow due to elevated CCL11 levels in ACKR2-deficient mice. The absence of eosinophil-mediated transcriptional programming alters monocyte development, although the precise molecular mechanisms remain to be elucidated. However, the selective impact on only specific macrophage populations, such as SPMs and MHCII⁺ lung macrophages, is not yet fully understood.

6.1 Conclusion

Ultimately, this thesis has defined chemokine expression in detail across multiple embryonic tissues during late gestation for the first time. The detection of inflammatory chemokines in the lung and skin, alongside iCCR expression in embryonic macrophages, suggests that these chemokines may play underappreciated roles in embryogenesis. Future work should focus on the precise localisation of chemokine expression in embryo tissues as well as elucidation of specific cellular targets and functional roles.

Additionally, this thesis defined for the first time, changes in specific macrophage populations in naïve ACKR2-deficient mice. The data revealed that certain macrophage subsets were significantly decreased across various adult tissues in ACKR2-deficient mice. This reduction was not due to disturbed cell recruitment from disrupted inflammatory chemokine signalling but from ACKR2 controlled cell interactions in the bone marrow (Wilson et al., 2024), highlighting a previously unrecognised role for ACKR2 in shaping

adult macrophage populations and underscoring its importance in immune function and development. Together, the findings presented throughout this study highlight that inflammatory CC-chemokines and ACKR2 are likely to play a range of underappreciated roles across both embryonic development and adult immune cell homeostasis.

List of References

- ADAMSKI, V., MENTLEIN, R., LUCIUS, R., SYNOWITZ, M., HELD-FEINDT, J. & HATTERMANN, K. 2017. The Chemokine Receptor CXCR6 Evokes Reverse Signaling via the Transmembrane Chemokine CXCL16. *Int J Mol Sci*, 18.
- ADDISON, C. L., DANIEL, T. O., BURDICK, M. D., LIU, H., EHLERT, J. E., XUE, Y. Y., BUECHI, L., WALZ, A., RICHMOND, A. & STRIETER, R. M. 2000. The CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR+ CXC chemokine-induced angiogenic activity. *J Immunol*, 165, 5269-77.
- AIELLO, R. J., BOURASSA, P. A., LINDSEY, S., WENG, W., NATOLI, E., ROLLINS, B. J. & MILOS, P. M. 1999. Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol*, 19, 1518-25.
- ALAMPOUR-RAJABI, S., EL BOUNKARI, O., ROT, A., MÜLLER-NEUEN, G., BACHELERIE, F., GAWAZ, M., WEBER, C., SCHÖBER, A. & BERNHAGEN, J. 2015. MIF interacts with CXCR7 to promote receptor internalization, ERK1/2 and ZAP-70 signaling, and lymphocyte chemotaxis. *Faseb Journal*, 29, 4497-4511.
- ANSEL, K. M., HARRIS, R. B. & CYSTER, J. G. 2002. CXCL13 is required for B1 cell homing, natural antibody production, and body cavity immunity. *Immunity*, 16, 67-76.
- ARIMONT, M., SUN, S. L., LEURS, R., SMIT, M., DE ESCH, I. J. P. & DE GRAAF, C. 2017. Structural Analysis of Chemokine Receptor-Ligand

Interactions. *Journal of Medicinal Chemistry*, 60, 4735-4779.

- ARMAS-GONZALEZ, E., DOMINGUEZ-LUIS, M. J., DIAZ-MARTIN, A., ARCE-FRANCO, M., CASTRO-HERNANDEZ, J., DANELON, G., HERNANDEZ-HERNANDEZ, V., BUSTABAD-REYES, S., CANTABRANA, A., UGUCCIONI, M. & DIAZ-GONZALEZ, F. 2018. Role of CXCL13 and CCL20 in the recruitment of B cells to inflammatory foci in chronic arthritis. *Arthritis Res Ther*, 20, 114.
- ARNOLD, I. C. & MUNITZ, A. 2024. Spatial adaptation of eosinophils and their emerging roles in homeostasis, infection and disease. *Nature Reviews Immunology*, 24, 858-877.
- ASHIZAWA, N., MIYAZAKI, T., ABE, S., TAKAZONO, T., SAIJO, T., OBATA, Y., SHIMAMURA, S., YAMAMOTO, K., IMAMURA, Y., KOJI, T., NISHINO, T., IZUMIKAWA, K., YANAGIHARA, K., KOHNO, S. & MUKAE, H. 2019. Evaluation of *Candida* peritonitis with underlying peritoneal fibrosis and efficacy of micafungin in murine models of intra-abdominal candidiasis. *Sci Rep*, 9, 9331.
- AUFFRAY, C., FOGG, D., GARFA, M., ELAIN, G., JOIN-LAMBERT, O., KAYAL, S., SARNACKI, S., CUMANO, A., LAUVAU, G. & GEISSMANN, F. 2007. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science*, 317, 666-70.
- BACHELERIE, F., BEN-BARUCH, A., BURKHARDT, A. M., COMBADIÈRE, C., FARBER, J. M., GRAHAM, G. J., HORUK, R., SPARRE-ULRICH, A. H., LOCATI, M., LUSTER, A. D., MANTOVANI, A., MATSUSHIMA, K., MURPHY, P. M., NIBBS,

- R., NOMIYAMA, H., POWER, C. A., PROUDFOOT, A. E. I., ROSENKILDE, M. M., ROT, A., SOZZANI, S., THELEN, M., YOSHIE, O. & ZLOTNIK, A. 2014. International Union of Pharmacology. LXXXIX. Update on the Extended Family of Chemokine Receptors and Introducing a New Nomenclature for Atypical Chemokine Receptors (vol 66, pg 1, 2014). *Pharmacological Reviews*, 66, 467-467.
- BACHMANN, M. F., KOPF, M. & MARSLAND, B. J. 2006. Chemokines: more than just road signs. *Nature Reviews Immunology*, 6, 159-164.
- BAGGIOLINI, M. 1998. Chemokines and leukocyte traffic. *Nature*, 392, 565-8.
- BAGHERI, V., KHORRAMDELAZAD, H., KAFI, M. & ABBASIFARD, M. 2024. Chemokine CCL2 and its receptor CCR2 in different age groups of patients with COVID-19. *BMC Immunol*, 25, 72.
- BAIN, C. C., BRAVO-BLAS, A., SCOTT, C. L., PERDIGUERO, E. G., GEISSMANN, F., HENRI, S., MALISSEN, B., OSBORNE, L. C., ARTIS, D. & MOWAT, A. M. 2014. Constant replenishment from circulating monocytes maintains the macrophage pool in the intestine of adult mice. *Nat Immunol*, 15, 929-937.
- BAIN, C. C., HAWLEY, C. A., GARNER, H., SCOTT, C. L., SCHRIDDE, A., STEERS, N. J., MACK, M., JOSHI, A., GUILLIAMS, M., MOWAT, A. M., GEISSMANN, F. & JENKINS, S. J. 2016. Long-lived self-renewing bone marrow-derived macrophages displace embryo-derived cells to inhabit adult serous cavities. *Nat Commun*, 7, ncomms11852.
- BAIN, C. C. & MACDONALD, A. S. 2022. The impact of the lung environment on macrophage

development, activation and function: diversity in the face of adversity. *Mucosal Immunol*, 15, 223-234.

- BAJOGLI, B., AGHAALLAEI, N., HESS, I., RODE, I., NETUSCHIL, N., TAY, B. H., VENKATESH, B., YU, J. K., KALTENBACH, S. L., HOLLAND, N. D., DIEKHOF, D., HAPPE, C., SCHORPP, M. & BOEHM, T. 2009. Evolution of genetic networks underlying the emergence of thymopoiesis in vertebrates. *Cell*, 138, 186-97.
- BARGATZE, R. F., KURK, S., BUTCHER, E. C. & JUTILA, M. A. 1994. Neutrophils roll on adherent neutrophils bound to cytokine-induced endothelial cells via L-selectin on the rolling cells. *J Exp Med*, 180, 1785-92.
- BARNES, J. L., YOSHIDA, M., HE, P., WORLOCK, K. B., LINDEBOOM, R. G. H., SUO, C., PETT, J. P., WILBREY-CLARK, A., DANN, E., MAMANOVA, L., RICHARDSON, L., POLANSKI, K., PENNYCUICK, A., ALLEN-HYTTINEN, J., HERCZEG, I. T., ARZILI, R., HYND, R. E., TEIXEIRA, V. H., HANIFFA, M., LIM, K., SUN, D., RAWLINS, E. L., OLIVER, A. J., LYONS, P. A., MARIONI, J. C., RUHRBERG, C., TUONG, Z. K., CLATWORTHY, M. R., READING, J. L., JANES, S. M., TEICHMANN, S. A., MEYER, K. B. & NIKOLIC, M. Z. 2023. Early human lung immune cell development and its role in epithelial cell fate. *Sci Immunol*, 8, eadf9988.
- BARRIENTOS, S., STOJADINOVIC, O., GOLINKO, M. S., BREM, H. & TOMIC-CANIC, M. 2008. Growth factors and cytokines in wound healing. *Wound Repair Regen*, 16, 585-601.
- BARTH, M. W., HENDRZAK, J. A., MELNICOFF, M. J. & MORAHAN, P. S. 1995. Review of the

- macrophage disappearance reaction. *J Leukoc Biol*, 57, 361-7.
- BEDORET, D., WALLEMACQ, H., MARICHAL, T., DESMET, C., QUESADA CALVO, F., HENRY, E., CLOSSET, R., DEWALS, B., THIELEN, C., GUSTIN, P., DE LEVAL, L., VAN ROOIJEN, N., LE MOINE, A., VANDERPLASSCHEN, A., CATALDO, D., DRION, P. V., MOSER, M., LEKEUX, P. & BUREAU, F. 2009. Lung interstitial macrophages alter dendritic cell functions to prevent airway allergy in mice. *J Clin Invest*, 119, 3723-38.
- BELMADANI, A., TRAN, P. B., REN, D. J., ASSIMACOPOULOS, S., GROVE, E. A. & MILLER, R. J. 2005. The chemokine stromal cell-derived factor-1 regulates the migration of sensory neuron progenitors. *Journal of Neuroscience*, 25, 3995-4003.
- BELPERIO, J. A., DY, M., BURDICK, M. D., XUE, Y. Y., LI, K., ELIAS, J. A. & KEANE, M. P. 2002. Interaction of IL-13 and C10 in the pathogenesis of bleomycin-induced pulmonary fibrosis. *Am J Respir Cell Mol Biol*, 27, 419-27.
- BERLIN, C., BARGATZE, R. F., CAMPBELL, J. J., VON ANDRIAN, U. H., SZABO, M. C., HASSLEN, S. R., NELSON, R. D., BERG, E. L., ERLANDSEN, S. L. & BUTCHER, E. C. 1995. alpha 4 integrins mediate lymphocyte attachment and rolling under physiologic flow. *Cell*, 80, 413-22.
- BERTRAND, J. Y., CHI, N. C., SANTOSO, B., TENG, S., STAINIER, D. Y. & TRAVER, D. 2010. Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature*, 464, 108-11.

- BHUSAL, R. P., EATON, J. R. O., CHOWDHURY, S. T., POWER, C. A., PROUDFOOT, A. E. I., STONE, M. J. & BHATTACHARYA, S. 2020. Evasins: Tick Salivary Proteins that Inhibit Mammalian Chemokines. *Trends Biochem Sci*, 45, 108-122.
- BLANCHARD, C. & ROTHENBERG, M. E. 2009. Biology of the eosinophil. *Adv Immunol*, 101, 81-121.
- BONECCHI, R. & GRAHAM, G. J. 2016. Atypical Chemokine Receptors and Their Roles in the Resolution of the inflammatory Response. *Frontiers in Immunology*, 7.
- BONECCHI, R., MASSARA, M. & LOCATI, M. 2016. Atypical Chemokine Receptors. *Encyclopedia of Immunobiology, Vol 2: Molecular Immunology*, 579-585.
- BONNAVION, R., LIU, S., KAWASE, H., ROQUID, K. A. & OFFERMANN, S. 2023. Large chemokine binding spectrum of human and mouse atypical chemokine receptor GPR182 (ACKR5). *Front Pharmacol*, 14, 1297596.
- BOT, I., ORTIZ ZACARIAS, N. V., DE WITTE, W. E., DE VRIES, H., VAN SANTBRINK, P. J., VAN DER VELDEN, D., KRONER, M. J., VAN DER BERG, D. J., STAMOS, D., DE LANGE, E. C., KUIPER, J., AP, I. J. & HEITMAN, L. H. 2017. A novel CCR2 antagonist inhibits atherogenesis in apoE deficient mice by achieving high receptor occupancy. *Sci Rep*, 7, 52.
- BRAUNERSREUTHER, V., MONTECUCCO, F., PELLI, G., GALAN, K., PROUDFOOT, A. E., BELIN, A., VUILLEUMIER, N., BURGER, F., LENGLET, S., CAFFA, I., SONCINI, D., NENCIONI, A., VALLEE, J. P. & MACH, F. 2013.

- Treatment with the CC chemokine-binding protein Evasin-4 improves post-infarction myocardial injury and survival in mice. *Thromb Haemost*, 110, 807-25.
- BREEN, E. J., TAN, W. & KHAN, A. 2016. The Statistical Value of Raw Fluorescence Signal in Luminex xMAP Based Multiplex Immunoassays. *Sci Rep*, 6, 26996.
- BRINKMANN, V., REICHARD, U., GOOSMANN, C., FAULER, B., UHLEMANN, Y., WEISS, D. S., WEINRAUCH, Y. & ZYCHLINSKY, A. 2004. Neutrophil extracellular traps kill bacteria. *Science*, 303, 1532-5.
- BROXMEYER, H. E., COOPER, S. H. & ROPA, J. 2021. CXCL15/Lungkine has suppressive activity on proliferation and expansion of multi-potential, erythroid, granulocyte and macrophage progenitors in S-phase specific manner. *Blood Cells Mol Dis*, 91, 102594.
- BRY, K., WHITSETT, J. A. & LAPPALAINEN, U. 2007. IL-1beta disrupts postnatal lung morphogenesis in the mouse. *Am J Respir Cell Mol Biol*, 36, 32-42.
- BURNS, J. M., SUMMERS, B. C., WANG, Y., MELIKIAN, A., BERAHOVICH, R., MIAO, Z. H., PENFOLD, M. E. T., SUNSHINE, M. J., LITTMAN, D. R., KUO, C. J., WEI, K., MCMASTER, B. E., WRIGHT, K., HOWARD, M. C. & SCHALL, T. J. 2006. A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *Journal of Experimental Medicine*, 203, 2201-2213.
- BYRNE, A. J., POWELL, J. E., O'SULLIVAN, B. J., OGGER, P. P., HOFFLAND, A., COOK, J., BONNER, K. L., HEWITT, R. J., WOLF, S., GHAI, P., WALKER, S. A., LUKOWSKI, S. W.,

- MOLYNEAUX, P. L., SAGLANI, S., CHAMBERS, D. C., MAHER, T. M. & LLOYD, C. M. 2020. Dynamics of human monocytes and airway macrophages during healthy aging and after transplant. *J Exp Med*, 217.
- CACALANO, G., LEE, J., KIKLY, K., RYAN, A. M., PITTS-MEEK, S., HULTGREN, B., WOOD, W. I. & MOORE, M. W. 1994. Neutrophil and B cell expansion in mice that lack the murine IL-8 receptor homolog. *Science*, 265, 682-4.
- CAMBIER, S., GOUWY, M. & PROOST, P. 2023. The chemokines CXCL8 and CXCL12: molecular and functional properties, role in disease and efforts towards pharmacological intervention. *Cellular & Molecular Immunology*, 20, 217-251.
- CAPOBIANCO, A., COTTONE, L., MONNO, A., MANFREDI, A. A. & ROVERE-QUERINI, P. 2017. The peritoneum: healing, immunity, and diseases. *J Pathol*, 243, 137-147.
- CAREY, P., LOW, E., HARPER, E. & STACK, M. S. 2021. Metalloproteinases in Ovarian Cancer. *Int J Mol Sci*, 22.
- CARLIN, L. M., STAMATIADES, E. G., AUFRAY, C., HANNA, R. N., GLOVER, L., VIZCAY-BARRENA, G., HEDRICK, C. C., COOK, H. T., DIEBOLD, S. & GEISSMANN, F. 2013. Nr4a1-dependent Ly6C(low) monocytes monitor endothelial cells and orchestrate their disposal. *Cell*, 153, 362-75.
- CASSADO ADOS, A., DE ALBUQUERQUE, J. A., SARDINHA, L. R., BUZZO CDE, L., FAUSTINO, L., NASCIMENTO, R., GHOSN, E. E., LIMA, M. R., ALVAREZ, J. M. & BORTOLUCI, K. R. 2011. Cellular renewal and improvement of local cell effector activity in peritoneal cavity in response to infectious stimuli. *PLoS One*, 6, e22141.

- CAVALLERO, S., SHEN, H., YI, C., LIEN, C. L., KUMAR, S. R. & SUCOV, H. M. 2015. CXCL12 Signaling Is Essential for Maturation of the Ventricular Coronary Endothelial Plexus and Establishment of Functional Coronary Circulation. *Dev Cell*, 33, 469-77.
- CEBINELLI, G. C. M., DE LIMA, K. A., SILVA CASTANHEIRA, F. V. E., HIROKI, C. H., MONTEIRO, V. V. S., DE LIMA, M. H. F., NASCIMENTO, D. C. B., ALVES FILHO, J. C., CUNHA, T. M. & CUNHA, F. Q. 2021. CCR2-deficient mice are protected to sepsis by the disruption of the inflammatory monocytes emigration from the bone marrow. *J Leukoc Biol*, 109, 1063-1070.
- CHAKAROV, S., LIM, H. Y., TAN, L., LIM, S. Y., SEE, P., LUM, J., ZHANG, X. M., FOO, S., NAKAMIZO, S., DUAN, K., KONG, W. T., GENTEK, R., BALACHANDER, A., CARBAJO, D., BLERIOT, C., MALLERET, B., TAM, J. K. C., BAIG, S., SHABEER, M., TOH, S. E. S., SCHLITZER, A., LARBI, A., MARICHAL, T., MALISSEN, B., CHEN, J., POIDINGER, M., KABASHIMA, K., BAJENOFF, M., NG, L. G., ANGELI, V. & GINHOUX, F. 2019. Two distinct interstitial macrophage populations coexist across tissues in specific subtissular niches. *Science*, 363.
- CHANDRASEKARAN, P., NEGRETTI, N. M., SIVAKUMAR, A., LIBERTI, D. C., WEN, H., PEERS DE NIEUWBURGH, M., WANG, J. Y., MICHKI, S. N., CHAUDHRY, F. N., KAUR, S., LU, M., JIN, A., ZEPP, J. A., YOUNG, L. R., SUCRE, J. M. S. & FRANK, D. B. 2022. CXCL12 defines lung endothelial heterogeneity and promotes distal vascular growth. *Development*, 149.

- CHATTERJEE, M., VON UNGERN-STERMBERG, S. N., SEIZER, P., SCHLEGEL, F., BUTTCHER, M., SINDHU, N. A., MULLER, S., MACK, A. & GAWAZ, M. 2015. Platelet-derived CXCL12 regulates monocyte function, survival, differentiation into macrophages and foam cells through differential involvement of CXCR4-CXCR7. *Cell Death Dis*, 6, e1989.
- CHEN, F., LIU, Z., WU, W., ROZO, C., BOWDRIDGE, S., MILLMAN, A., VAN ROOIJEN, N., URBAN, J. F., JR., WYNN, T. A. & GAUSE, W. C. 2012. An essential role for TH2-type responses in limiting acute tissue damage during experimental helminth infection. *Nat Med*, 18, 260-6.
- CHEN, K. Q., BAO, Z. Y., TANG, P., GONG, W. H., YOSHIMURA, T. & WANG, J. M. 2018. Chemokines in homeostasis and diseases. *Cellular & Molecular Immunology*, 15, 324-334.
- CHEVIGNÉ, A., JANJI, B., MEYRATH, M., REYNDERS, N., D'UONNOLO, G., UCHANSKI, T., XIAO, M. L. N., BERCHEM, G., OLLERT, M., KWON, Y. J., NOMAN, M. Z. & SZPAKOWSKA, M. 2021. CXCL10 Is an Agonist of the CC Family Chemokine Scavenger Receptor ACKR2/D6. *Cancers*, 13.
- CHO, G. J., LEE, E. S., JIN, H. M., LEE, J. H., KIM, Y. S., OH, M. J., SEOL, H. J., HONG, S. C. & KIM, H. J. 2015. Placental expression of D6 decoy receptor in preeclampsia. *Obstet Gynecol Sci*, 58, 333-9.
- CHO, M. L., YOON, B. Y., JU, J. H., JUNG, Y. O., JHUN, J. Y., PARK, M. K., PARK, S. H., CHO, C. S. & KIM, H. Y. 2007. Expression of CCR2A, an isoform of MCP-1 receptor, is increased by MCP-1, CD40 ligand and TGF-beta in fibroblast like

- synoviocytes of patients with RA. *Exp Mol Med*, 39, 499-507.
- CHOE, H., FARZAN, M., SUN, Y., SULLIVAN, N., ROLLINS, B., PONATH, P. D., WU, L., MACKAY, C. R., LAROSA, G., NEWMAN, W., GERARD, N., GERARD, C. & SODROSKI, J. 1996. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell*, 85, 1135-48.
- CHORRO, L., SARDE, A., LI, M., WOOLLARD, K. J., CHAMBON, P., MALISSEN, B., KISSENPFENNIG, A., BARBAROUX, J. B., GROVES, R. & GEISSMANN, F. 2009. Langerhans cell (LC) proliferation mediates neonatal development, homeostasis, and inflammation-associated expansion of the epidermal LC network. *J Exp Med*, 206, 3089-100.
- CHOU, R. C., KIM, N. D., SADIK, C. D., SEUNG, E., LAN, Y., BYRNE, M. H., HARIBABU, B., IWAKURA, Y. & LUSTER, A. D. 2010. Lipid-cytokine-chemokine cascade drives neutrophil recruitment in a murine model of inflammatory arthritis. *Immunity*, 33, 266-78.
- CHTANOVA, T., SCHAEFFER, M., HAN, S. J., VAN DOOREN, G. G., NOLLMANN, M., HERZMARK, P., CHAN, S. W., SATIJA, H., CAMFIELD, K., AARON, H., STRIEPEN, B. & ROBEY, E. A. 2008. Dynamics of neutrophil migration in lymph nodes during infection. *Immunity*, 29, 487-96.
- CHU, V. T., BELLER, A., RAUSCH, S., STRANDMARK, J., ZANKER, M., ARBACH, O., KRUGLOV, A. & BEREK, C. 2014. Eosinophils promote generation and maintenance of immunoglobulin-A-expressing plasma cells and

- contribute to gut immune homeostasis. *Immunity*, 40, 582-93.
- CHU, V. T., FROHLICH, A., STEINHAUSER, G., SCHEEL, T., ROCH, T., FILLATREAU, S., LEE, J. J., LOHNING, M. & BEREK, C. 2011. Eosinophils are required for the maintenance of plasma cells in the bone marrow. *Nat Immunol*, 12, 151-9.
- COATES, P. T., COLVIN, B. L., RANGANATHAN, A., DUNCAN, F. J., LAN, Y. Y., SHUFESKY, W. J., ZAHORCHAK, A. F., MORELLI, A. E. & THOMSON, A. W. 2004. CCR and CC chemokine expression in relation to Flt3 ligand-induced renal dendritic cell mobilization. *Kidney Int*, 66, 1907-17.
- COLE, J. J., FAYDACI, B. A., MCGUINNESS, D., SHAW, R., MACIEWICZ, R. A., ROBERTSON, N. A. & GOODYEAR, C. S. 2021. Searchlight: automated bulk RNA-seq exploration and visualisation using dynamically generated R scripts. *BMC Bioinformatics*, 22, 411.
- COLOM, B., BODKIN, J. V., BEYRAU, M., WOODFIN, A., ODY, C., ROURKE, C., CHAVAKIS, T., BROHI, K., IMHOF, B. A. & NOURSHARGH, S. 2015. Leukotriene B4-Neutrophil Elastase Axis Drives Neutrophil Reverse Transendothelial Cell Migration In Vivo. *Immunity*, 42, 1075-86.
- COMBADIÈRE, C., AHUJA, S. K. & MURPHY, P. M. 1996. Cloning and functional expression of a human eosinophil CC chemokine receptor. *J Biol Chem*, 271, 11034.
- COMERFORD, I. & MCCOLL, S. R. 2024. Atypical chemokine receptors in the immune system. *Nature Reviews Immunology*, 24, 753-769.
- COMERFORD, I., MILASTA, S., MORROW, V., MILLIGAN, G. & NIBBS, R. 2006. The chemokine

receptor CCX-CKR mediates effective scavenging of CCL19 in vitro. *Eur J Immunol*, 36, 1904-16.

- CONNELL, B. J., HERMANS, L. E., WENSING, A. M. J., SCHELLENS, I., SCHIPPER, P. J., VAN HAM, P. M., DE JONG, D., OTTO, S., MATHE, T., MORABA, R., BORGHANS, J. A. M., PAPATHANASOPOULOS, M. A., KRUIZE, Z., VENTER, F. W. D., KOOTSTRA, N. A., TEMPELMAN, H., TESSELAAR, K. & NIJHUIS, M. 2020. Immune activation correlates with and predicts CXCR4 co-receptor tropism switch in HIV-1 infection. *Sci Rep*, 10, 15866.
- COSSARIZZA, A., CHANG, H. D., RADBRUCH, A., ABRIGNANI, S., ADDO, R., AKDIS, M., ANDRA, I., ANDREATA, F., ANNUNZIATO, F., ARRANZ, E., BACHER, P., BARI, S., BARNABA, V., BARROS-MARTINS, J., BAUMJOHANN, D., BECCARIA, C. G., BERNARDO, D., BOARDMAN, D. A., BORGER, J., BOTTCHER, C., BROCKMANN, L., BURNS, M., BUSCH, D. H., CAMERON, G., CAMMARATA, I., CASSOTTA, A., CHANG, Y., CHIRDO, F. G., CHRISTAKOU, E., CICIN-SAIN, L., COOK, L., CORBETT, A. J., CORNELIS, R., COSMI, L., DAVEY, M. S., DE BIASI, S., DE SIMONE, G., DEL ZOTTO, G., DELACHER, M., DI ROSA, F., DI SANTO, J., DIEFENBACH, A., DONG, J., DORNER, T., DRESS, R. J., DUTERTRE, C. A., ECKLE, S. B. G., EEDE, P., EVRARD, M., FALK, C. S., FEUERER, M., FILLATREAU, S., FIZ-LOPEZ, A., FOLLO, M., FOULDS, G. A., FROBEL, J., GAGLIANI, N., GALLETTI, G., GANGAEV, A., GARBI, N., GARROTE, J. A., GEGINAT, J., GHERARDIN, N. A., GIBELLINI, L., GINHOUX, F., GODFREY, D. I., GRUARIN, P., HAFTMANN, C., HANSMANN, L.,

- HARPUR, C. M., HAYDAY, A. C., HEINE, G., HERNANDEZ, D. C., HERRMANN, M., HOELSKEN, O., HUANG, Q., HUBER, S., HUBER, J. E., HUEHN, J., HUNDEMER, M., HWANG, W. Y. K., IANNAcone, M., IVISON, S. M., JACK, H. M., JANI, P. K., KELLER, B., KESSLER, N., KETELAARS, S., KNOP, L., KNOPF, J., KOAY, H. F., KOBOW, K., KRIEGSMANN, K., KRISTYANTO, H., KRUEGER, A., KUEHNE, J. F., KUNZE-SCHUMACHER, H., KVISTBORG, P., KWOK, I., LATORRE, D., et al. 2021. Guidelines for the use of flow cytometry and cell sorting in immunological studies (third edition). *Eur J Immunol*, 51, 2708-3145.
- CRAWFORD, K. S. & VOLKMAN, B. F. 2023. Prospects for targeting ACKR1 in cancer and other diseases. *Frontiers in Immunology*, 14.
- CRUZ-ORENGO, L., CHEN, Y. J., KIM, J. H., DORSEY, D., SONG, S. K. & KLEIN, R. S. 2011. CXCR7 antagonism prevents axonal injury during experimental autoimmune encephalomyelitis as revealed by in vivo axial diffusivity. *J Neuroinflammation*, 8, 170.
- CUI, J., SHIPLEY, F. B., SHANNON, M. L., ALTURKISTANI, O., DANI, N., WEBB, M. D., SUGDEN, A. U., ANDERMANN, M. L. & LEHTINEN, M. K. 2020. Inflammation of the Embryonic Choroid Plexus Barrier following Maternal Immune Activation. *Dev Cell*, 55, 617-628 e6.
- DAI, X. M., RYAN, G. R., HAPEL, A. J., DOMINGUEZ, M. G., RUSSELL, R. G., KAPP, S., SYLVESTRE, V. & STANLEY, E. R. 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, 111-20.

- DAVIES, L. C., ROSAS, M., JENKINS, S. J., LIAO, C. T., SCURR, M. J., BROMBACHER, F., FRASER, D. J., ALLEN, J. E., JONES, S. A. & TAYLOR, P. R. 2013. Distinct bone marrow-derived and tissue-resident macrophage lineages proliferate at key stages during inflammation. *Nat Commun*, 4, 1886.
- DAWSON, T. C., LENTSCH, A. B., WANG, Z., COWHIG, J. E., ROT, A., MAEDA, N. & PEIPER, S. C. 2000. Exaggerated response to endotoxin in mice lacking the Duffy antigen/receptor for chemokines (DARC). *Blood*, 96, 1681-4.
- DE LA TORRE, Y. M., LOCATI, M., BURACCHI, C., DUPOR, J., COOK, D. N., BONECCHI, R., NEBULONI, M., RUKAVINA, D., VAGO, L., VECCHI, A., LIRA, S. A. & MANTOVANI, A. 2005. Increased inflammation in mice deficient for the chemokine decoy receptor D6. *European Journal of Immunology*, 35, 1342-1346.
- DEAN, M., CARRINGTON, M., WINKLER, C., HUTTLEY, G. A., SMITH, M. W., ALLIKMETS, R., GOEDERT, J. J., BUCHBINDER, S. P., VITTINGHOFF, E., GOMPERTS, E., DONFIELD, S., VLAHOV, D., KASLOW, R., SAAH, A., RINALDO, C., DETELS, R. & O'BRIEN, S. J. 1996. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San

- Francisco City Cohort, ALIVE Study. *Science*, 273, 1856-62.
- DENG, H., LIU, R., ELLMEIER, W., CHOE, S., UNUTMAZ, D., BURKHART, M., DI MARZIO, P., MARMON, S., SUTTON, R. E., HILL, C. M., DAVIS, C. B., PEIPER, S. C., SCHALL, T. J., LITTMAN, D. R. & LANDAU, N. R. 1996. Identification of a major co-receptor for primary isolates of HIV-1. *Nature*, 381, 661-6.
- DEVINE, S. M., FLOMENBERG, N., VESOLE, D. H., LIESVELD, J., WEISDORF, D., BADEL, K., CALANDRA, G. & DIPERSIO, J. F. 2004. Rapid mobilization of CD34+ cells following administration of the CXCR4 antagonist AMD3100 to patients with multiple myeloma and non-Hodgkin's lymphoma. *J Clin Oncol*, 22, 1095-102.
- DEVKOTA, S. R., ARYAL, P., POKHREL, R., JIAO, W., PERRY, A., PANJIKAR, S., PAYNE, R. J., WILCE, M. C. J., BHUSAL, R. P. & STONE, M. J. 2023. Engineering broad-spectrum inhibitors of inflammatory chemokines from subclass A3 tick evasins. *Nat Commun*, 14, 4204.
- DEVRIES, M. E., KELVIN, A. A., XU, L. L., RAN, L. S., ROBINSON, J. & KELVIN, D. J. 2006. Defining the origins and evolution of the chemokine/chemokine receptor system. *Journal of Immunology*, 176, 401-415.
- DI LIBERTO, D., LOCATI, M., CACCAMO, N., VECCHI, A., MERAVIDGLIA, S., SALERNO, A., SIRECI, G., NEBULONI, M., CACERES, N., CARDONA, P. J., DIELI, F. & MANTOVANI, A. 2008. Role of the chemokine decoy receptor D6 in balancing inflammation, immune activation, and antimicrobial resistance in *Mycobacterium tuberculosis* infection. *J Exp Med*, 205, 2075-84.

- DIEZ-ROUX, G., BANFI, S., SULTAN, M., GEFFERS, L., ANAND, S., ROZADO, D., MAGEN, A., CANIDIO, E., PAGANI, M., PELUSO, I., LINMARQ, N., KOCH, M., BILIO, M., CANTIELLO, I., VERDE, R., DE MASI, C., BIANCHI, S. A., CICCHINI, J., PERROUD, E., MEHMETI, S., DAGAND, E., SCHRINNER, S., NURNBERGER, A., SCHMIDT, K., METZ, K., ZWINGMANN, C., BRIESKE, N., SPRINGER, C., HERNANDEZ, A. M., HERZOG, S., GRABBE, F., SIEVERDING, C., FISCHER, B., SCHRADER, K., BROCKMEYER, M., DETTMER, S., HELBIG, C., ALUNNI, V., BATTAINI, M. A., MURA, C., HENRICHSEN, C. N., GARCIA-LOPEZ, R., ECHEVARRIA, D., PUELLES, E., GARCIA-CALERO, E., KRUSE, S., UHR, M., KAUCK, C., FENG, G., MILYAEV, N., ONG, C. K., KUMAR, L., LAM, M., SEMPLE, C. A., GYENESEI, A., MUNDLOS, S., RADELOF, U., LEHRACH, H., SARMIENTOS, P., REYMOND, A., DAVIDSON, D. R., DOLLE, P., ANTONARAKIS, S. E., YASPO, M. L., MARTINEZ, S., BALDOCK, R. A., EICHELE, G. & BALLABIO, A. 2011. A high-resolution anatomical atlas of the transcriptome in the mouse embryo. *PLoS Biol*, 9, e1000582.
- DORR, P., WESTBY, M., DOBBS, S., GRIFFIN, P., IRVINE, B., MACARTNEY, M., MORI, J., RICKETT, G., SMITH-BURCHNELL, C., NAPIER, C., WEBSTER, R., ARMOUR, D., PRICE, D., STAMMEN, B., WOOD, A. & PERROS, M. 2005. Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. *Antimicrob Agents Chemother*, 49, 4721-32.

- DOTTA, L., TASSONE, L. & BADOLATO, R. 2011. Clinical and genetic features of Warts, Hypogammaglobulinemia, Infections and Myelokathexis (WHIM) syndrome. *Curr Mol Med*, 11, 317-25.
- DUCHENE, J., NOVITZKY-BASSO, I., THIRIOT, A., CASANOVA-ACEBES, M., BIANCHINI, M., ETHERIDGE, S. L., HUB, E., NITZ, K., ARTINGER, K., ELLER, K., CAAMAÑO, J., RÜLICHE, T., MOSS, P., MEGENS, R. T. A., VON ANDRIAN, U. H., HIDALGO, A., WEBER, C. & ROT, A. 2017. Atypical chemokine receptor 1 on nucleated erythroid cells regulates hematopoiesis. *Nature Immunology*, 18, 753-+.
- DUVIC, M., EVANS, M. & WANG, C. 2016. Mogamulizumab for the treatment of cutaneous T-cell lymphoma: recent advances and clinical potential. *Ther Adv Hematol*, 7, 171-4.
- DYER, D. P. 2020. Understanding the mechanisms that facilitate specificity, not redundancy, of chemokine-mediated leukocyte recruitment. *Immunology*, 160, 336-344.
- DYER, D. P., MEDINA-RUIZ, L., BARTOLINI, R., SCHUETTE, F., HUGHES, C. E., PALLAS, K., VIDLER, F., MACLEOD, M. K. L., KELLY, C. J., LEE, K. M., HANSELL, C. A. H. & GRAHAM, G. J. 2019a. Chemokine Receptor Redundancy and Specificity Are Context Dependent. *Immunity*, 50, 378-+.
- DYER, D. P., NEBOT, J. B., KELLY, C. J., MEDINA-RUIZ, L., SCHUETTE, F. & GRAHAM, G. J. 2019b. The chemokine receptor CXCR2 contributes to murine adipocyte development. *J Leukoc Biol*, 105, 497-506.

- DYER, D. P., SALANGA, C. L., JOHNS, S. C., VALDAMBRINI, E., FUSTER, M. M., MILNER, C. M., DAY, A. J. & HANDEL, T. M. 2016. The Anti-inflammatory Protein TSG-6 Regulates Chemokine Function by Inhibiting Chemokine/Glycosaminoglycan Interactions. *J Biol Chem*, 291, 12627-12640.
- DZIERZAK, E. & BIGAS, A. 2018. Blood Development: Hematopoietic Stem Cell Dependence and Independence. *Cell Stem Cell*, 22, 639-651.
- EKSTEEN, B., GRANT, A. J., MILES, A., CURBISHLEY, S. M., LALOR, P. F., HÜBSCHER, S. G., BRISKIN, M., SALMON, M. & ADAMS, D. H. 2004. Hepatic endothelial CCL25 mediates the recruitment of CCR9 gut-homing lymphocytes to the liver in primary sclerosing cholangitis. *Journal of Experimental Medicine*, 200, 1511-1517.
- EVANS, T. J., BASU, B., HUBNER, R., MA, Y. T., MEYER, T., PALMER, D. H., PINATO, D. J. J., PLUMMER, E. R., ROSS, P. J. & SAMSON, A. 2023. A phase I/II study of the CXCR2 inhibitor, AZD5069, in combination with durvalumab, in patients (pts) with advanced hepatocellular carcinoma (HCC). American Society of Clinical Oncology.
- FANTUZZI, L., TAGLIAMONTE, M., GAUZZI, M. C. & LOPALCO, L. 2019. Dual CCR5/CCR2 targeting: opportunities for the cure of complex disorders. *Cell Mol Life Sci*, 76, 4869-4886.
- FEEHAN, K. T. & GILROY, D. W. 2019. Is Resolution the End of Inflammation? *Trends Mol Med*, 25, 198-214.
- FENG, Y., BRODER, C. C., KENNEDY, P. E. & BERGER, E. A. 1996. HIV-1 entry cofactor:

functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science*, 272, 872-7.

- FERRIZ, M., VEGA-PEREZ, A., GUTIERREZ-GONZALEZ, A., ALVAREZ-LADRON, N. & ARDAVIN, C. 2023. Whole-mount immunofluorescence imaging and isolation of mesothelium-bound immune cell aggregates during mouse peritoneal inflammation. *STAR Protoc*, 4, 102561.
- FRA, A. M., LOCATI, M., OTERO, K., SIRONI, M., SIGNORELLI, P., MASSARDI, M. L., GOBBI, M., VECCHI, A., SOZZANI, S. & MANTOVANI, A. 2003. Cutting edge: scavenging of inflammatory CC chemokines by the promiscuous putatively silent chemokine receptor D6. *J Immunol*, 170, 2279-82.
- FUKUOKA, A., WILSON, G. J., PITMON, E., KOUMBAS FOLEY, L., JOHNSON, H., PINGEN, M. & GRAHAM, G. J. 2024. Disruption of placental ACKR3 impairs growth and hematopoietic development of offspring. *Development*, 151.
- GALLIERA, E., JALA, V. R., TRENT, J. O., BONECCHI, R., SIGNORELLI, P., LEFKOWITZ, R. J., MANTOVANI, A., LOCATI, M. & HARIBABU, B. 2004. beta-Arrestin-dependent constitutive internalization of the human chemokine decoy receptor D6. *J Biol Chem*, 279, 25590-7.
- GAO, J. L., WYNN, T. A., CHANG, Y., LEE, E. J., BROXMEYER, H. E., COOPER, S., TIFFANY, H. L., WESTPHAL, H., KWON-CHUNG, J. & MURPHY, P. M. 1997. Impaired host defense, hematopoiesis, granulomatous inflammation and type 1-type 2 cytokine balance in mice lacking CC chemokine receptor 1. *J Exp Med*, 185, 1959-68.

- GAO, X., XU, C., ASADA, N. & FRENETTE, P. S. 2018. The hematopoietic stem cell niche: from embryo to adult. *Development*, 145.
- GARDNER, L., PATTERSON, A. M., ASHTON, B. A., STONE, M. A. & MIDDLETON, J. 2004. The human Duffy antigen binds selected inflammatory but not homeostatic chemokines. *Biochem Biophys Res Commun*, 321, 306-12.
- GASPAR, K., KUKOVA, G., BUNEMANN, E., BUHREN, B. A., SONKOLY, E., SZOLLOSI, A. G., MULLER, A., SAVINKO, T., LAUERMA, A. I., ALENIUS, H., KEMENY, L., DIEU-NOSJEAN, M. C., STANDER, S., FISCHER, J. W., RUZICKA, T., ZLOTNIK, A., SZEGEDI, A. & HOMEY, B. 2013. The chemokine receptor CCR3 participates in tissue remodeling during atopic skin inflammation. *J Dermatol Sci*, 71, 12-21.
- GEISSMANN, F., JUNG, S. & LITTMAN, D. R. 2003. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*, 19, 71-82.
- GERRITS, H., SCHENAU, D. S. V. I., BAKKER, N. E. C., VAN DISSELDORP, A. J. M., STRIK, A., HERMENS, L. S., KOENEN, T. B., KRAJNC-FRANKEN, M. A. M. & GOSSEN, J. A. 2008. Early postnatal lethality and cardiovascular defects in CXCR7-deficient mice. *Genesis*, 46, 235-245.
- GHOSH, E. E., CASSADO, A. A., GOVONI, G. R., FUKUHARA, T., YANG, Y., MONACK, D. M., BORTOLUCI, K. R., ALMEIDA, S. R., HERZENBERG, L. A. & HERZENBERG, L. A. 2010. Two physically, functionally, and developmentally distinct peritoneal macrophage subsets. *Proc Natl Acad Sci U S A*, 107, 2568-73.

- GIBBINGS, S. L., THOMAS, S. M., ATIF, S. M., MCCUBBREY, A. L., DESCH, A. N., DANHORN, T., LEACH, S. M., BRATTON, D. L., HENSON, P. M., JANSSEN, W. J. & JAKUBZICK, C. V. 2017. Three Unique Interstitial Macrophages in the Murine Lung at Steady State. *Am J Respir Cell Mol Biol*, 57, 66-76.
- GILLILAND, C. T., SALANGA, C. L., KAWAMURA, T., TREJO, J. & HANDEL, T. M. 2013. The chemokine receptor CCR1 is constitutively active, which leads to G protein-independent, beta-arrestin-mediated internalization. *J Biol Chem*, 288, 32194-32210.
- GINHOUX, F. & JUNG, S. 2014. Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nat Rev Immunol*, 14, 392-404.
- GOMEZ PERDIGUERO, E., KLAPPROTH, K., SCHULZ, C., BUSCH, K., AZZONI, E., CROZET, L., GARNER, H., TROUILLET, C., DE BRUIJN, M. F., GEISSMANN, F. & RODEWALD, H. R. 2015. Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. *Nature*, 518, 547-51.
- GOSLING, J., DAIRAGHI, D. J., WANG, Y., HANLEY, M., TALBOT, D., MIAO, Z. & SCHALL, T. J. 2000. Cutting edge: identification of a novel chemokine receptor that binds dendritic cell- and T cell-active chemokines including ELC, SLC, and TECK. *J Immunol*, 164, 2851-6.
- GRAHAM, G. J., HANDEL, T. M. & PROUDFOOT, A. E. I. 2019. Leukocyte Adhesion: Reconceptualizing Chemokine Presentation by Glycosaminoglycans. *Trends Immunol*, 40, 472-481.

- GRAHAM, G. J., LOCATI, M., MANTOVANI, A., ROT, A. & THELEN, M. 2012. The biochemistry and biology of the atypical chemokine receptors. *Immunology Letters*, 145, 30-38.
- GRAY, A. L., KARLSSON, R., ROBERTS, A. R. E., RIDLEY, A. J. L., PUN, N., KHAN, B., LAWLESS, C., LUIS, R., SZPAKOWSKA, M., CHEVIGNE, A., HUGHES, C. E., MEDINA-RUIZ, L., BIRCHENOUGH, H. L., MULHOLLAND, I. Z., SALANGA, C. L., YATES, E. A., TURNBULL, J. E., HANDEL, T. M., GRAHAM, G. J., JOWITT, T. A., SCHIESSL, I., RICHTER, R. P., MILLER, R. L. & DYER, D. P. 2023. Chemokine CXCL4 interactions with extracellular matrix proteoglycans mediate widespread immune cell recruitment independent of chemokine receptors. *Cell Rep*, 42, 111930.
- GRECH, V. & CALLEJA, N. 2018. WASP (Write a Scientific Paper): Parametric vs. non-parametric tests. *Early Hum Dev*, 123, 48-49.
- GUILLIAMS, M., DE KLEER, I., HENRI, S., POST, S., VANHOUTTE, L., DE PRIJCK, S., DESWARTE, K., MALISSEN, B., HAMMAD, H. & LAMBRECHT, B. N. 2013. Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF. *J Exp Med*, 210, 1977-92.
- GUILLIAMS, M., THIERRY, G. R., BONNARDEL, J. & BAJENOFF, M. 2020. Establishment and Maintenance of the Macrophage Niche. *Immunity*, 52, 434-451.
- GUPTA, S. K. & PILLARISETTI, K. 1999. Cutting edge: CXCR4-Lo: Molecular cloning and functional expression of a novel human CXCR4 splice variant. *Journal of Immunology*, 163, 2368-2372.

- HADLAND, B. & YOSHIMOTO, M. 2018. Many layers of embryonic hematopoiesis: new insights into B-cell ontogeny and the origin of hematopoietic stem cells. *Exp Hematol*, 60, 1-9.
- HANDEL, T. M. & DYER, D. P. 2021. Perspectives on the Biological Role of Chemokine:Glycosaminoglycan Interactions. *J Histochem Cytochem*, 69, 87-91.
- HANNA, J., GOLDMAN-WOHL, D., HAMANI, Y., AVRAHAM, I., GREENFIELD, C., NATANSON-YARON, S., PRUS, D., COHEN-DANIEL, L., ARNON, T. I., MANASTER, I., GAZIT, R., YUTKIN, V., BENHARROCH, D., PORGADOR, A., KESHET, E., YAGEL, S. & MANDELBOIM, O. 2006. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat Med*, 12, 1065-74.
- HANSELL, C. A., SCHIERING, C., KINSTRIE, R., FORD, L., BORDON, Y., MCINNES, I. B., GOODYEAR, C. S. & NIBBS, R. J. 2011. Universal expression and dual function of the atypical chemokine receptor D6 on innate-like B cells in mice. *Blood*, 117, 5413-24.
- HANSELL, C. A. H., FRASER, A. R., HAYES, A. J., PINGEN, M., BURT, C. L., LEE, K. M., MEDINA-RUIZ, L., BROWNLIE, D., MACLEOD, M. K. L., BURGOYNE, P., WILSON, G. J., NIBBS, R. J. B. & GRAHAM, G. J. 2018. The Atypical Chemokine Receptor Ackr2 Constrains NK Cell Migratory Activity and Promotes Metastasis. *J Immunol*, 201, 2510-2519.
- HANSELL, C. A. H., LOVE, S., PINGEN, M., WILSON, G. J., MACLEOD, M. & GRAHAM, G. J. 2020. Analysis of lung stromal expression of the atypical chemokine receptor ACKR2 reveals unanticipated

- expression in murine blood endothelial cells. *Eur J Immunol*, 50, 666-675.
- HASAN, A., REPICI, A., CAPRA, A. P., MANNINO, D., BOVA, V., CATALFAMO, A., CAMPOLO, M., PATERNITI, I., ESPOSITO, E. & ARDIZZONE, A. 2025. CCR1 antagonist as a potential modulator of inflammatory, autophagic, and apoptotic markers in spinal cord injury. *Neuropharmacology*, 264, 110239.
- HASHIMOTO, D., CHOW, A., NOIZAT, C., TEO, P., BEASLEY, M. B., LEBOEUF, M., BECKER, C. D., SEE, P., PRICE, J., LUCAS, D., GRETER, M., MORTHA, A., BOYER, S. W., FORSBERG, E. C., TANAKA, M., VAN ROOIJEN, N., GARCIA-SASTRE, A., STANLEY, E. R., GINHOUX, F., FRENETTE, P. S. & MERAD, M. 2013. Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity*, 38, 792-804.
- HAYES, A. J., PINGEN, M., WILSON, G., HANSELL, C., LOVE, S., BURGOYNE, P., MCELROY, D., BARTOLINI, R., VIDLER, F., SCHUETTE, F., GAMBLE, A., CAMPBELL, J., GALATIS, D., CAMPBELL, J. D. M. & GRAHAM, G. J. 2024. Enhanced CCR2 expression by ACKR2-deficient NK cells increases tumoricidal cell therapy efficacy. *J Leukoc Biol*, 116, 1544-1553.
- HILDYARD, J. C. W., WELLS, D. J. & PIERCY, R. J. 2021. Identification of qPCR reference genes suitable for normalising gene expression in the developing mouse embryo. *Wellcome Open Res*, 6, 197.
- HOEFFEL, G., CHEN, J., LAVIN, Y., LOW, D., ALMEIDA, F. F., SEE, P., BEAUDIN, A. E., LUM, J., LOW, I., FORSBERG, E. C., POIDINGER, M.,

- ZOLEZZI, F., LARBI, A., NG, L. G., CHAN, J. K., GRETER, M., BECHER, B., SAMOKHVALOV, I. M., MERAD, M. & GINHOUX, F. 2015. C-Myb(+) erythro-myeloid progenitor-derived fetal monocytes give rise to adult tissue-resident macrophages. *Immunity*, 42, 665-78.
- HOEFFEL, G., WANG, Y., GRETER, M., SEE, P., TEO, P., MALLERET, B., LEBOEUF, M., LOW, D., OLLER, G., ALMEIDA, F., CHOY, S. H., GRISOTTO, M., RENIA, L., CONWAY, S. J., STANLEY, E. R., CHAN, J. K., NG, L. G., SAMOKHVALOV, I. M., MERAD, M. & GINHOUX, F. 2012. Adult Langerhans cells derive predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac-derived macrophages. *J Exp Med*, 209, 1167-81.
- HOSSAIN, Z., REZA, A., QASEM, W. A., FRIEL, J. K. & OMRI, A. 2022. Development of the immune system in the human embryo. *Pediatr Res*, 92, 951-955.
- HSIEH, C. L., NIEMI, E. C., WANG, S. H., LEE, C. C., BINGHAM, D., ZHANG, J., COZEN, M. L., CHARO, I., HUANG, E. J., LIU, J. & NAKAMURA, M. C. 2014. CCR2 deficiency impairs macrophage infiltration and improves cognitive function after traumatic brain injury. *J Neurotrauma*, 31, 1677-88.
- HU, Y. H., ZHANG, Y., JIANG, L. Q., WANG, S., LEI, C. Q., SUN, M. S., SHU, H. B. & LIU, Y. 2015. WDFY1 mediates TLR3/4 signaling by recruiting TRIF. *EMBO Rep*, 16, 447-55.
- HUANG, L., GEBRESELASSIE, N. G., GAGLIARDO, L. F., RUYECHAN, M. C., LEE, N. A., LEE, J. J. & APPLETON, J. A. 2014. Eosinophil-derived IL-10

- supports chronic nematode infection. *J Immunol*, 193, 4178-87.
- HUANG, Y., PAXTON, W. A., WOLINSKY, S. M., NEUMANN, A. U., ZHANG, L., HE, T., KANG, S., CERADINI, D., JIN, Z., YAZDANBAKHSH, K., KUNSTMAN, K., ERICKSON, D., DRAGON, E., LANDAU, N. R., PHAIR, J., HO, D. D. & KOUP, R. A. 1996. The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. *Nat Med*, 2, 1240-3.
- HUGHES, C. E. & NIBBS, R. J. B. 2018. A guide to chemokines and their receptors. *Febs Journal*, 285, 2944-2971.
- HUISING, M. O., VAN DER MEULEN, T., FLIK, G. & VERBURG-VAN KEMENADE, B. M. 2004. Three novel carp CXC chemokines are expressed early in ontogeny and at nonimmune sites. *Eur J Biochem*, 271, 4094-106.
- HUMBLES, A. A., LU, B., FRIEND, D. S., OKINAGA, S., LORA, J., AL-GARAWI, A., MARTIN, T. R., GERARD, N. P. & GERARD, C. 2002. The murine CCR3 receptor regulates both the role of eosinophils and mast cells in allergen-induced airway inflammation and hyperresponsiveness. *Proc Natl Acad Sci U S A*, 99, 1479-84.
- HUMPERT, M. L., PINTO, D., JARROSSAY, D. & THELEN, M. 2014. CXCR7 influences the migration of B cells during maturation. *Eur J Immunol*, 44, 694-705.
- HUMPERT, M. L., TZOUROS, M., THELEN, S., BIGNON, A., LEVOYE, A., ARENZANA-SEISDEDOS, F., BALABANIAN, K., BACHELERIE, F., LANGEN, H. & THELEN, M. 2012. Complementary methods provide evidence

- for the expression of CXCR7 on human B cells. *Proteomics*, 12, 1938-48.
- HYSONG, M. R., SHUEY, M. M., HUFFMAN, J. E., AUER, P., REINER, A. & RAFFIELD, L. M. 2025. Characterization of the phenotypic consequences of the Duffy-null genotype. *Blood Adv*, 9, 1452-1462.
- INGERSOLL, M. A., SPANBROEK, R., LOTTAZ, C., GAUTIER, E. L., FRANKENBERGER, M., HOFFMANN, R., LANG, R., HANIFFA, M., COLLIN, M., TACKE, F., HABENICHT, A. J., ZIEGLER-HEITBROCK, L. & RANDOLPH, G. J. 2010. Comparison of gene expression profiles between human and mouse monocyte subsets. *Blood*, 115, e10-9.
- INGULLI, E., MONDINO, A., KHORUTS, A. & JENKINS, M. K. 1997. In vivo detection of dendritic cell antigen presentation to CD4(+) T cells. *J Exp Med*, 185, 2133-41.
- ISHII, T., ISHIDA, T., UTSUNOMIYA, A., INAGAKI, A., YANO, H., KOMATSU, H., IIDA, S., IMADA, K., UCHIYAMA, T., AKINAGA, S., SHITARA, K. & UEDA, R. 2010. Defucosylated humanized anti-CCR4 monoclonal antibody KW-0761 as a novel immunotherapeutic agent for adult T-cell leukemia/lymphoma. *Clin Cancer Res*, 16, 1520-31.
- IWASAKI, A. & MEDZHITOV, R. 2015. Control of adaptive immunity by the innate immune system. *Nat Immunol*, 16, 343-53.
- JACOB, T., ANNUSVER, K., CZARNEWSKI, P., DALESSANDRI, T., KALK, C., LEVRA LEVRON, C., CAMPAMA SANZ, N., KASTRITI, M. E., MIKKOLA, M. L., RENDL, M., LICHTENBERGER, B. M., DONATI, G., BJORKLUND, A. K. &

- KASPER, M. 2023. Molecular and spatial landmarks of early mouse skin development. *Dev Cell*, 58, 2140-2162 e5.
- JACOBSEN, E. A., JACKSON, D. J., HEFFLER, E., MATHUR, S. K., BREDENOORD, A. J., PAVORD, I. D., AKUTHOTA, P., ROUFOSSE, F. & ROTHENBERG, M. E. 2021. Eosinophil Knockout Humans: Uncovering the Role of Eosinophils Through Eosinophil-Directed Biological Therapies. *Annu Rev Immunol*, 39, 719-757.
- JAKUBZICK, C., GAUTIER, E. L., GIBBINGS, S. L., SOJKA, D. K., SCHLITZER, A., JOHNSON, T. E., IVANOV, S., DUAN, Q., BALA, S., CONDON, T., VAN ROOIJEN, N., GRAINGER, J. R., BELKAID, Y., MA'AYAN, A., RICHES, D. W., YOKOYAMA, W. M., GINHOUX, F., HENSON, P. M. & RANDOLPH, G. J. 2013. Minimal differentiation of classical monocytes as they survey steady-state tissues and transport antigen to lymph nodes. *Immunity*, 39, 599-610.
- JAKUBZICK, C. V., RANDOLPH, G. J. & HENSON, P. M. 2017. Monocyte differentiation and antigen-presenting functions. *Nat Rev Immunol*, 17, 349-362.
- JAMES, K. D., LEGLER, D. F., PURVANOV, V., OHIGASHI, I., TAKAHAMA, Y., PARNELL, S. M., WHITE, A. J., JENKINSON, W. E. & ANDERSON, G. 2021. Medullary stromal cells synergize their production and capture of CCL21 for T-cell emigration from neonatal mouse thymus. *Blood Adv*, 5, 99-112.
- JAMIESON, T., COOK, D. N., NIBBS, R. J., ROT, A., NIXON, C., MCLEAN, P., ALCAMI, A., LIRA, S. A., WIEKOWSKI, M. & GRAHAM, G. J. 2005. The

- chemokine receptor D6 limits the inflammatory response in vivo. *Nat Immunol*, 6, 403-11.
- JANSSENS, R., MORTIER, A., BOFF, D., RUYTINX, P., GOUWY, M., VANTILT, B., LARSEN, O., DAUGVILAITE, V., ROSENKILDE, M. M., PARMENTIER, M., NOPPEN, S., LIEKENS, S., VAN DAMME, J., STRUYF, S., TEIXEIRA, M. M., AMARAL, F. A. & PROOST, P. 2017. Truncation of CXCL12 by CD26 reduces its CXC chemokine receptor 4- and atypical chemokine receptor 3-dependent activity on endothelial cells and lymphocytes. *Biochem Pharmacol*, 132, 92-101.
- JENKINS, B. D., MARTINI, R. N., HIRE, R., BROWN, A., BENNETT, B., BROWN, I., HOWERTH, E. W., EGAN, M., HODGSON, J., YATES, C., KITTLES, R., CHITALE, D., ALI, H., NATHANSON, D., NIKOLINAKOS, P., NEWMAN, L., MONTEIL, M. & DAVIS, M. B. 2019. Atypical Chemokine Receptor 1 (DARC/ACKR1) in Breast Tumors Is Associated with Survival, Circulating Chemokines, Tumor-Infiltrating Immune Cells, and African Ancestry. *Cancer Epidemiol Biomarkers Prev*, 28, 690-700.
- JI, R. P., PHOON, C. K., ARISTIZABAL, O., MCGRATH, K. E., PALIS, J. & TURNBULL, D. H. 2003. Onset of cardiac function during early mouse embryogenesis coincides with entry of primitive erythroblasts into the embryo proper. *Circ Res*, 92, 133-5.
- JOHNSTON, L. K., HSU, C. L., KRIER-BURRIS, R. A., CHHIBA, K. D., CHIEN, K. B., MCKENZIE, A., BERDNIKOV, S. & BRYCE, P. J. 2016. IL-33 Precedes IL-5 in Regulating Eosinophil Commitment and Is Required for Eosinophil Homeostasis. *J Immunol*, 197, 3445-3453.

- KAESLER, S., REGENBOGEN, J., DURKA, S., GOPPELT, A. & WERNER, S. 2002. The healing skin wound: a novel site of action of the chemokine C10. *Cytokine*, 17, 157-63.
- KANY, S., VOLLRATH, J. T. & RELJA, B. 2019. Cytokines in Inflammatory Disease. *Int J Mol Sci*, 20.
- KARA, E. E., BASTOW, C. R., MCKENZIE, D. R., GREGOR, C. E., FENIX, K. A., BABB, R., NORTON, T. S., ZOTOS, D., RODDA, L. B., HERMES, J. R., BOURNE, K., GILCHRIST, D. S., NIBBS, R. J., ALSHARIFI, M., VINUESA, C. G., TARLINTON, D. M., BRINK, R., HILL, G. R., CYSTER, J. G., COMERFORD, I. & MCCOLL, S. R. 2018. Atypical chemokine receptor 4 shapes activated B cell fate. *Journal of Experimental Medicine*, 215, 801-813.
- KAWANO, H., KAYAMA, H., NAKAMA, T., HASHIMOTO, T., UMEMOTO, E. & TAKEDA, K. 2016. IL-10-producing lung interstitial macrophages prevent neutrophilic asthma. *Int Immunol*, 28, 489-501.
- KERSTJENS, H. A., BJERMER, L., ERIKSSON, L., DAHLSTROM, K. & VESTBO, J. 2010. Tolerability and efficacy of inhaled AZD4818, a CCR1 antagonist, in moderate to severe COPD patients. *Respir Med*, 104, 1297-303.
- KHAN, I. A., MURPHY, P. M., CASCIOTTI, L., SCHWARTZMAN, J. D., COLLINS, J., GAO, J. L. & YEAMAN, G. R. 2001. Mice lacking the chemokine receptor CCR1 show increased susceptibility to *Toxoplasma gondii* infection. *J Immunol*, 166, 1930-7.
- KIDOYA, H., NAITO, H., MURAMATSU, F., YAMAKAWA, D., JIA, W., IKAWA, M., SONOBE,

- T., TSUCHIMOCHI, H., SHIRAI, M., ADAMS, R. H., FUKAMIZU, A. & TAKAKURA, N. 2015. APJ Regulates Parallel Alignment of Arteries and Veins in the Skin. *Dev Cell*, 33, 247-59.
- KIM, K. W., WILLIAMS, J. W., WANG, Y. T., IVANOV, S., GILFILLAN, S., COLONNA, M., VIRGIN, H. W., GAUTIER, E. L. & RANDOLPH, G. J. 2016. MHC II⁺ resident peritoneal and pleural macrophages rely on IRF4 for development from circulating monocytes. *J Exp Med*, 213, 1951-9.
- KINOSHITA, M., UCHIDA, T., SATO, A., NAKASHIMA, M., NAKASHIMA, H., SHONO, S., HABU, Y., MIYAZAKI, H., HIROI, S. & SEKI, S. 2010. Characterization of two F4/80-positive Kupffer cell subsets by their function and phenotype in mice. *J Hepatol*, 53, 903-10.
- KITAMURA, T., FUJISHITA, T., LOETSCHER, P., REVESZ, L., HASHIDA, H., KIZAKA-KONDOH, S., AOKI, M. & TAKETO, M. M. 2010. Inactivation of chemokine (C-C motif) receptor 1 (CCR1) suppresses colon cancer liver metastasis by blocking accumulation of immature myeloid cells in a mouse model. *Proc Natl Acad Sci U S A*, 107, 13063-8.
- KITAURA, M., NAKAJIMA, T., IMAI, T., HARADA, S., COMBADIÈRE, C., TIFFANY, H. L., MURPHY, P. M. & YOSHIE, O. 1996. Molecular cloning of human eotaxin, an eosinophil-selective CC chemokine, and identification of a specific eosinophil eotaxin receptor, CC chemokine receptor 3. *J Biol Chem*, 271, 7725-30.
- LAM, N., LEE, Y. & FARBER, D. L. 2024. A guide to adaptive immune memory. *Nat Rev Immunol*, 24, 810-829.

- LAMMERMAN, T., AFONSO, P. V., ANGERMANN, B. R., WANG, J. M., KASTENMULLER, W., PARENT, C. A. & GERMAIN, R. N. 2013. Neutrophil swarms require LTB₄ and integrins at sites of cell death in vivo. *Nature*, 498, 371-5.
- LASAGNI, L., FRANICALANCI, M., ANNUNZIATO, F., LAZZERI, E., GIANNINI, S., COSMI, L., SAGRINATI, C., MAZZINGHI, B., ORLANDO, C., MAGGI, E., MARRA, F., ROMAGNANI, S., SERIO, M. & ROMAGNANI, P. 2003. An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as functional receptor for platelet factor 4. *Journal of Experimental Medicine*, 197, 1537-1549.
- LAZENNEC, G., RAJARATHNAM, K. & RICHMOND, A. 2024. CXCR2 chemokine receptor - a master regulator in cancer and physiology. *Trends Mol Med*, 30, 37-55.
- LE, K., SUN, J., GHAEMMAGHAMI, J., SMITH, M. R., IP, W. K. E., PHILLIPS, T. & GUPTA, M. 2023. Blockade of CCR1 induces a phenotypic shift in macrophages and triggers a favorable antilymphoma activity. *Blood Adv*, 7, 3952-3967.
- LE MERCIER, A., BONNAVION, R., YU, W. J., ALNOURI, M. W., RAMAS, S., ZHANG, Y., JÄGER, Y., ROQUID, K. A., JEONG, H. W., SIVARAJ, K. K., CHO, H., CHEN, X. Y., STRILIC, B., SIJMONSMA, T., ADAMS, R., SCHROEDER, T., RIEGER, M. A. & OFFEMANNS, S. 2021. GPR182 is an endothelium-specific atypical chemokine receptor that maintains hematopoietic stem cell homeostasis. *Proceedings of the National Academy of Sciences of the United States of America*, 118.

- LEE, K. M., DANUSER, R., STEIN, J. V., GRAHAM, D., NIBBS, R. J. & GRAHAM, G. J. 2014. The chemokine receptors ACKR2 and CCR2 reciprocally regulate lymphatic vessel density. *EMBO J*, 33, 2564-80.
- LEE, K. M., WILSON, G. J., PINGEN, M., FUKUOKA, A., HANSELL, C. A. H., BARTOLINI, R., MEDINA-RUIZ, L. & GRAHAM, G. J. 2019. Placental chemokine compartmentalisation: A novel mammalian molecular control mechanism. *PLoS Biol*, 17, e3000287.
- LEGLER, D. F. & THELEN, M. 2018. New insights in chemokine signaling. *F1000Res*, 7, 95.
- LEWIS, N. D., MUTHUKUMARANA, A., FOGAL, S. E., CORRADINI, L., STEFANOPOULOS, D. E., ADUSUMALLI, P., PELLETIER, J., PANZENBECK, M., BERG, K., CANFIELD, M., COOK, B. N., RAZAVI, H., KUZMICH, D., ANDERSON, S., ALLARD, D., HARRISON, P., GRIMALDI, C., SOUZA, D., HARCKEN, C., FRYER, R. M., MODIS, L. K. & BROWN, M. L. 2014. CCR1 plays a critical role in modulating pain through hematopoietic and non-hematopoietic cells. *PLoS One*, 9, e105883.
- LEY, K., LAUDANNA, C., CYBULSKY, M. I. & NOURSHARGH, S. 2007. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol*, 7, 678-89.
- LI, D. D., YANG, C., SHAO, Z. M. & YU, K. D. 2018. Effect of functional genetic variants in chemokine decoy receptors on the recurrence risk of breast cancer. *Cancer Med*, 7, 5497-5504.
- LI, J., YAO, Z., LIU, X., DUAN, R., YI, X., AYOUB, A., SANDERS, J. O., MESFIN, A., XING, L. & BOYCE, B. F. 2023. TGFbeta1(+)CCR5(+)

neutrophil subset increases in bone marrow and causes age-related osteoporosis in male mice. *Nat Commun*, 14, 159.

- LI, X., MARA, A. B., MUSIAL, S. C., KOLLING, F. W., GIBBINGS, S. L., GEREBTSOV, N. & JAKUBZICK, C. V. 2024. Coordinated chemokine expression defines macrophage subsets across tissues. *Nat Immunol*, 25, 1110-1122.
- LI, X., YAO, W., YUAN, Y., CHEN, P., LI, B., LI, J., CHU, R., SONG, H., XIE, D., JIANG, X. & WANG, H. 2017. Targeting of tumour-infiltrating macrophages via CCL2/CCR2 signalling as a therapeutic strategy against hepatocellular carcinoma. *Gut*, 66, 157-167.
- LIANG, M., LV, J., JIANG, Z., HE, H., CHEN, C., XIONG, Y., ZHU, X., XUE, Y., YU, Y., YANG, S., WANG, L., LI, W., GUAN, M., WAN, W., HE, R. & ZOU, H. 2020. Promotion of Myofibroblast Differentiation and Tissue Fibrosis by the Leukotriene B(4) -Leukotriene B(4) Receptor Axis in Systemic Sclerosis. *Arthritis Rheumatol*, 72, 1013-1025.
- LIEBICK, M., SCHLÄGER, C. & OPPERMANN, M. 2016. Analysis of Chemokine Receptor Trafficking by Site-Specific Biotinylation. *Plos One*, 11.
- LIM, J. K., MCDERMOTT, D. H., LISCO, A., FOSTER, G. A., KRYSZTOF, D., FOLLMANN, D., STRAMER, S. L. & MURPHY, P. M. 2010. CCR5 deficiency is a risk factor for early clinical manifestations of West Nile virus infection but not for viral transmission. *J Infect Dis*, 201, 178-85.
- LIOU, L. B., FANG, Y. F., TAN, C. F., LAI, J. H., JANG, S. S., TSAI, P. H. & YEH, T. C. 2020. A new laboratory surrogate (Monocyte Chemotactic Protein-1) for Disease Activity Score₂₈: a

- favourable indicator for remission in rheumatoid arthritis. *Sci Rep*, 10, 8238.
- LIU, M., CAO, S., HE, L., GAO, J., ARAB, J. P., CUI, H., XUAN, W., GAO, Y., SEHRAWAT, T. S., HAMDAN, F. H., VENTURA-COTS, M., ARGEMI, J., POMERANTZ, W. C. K., JOHNSEN, S. A., LEE, J. H., GAO, F., ORDOG, T., MATHURIN, P., REVZIN, A., BATALLER, R., YAN, H. & SHAH, V. H. 2021a. Super enhancer regulation of cytokine-induced chemokine production in alcoholic hepatitis. *Nat Commun*, 12, 4560.
- LIU, M. Y., SILVA-SANCHEZ, A., RANDALL, T. D. & MEZA-PEREZ, S. 2021b. Specialized immune responses in the peritoneal cavity and omentum. *Journal of Leukocyte Biology*, 109, 717-729.
- LIU, R., PAXTON, W. A., CHOE, S., CERADINI, D., MARTIN, S. R., HORUK, R., MACDONALD, M. E., STUHLMANN, H., KOUP, R. A. & LANDAU, N. R. 1996. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell*, 86, 367-77.
- LIU, Z., GU, Y., CHAKAROV, S., BLERIOT, C., KWOK, I., CHEN, X., SHIN, A., HUANG, W., DRESS, R. J., DUTERTRE, C. A., SCHLITZER, A., CHEN, J., NG, L. G., WANG, H., LIU, Z., SU, B. & GINHOUX, F. 2019. Fate Mapping via Ms4a3-Expression History Traces Monocyte-Derived Cells. *Cell*, 178, 1509-1525 e19.
- LOFFREDO, L. F., CODEN, M. E., JEONG, B. M., WALKER, M. T., ANEKALLA, K. R., DOAN, T. C., RODRIGUEZ, R., BROWNING, M., NAM, K., LEE, J. J., ABDALA-VALENCIA, H. & BERDNIKOV, S. 2020. Eosinophil accumulation in postnatal lung is specific to the primary septation phase of development. *Sci Rep*, 10, 4425.

- LOUWE, P. A., GOMEZ, L. B., WEBSTER, H., PERONA-WRIGHT, G., BAIN, C. C., FORBES, S. J. & JENKINS, S. J. 2021. Recruited macrophages that colonize the post-inflammatory peritoneal niche convert into functionally divergent resident cells. *Nature Communications*, 12.
- LUO, H., CHAUDHURI, A., ZBRZEZNA, V., HE, Y. & POGO, A. O. 2000. Deletion of the murine Duffy gene (Dfy) reveals that the Duffy receptor is functionally redundant. *Mol Cell Biol*, 20, 3097-101.
- MACHIELS, B., DOURCY, M., XIAO, X., JAVAUX, J., MESNIL, C., SABATEL, C., DESMECHT, D., LALLEMAND, F., MARTINIVE, P., HAMMAD, H., GUILLIAMS, M., DEWALS, B., VANDERPLASSCHEN, A., LAMBRECHT, B. N., BUREAU, F. & GILLET, L. 2018. Author Correction: A gammaherpesvirus provides protection against allergic asthma by inducing the replacement of resident alveolar macrophages with regulatory monocytes. *Nat Immunol*, 19, 1035.
- MADIGAN, J., FREEMAN, D. J., MENZIES, F., FORROW, S., NELSON, S. M., YOUNG, A., SHARKEY, A., MOFFETT, A., GRAHAM, G. J., GREER, I. A., ROT, A. & NIBBS, R. J. 2010. Chemokine scavenger D6 is expressed by trophoblasts and aids the survival of mouse embryos transferred into allogeneic recipients. *J Immunol*, 184, 3202-12.
- MANIK, M. & SINGH, R. K. 2022. Role of toll-like receptors in modulation of cytokine storm signaling in SARS-CoV-2-induced COVID-19. *J Med Virol*, 94, 869-877.

- MARION, C. R., WANG, J., SHARMA, L., LOSIER, A., LUI, W., ANDREWS, N., ELIAS, J. A., KAZMIERCZAK, B. I., ROY, C. R. & DELA CRUZ, C. S. 2016. Chitinase 3-Like 1 (Chil1) Regulates Survival and Macrophage-Mediated Interleukin-1 β and Tumor Necrosis Factor Alpha during *Pseudomonas aeruginosa* Pneumonia. *Infect Immun*, 84, 2094-2104.
- MARSHALL, J. S., WARRINGTON, R., WATSON, W. & KIM, H. L. 2018. An introduction to immunology and immunopathology. *Allergy Asthma Clin Immunol*, 14, 49.
- MARTINEZ DE LA TORRE, Y., BURACCHI, C., BORRONI, E. M., DUPOR, J., BONECCHI, R., NEBULONI, M., PASQUALINI, F., DONI, A., LAURI, E., AGOSTINIS, C., BULLA, R., COOK, D. N., HARIBABU, B., MERONI, P., RUKAVINA, D., VAGO, L., TEDESCO, F., VECCHI, A., LIRA, S. A., LOCATI, M. & MANTOVANI, A. 2007. Protection against inflammation- and autoantibody-caused fetal loss by the chemokine decoy receptor D6. *Proc Natl Acad Sci U S A*, 104, 2319-24.
- MARTINEZ DE LA TORRE, Y., LOCATI, M., BURACCHI, C., DUPOR, J., COOK, D. N., BONECCHI, R., NEBULONI, M., RUKAVINA, D., VAGO, L., VECCHI, A., LIRA, S. A. & MANTOVANI, A. 2005. Increased inflammation in mice deficient for the chemokine decoy receptor D6. *Eur J Immunol*, 35, 1342-6.
- MARUYAMA, K., KIDOYA, H., TAKEMURA, N., SUGISAWA, E., TAKEUCHI, O., KONDO, T., EID, M. M. A., TANAKA, H., MARTINO, M. M., TAKAKURA, N., TAKAYAMA, Y., AKIRA, S., VANDENBON, A. & KUMAGAI, Y. 2020. Zinc

Finger Protein St18 Protects against Septic Death by Inhibiting VEGF-A from Macrophages. *Cell Rep*, 32, 107906.

- MASS, E., BALLESTEROS, I., FARLIK, M., HALBRITTER, F., GUNTHER, P., CROZET, L., JACOME-GALARZA, C. E., HANDLER, K., KLUGHAMMER, J., KOBAYASHI, Y., GOMEZ-PERDIGUERO, E., SCHULTZE, J. L., BEYER, M., BOCK, C. & GEISSMANN, F. 2016. Specification of tissue-resident macrophages during organogenesis. *Science*, 353.
- MASS, E., NIMMERJAHN, F., KIERDORF, K. & SCHLITZER, A. 2023. Tissue-specific macrophages: how they develop and choreograph tissue biology. *Nat Rev Immunol*, 23, 563-579.
- MASUI, H., KAWADA, K., ITATANI, Y., HIRAI, H., NAKANISHI, Y., KIYASU, Y., HANADA, K., OKAMOTO, M., HIRATA, W., NISHIKAWA, Y., SUGIMOTO, N., TAMURA, T., SAKAI, Y. & OBAMA, K. 2024. Synergistic antitumor activity by dual blockade of CCR1 and CXCR2 expressed on myeloid cells within the tumor microenvironment. *Br J Cancer*, 131, 63-76.
- MATTI, C., D'UONNOLO, G., ARTINGER, M., MELGRATI, S., SALNIKOV, A., THELEN, S., PURVANOV, V., STROBEL, T. D., SPANNAGEL, L., THELEN, M. & LEGLER, D. F. 2020a. CCL20 is a novel ligand for the scavenging atypical chemokine receptor 4. *Journal of Leukocyte Biology*, 107, 1137-1154.
- MATTI, C., SALNIKOV, A., ARTINGER, M., D'AGOSTINO, G., KINDINGER, I., UGUCCIONI, M., THELEN, M. & LEGLER, D. F. 2020b. ACKR4 Recruits GRK3 Prior to β -Arrestins but Can

- Scavenge Chemokines in the Absence of β -Arrestins. *Frontiers in Immunology*, 11.
- MAZZINGHI, B., RONCONI, E., LAZZERI, E., SAGRINATI, C., BALLERINI, L., ANGELOTTI, M. L., PARENTE, E., MANCINA, R., NETTI, G. S., BECHERUCCI, F., GACCI, M., CARINI, M., GESUALDO, L., ROTONDI, M., MAGGI, E., LASAGNI, L., SERIO, M., ROMAGNANI, S. & ROMAGNANI, P. 2008. Essential but differential role for CXCR4 and CXCR7 in the therapeutic homing of human renal progenitor cells. *J Exp Med*, 205, 479-90.
- MCEVER, R. P. & CUMMINGS, R. D. 1997. Role of PSGL-1 binding to selectins in leukocyte recruitment. *J Clin Invest*, 100, S97-103.
- MCGRATH, K. E., FRAME, J. M., FEGAN, K. H., BOWEN, J. R., CONWAY, S. J., CATHERMAN, S. C., KINGSLEY, P. D., KONISKI, A. D. & PALIS, J. 2015. Distinct Sources of Hematopoietic Progenitors Emerge before HSCs and Provide Functional Blood Cells in the Mammalian Embryo. *Cell Rep*, 11, 1892-904.
- MCKIMMIE, C. S., SINGH, M. D., HEWIT, K., LOPEZ-FRANCO, O., LE BROCCQ, M., ROSE-JOHN, S., LEE, K. M., BAKER, A. H., WHEAT, R., BLACKBOURN, D. J., NIBBS, R. J. & GRAHAM, G. J. 2013. An analysis of the function and expression of D6 on lymphatic endothelial cells. *Blood*, 121, 3768-77.
- MEDINA-RUIZ, L., BARTOLINI, R., MATHIE, H., HALAWA, H. A., CUNNINGHAM, M. & GRAHAM, G. J. 2024. CCR1 and CCR2 Coexpression on Monocytes Is Nonredundant and Delineates a Distinct Monocyte Subpopulation. *J Immunol*, 213, 214-225.

- MEDINA-RUIZ, L., BARTOLINI, R., WILSON, G. J., DYER, D. P., VIDLER, F., HUGHES, C. E., SCHUETTE, F., LOVE, S., PINGEN, M., HAYES, A. J., FU, J., STEWART, A. F. & GRAHAM, G. J. 2022. Analysis of combinatorial chemokine receptor expression dynamics using multi-receptor reporter mice. *Elife*, 11.
- MELGRATI, S., GERKEN, O. J., ARTINGER, M., RADICE, E., SZPAKOWSKA, M., CHEVIGNE, A., D'UONNOLO, G., ANTONELLO, P., THELEN, S., PELCZAR, P., LEGLER, D. F. & THELEN, M. 2023. GPR182 is a broadly scavenging atypical chemokine receptor influencing T-independent immunity. *Front Immunol*, 14, 1242531.
- METZEMAEEKERS, M., MORTIER, A., JANSSENS, R., BOFF, D., VANBRABANT, L., LAMOEN, N., VAN DAMME, J., TEIXEIRA, M. M., DE MEESTER, I., AMARAL, F. A. & PROOST, P. 2017. Glycosaminoglycans Regulate CXCR3 Ligands at Distinct Levels: Protection against Processing by Dipeptidyl Peptidase IV/CD26 and Interference with Receptor Signaling. *Int J Mol Sci*, 18.
- MEYRATH, M., REYNDERS, N., UCHANSKI, T., CHEVIGNE, A. & SZPAKOWSKA, M. 2021. Systematic reassessment of chemokine-receptor pairings confirms CCL20 but not CXCL13 and extends the spectrum of ACKR4 agonists to CCL22. *J Leukoc Biol*, 109, 373-376.
- MEYRATH, M., SZPAKOWSKA, M., ZEINER, J., MASSOTTE, L., MERZ, M. P., BENKEL, T., SIMON, K., OHNMACHT, J., TURNER, J. D., KRÜGER, R., SEUTIN, V., OLLERT, M., KOSTENIS, E. & CHEVIGNÉ, A. 2020. The atypical chemokine receptor ACKR3/CXCR7 is a

- broad-spectrum scavenger for opioid peptides. *Nature Communications*, 11.
- MICHALCZYK, M., CELEWICZ, A., CELEWICZ, M., WOZNIAKOWSKA-GONDEK, P. & RZEPKA, R. 2020. The Role of Inflammation in the Pathogenesis of Preeclampsia. *Mediators Inflamm*, 2020, 3864941.
- MILLER, M. C. & MAYO, K. H. 2017. Chemokines from a Structural Perspective. *International Journal of Molecular Sciences*, 18.
- MITROULIS, I., ALEXAKI, V. I., KOURTZELIS, I., ZIOGAS, A., HAJISHENGALLIS, G. & CHAVAKIS, T. 2015. Leukocyte integrins: role in leukocyte recruitment and as therapeutic targets in inflammatory disease. *Pharmacol Ther*, 147, 123-135.
- MONTECUCCO, F., MACH, F., LENGLET, S., VONLAUFEN, A., GOMES QUINDERE, A. L., PELLI, G., BURGER, F., GALAN, K., DALLEGRI, F., CARBONE, F., PROUDFOOT, A. E., VUILLEUMIER, N. & FROSSARD, J. L. 2014. Treatment with Evasin-3 abrogates neutrophil-mediated inflammation in mouse acute pancreatitis. *Eur J Clin Invest*, 44, 940-50.
- MOSER, B., WOLF, M., WALZ, A. & LOETSCHER, P. 2004. Chemokines: multiple levels of leukocyte migration control. *Trends in Immunology*, 25, 75-84.
- MOSSER, D. M., HAMIDZADEH, K. & GONCALVES, R. 2021. Macrophages and the maintenance of homeostasis. *Cell Mol Immunol*, 18, 579-587.
- MU, T., XU, L., ZHONG, Y., LIU, X., ZHAO, Z., HUANG, C., LAN, X., LUFELI, C., ZHOU, Y., SU, Y., XU, L., JIANG, M., ZHOU, H., LIN, X., WU, L., PENG, S., LIU, S., BRIX, S., DEAN, M., DUNN, N.

- R., ZARET, K. S., FU, X. Y. & HOU, Y. 2020. Embryonic liver developmental trajectory revealed by single-cell RNA sequencing in the *Foxa2*(eGFP) mouse. *Commun Biol*, 3, 642.
- NAGASAWA, T., HIROTA, S., TACHIBANA, K., TAKAKURA, N., NISHIKAWA, S., KITAMURA, Y., YOSHIDA, N., KIKUTANI, H. & KISHIMOTO, T. 1996. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature*, 382, 635-8.
- NEMETH, T., SPERANDIO, M. & MOCSAI, A. 2020. Neutrophils as emerging therapeutic targets. *Nat Rev Drug Discov*, 19, 253-275.
- NEO, W. H., LIE, A. L. M., FADLULLAH, M. Z. H. & LACAUD, G. 2021. Contributions of Embryonic HSC-Independent Hematopoiesis to Organogenesis and the Adult Hematopoietic System. *Front Cell Dev Biol*, 9, 631699.
- NEVES, M., FUMAGALLI, A., VAN DEN BOR, J., MARIN, P., SMIT, M. J. & MAYOR, F. 2019. The Role of ACKR3 in Breast, Lung, and Brain Cancer. *Mol Pharmacol*, 96, 819-825.
- NGAMSRI, K. C., MULLER, A., BOSMULLER, H., GAMPER-TSIGARAS, J., REUTERSHAN, J. & KONRAD, F. M. 2017. The Pivotal Role of CXCR7 in Stabilization of the Pulmonary Epithelial Barrier in Acute Pulmonary Inflammation. *J Immunol*, 198, 2403-2413.
- NGUYEN, A. F., KUO, N. W., SHOWALTER, L. J., RAMOS, R., DUPUREUR, C. M., COLVIN, M. E. & LIWANG, P. J. 2017a. Biophysical and Computational Studies of the vCCI:vMIP-II Complex. *Int J Mol Sci*, 18.
- NGUYEN, A. F., M, S. S., JIAN, M. & P, J. L. 2017b. The Effect of N-Terminal Cyclization on the

Function of the HIV Entry Inhibitor 5P12-RANTES.
Int J Mol Sci, 18.

- NGUYEN, H. H., TRAN, B. T., MULLER, W. & JACK, R. S. 2012. IL-10 acts as a developmental switch guiding monocyte differentiation to macrophages during a murine peritoneal infection. *J Immunol*, 189, 3112-20.
- NIBBS, R., GRAHAM, G. & ROT, A. 2003. Chemokines on the move: control by the chemokine "interceptors" Duffy blood group antigen and D6. *Seminars in Immunology*, 15, 287-294.
- NIBBS, R. J., GILCHRIST, D. S., KING, V., FERRA, A., FORROW, S., HUNTER, K. D. & GRAHAM, G. J. 2007. The atypical chemokine receptor D6 suppresses the development of chemically induced skin tumors. *J Clin Invest*, 117, 1884-92.
- NIBBS, R. J., KRIEHLER, E., PONATH, P. D., PARENT, D., QIN, S., CAMPBELL, J. D., HENDERSON, A., KERJASCHKI, D., MAURER, D., GRAHAM, G. J. & ROT, A. 2001. The beta-chemokine receptor D6 is expressed by lymphatic endothelium and a subset of vascular tumors. *Am J Pathol*, 158, 867-77.
- NIBBS, R. J., WYLIE, S. M., YANG, J., LANDAU, N. R. & GRAHAM, G. J. 1997. Cloning and characterization of a novel promiscuous human beta-chemokine receptor D6. *J Biol Chem*, 272, 32078-83.
- NIBBS, R. J. B. & GRAHAM, G. J. 2013. Immune regulation by atypical chemokine receptors. *Nature Reviews Immunology*, 13, 815-829.
- NOMIYAMA, H., OSADA, N. & YOSHIE, O. 2011a. A family tree of vertebrate chemokine receptors for a unified nomenclature. *Developmental and Comparative Immunology*, 35, 705-715.

- NOMIYAMA, H., OSADA, N. & YOSHIE, O. 2011b. A family tree of vertebrate chemokine receptors for a unified nomenclature. *Dev Comp Immunol*, 35, 705-15.
- NOURSHARGH, S. & ALON, R. 2014. Leukocyte migration into inflamed tissues. *Immunity*, 41, 694-707.
- OGILVIE, P., BARDI, G., CLARK-LEWIS, I., BAGGIOLINI, M. & UGUCCIONI, M. 2001. Eotaxin is a natural antagonist for CCR2 and an agonist for CCR5. *Blood*, 97, 1920-4.
- OJEDA-FERNANDEZ, L., RECIO-POVEDA, L., ARISTORENA, M., LASTRES, P., BLANCO, F. J., SANZ-RODRIGUEZ, F., GALLARDO-VARA, E., DE LAS CASAS-ENGEL, M., CORBI, A., ARTHUR, H. M., BERNABEU, C. & BOTELLA, L. M. 2016. Mice Lacking Endoglin in Macrophages Show an Impaired Immune Response. *PLoS Genet*, 12, e1005935.
- OKABE, Y. & MEDZHITOV, R. 2014. Tissue-specific signals control reversible program of localization and functional polarization of macrophages. *Cell*, 157, 832-44.
- PACIFICO, F., LEPORE, A., MELLONE, S., SANGUIGNO, L., FEDERICO, G., GRECO, A., BRUNETTI, A. & LEONARDI, A. 2017. The chemokine scavenging receptor D6/ACKR2 is a target of miR-146a in thyroid cancer. *Genes Cancer*, 8, 577-588.
- PATEL, A. A., ZHANG, Y., FULLERTON, J. N., BOELEN, L., RONGVAUX, A., MAINI, A. A., BIGLEY, V., FLAVELL, R. A., GILROY, D. W., ASQUITH, B., MACALLAN, D. & YONA, S. 2017. The fate and lifespan of human monocyte subsets

- in steady state and systemic inflammation. *J Exp Med*, 214, 1913-1923.
- PHAM, C. T. 2006. Neutrophil serine proteases: specific regulators of inflammation. *Nat Rev Immunol*, 6, 541-50.
- PLAKS, V., BIRNBERG, T., BERKUTZKI, T., SELA, S., BENYASHAR, A., KALCHENKO, V., MOR, G., KESHET, E., DEKEL, N., NEEMAN, M. & JUNG, S. 2008. Uterine DCs are crucial for decidua formation during embryo implantation in mice. *J Clin Invest*, 118, 3954-65.
- PRADEU, T., THOMMA, B., GIRARDIN, S. E. & LEMAITRE, B. 2024. The conceptual foundations of innate immunity: Taking stock 30 years later. *Immunity*, 57, 613-631.
- PRINS, M., DUTTA, R., BASELMANS, B., BREVE, J. J., BOL, J. G., DECKARD, S. A., VAN DER VALK, P., AMOR, S., TRAPP, B. D., DE VRIES, H. E., DRUKARCH, B. & VAN DAM, A. M. 2014. Discrepancy in CCL2 and CCR2 expression in white versus grey matter hippocampal lesions of Multiple Sclerosis patients. *Acta Neuropathol Commun*, 2, 98.
- PROUDFOOT, A. E., HANDEL, T. M., JOHNSON, Z., LAU, E. K., LIWANG, P., CLARK-LEWIS, I., BORLAT, F., WELLS, T. N. & KOSCO-VILBOIS, M. H. 2003a. Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proc Natl Acad Sci U S A*, 100, 1885-90.
- PROUDFOOT, A. E. I., HANDEL, T. M., JOHNSON, Z., LAU, E. K., LIWANG, P., CLARK-LEWIS, I., BORLAT, F., WELLS, T. N. C. & KOSCO-VILBOIS, M. H. 2003b. Glycosaminoglycan binding and oligomerization are essential for the

- activity of certain chemokines. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 1885-1890.
- PROUDFOOT, A. E. I., JOHNSON, Z., BONVIN, P. & HANDEL, T. M. 2017. Glycosaminoglycan Interactions with Chemokines Add Complexity to a Complex System. *Pharmaceuticals (Basel)*, 10.
- PUXEDDU, I., BADER, R., PILIPONSKY, A. M., REICH, R., LEVI-SCHAFFER, F. & BERKMAN, N. 2006. The CC chemokine eotaxin/CCL11 has a selective profibrogenic effect on human lung fibroblasts. *J Allergy Clin Immunol*, 117, 103-10.
- QUINN, K. E., MATSON, B. C. & CARON, K. M. 2020. Deletion of atypical chemokine receptor 3 (ACKR3) increases immune cells at the fetal-maternal interface. *Placenta*, 95, 18-25.
- RADICE, E., AMETI, R., MELGRATI, S., FOGLIERINI, M., ANTONELLO, P., STAHL, R. A. K., THELEN, S., JARROSSAY, D. & THELEN, M. 2020. Marginal Zone Formation Requires ACKR3 Expression on B Cells. *Cell Rep*, 32, 107951.
- RAMOS, C., GERAKOPOULOS, V. & OEHLER, R. 2024. Metastasis-associated fibroblasts in peritoneal surface malignancies. *Br J Cancer*, 131, 407-419.
- REICH, D., NALLS, M. A., KAO, W. H., AKYLBKOV, E. L., TANDON, A., PATTERSON, N., MULLIKIN, J., HSUEH, W. C., CHENG, C. Y., CORESH, J., BOERWINKLE, E., LI, M., WALISZEWSKA, A., NEUBAUER, J., LI, R., LEAK, T. S., EKUNWE, L., FILES, J. C., HARDY, C. L., ZMUDA, J. M., TAYLOR, H. A., ZIV, E., HARRIS, T. B. & WILSON, J. G. 2009. Reduced neutrophil count in people of African descent is due to a regulatory

- variant in the Duffy antigen receptor for chemokines gene. *PLoS Genet*, 5, e1000360.
- RIDLEY, A. J. L., OU, Y., KARLSSON, R., PUN, N., BIRCHENOUGH, H. L., MULHOLLAND, I. Z., BIRCH, M. L., MACDONALD, A. S., JOWITT, T. A., LAWLESS, C., MILLER, R. L. & DYER, D. P. 2023. Chemokines form complex signals during inflammation and disease that can be decoded by extracellular matrix proteoglycans. *Sci Signal*, 16, eadf2537.
- ROSAS, M., DAVIES, L. C., GILES, P. J., LIAO, C. T., KHARFAN, B., STONE, T. C., O'DONNELL, V. B., FRASER, D. J., JONES, S. A. & TAYLOR, P. R. 2014. The transcription factor Gata6 links tissue macrophage phenotype and proliferative renewal. *Science*, 344, 645-648.
- ROSSANT, J. & CROSS, J. C. 2001. Placental development: lessons from mouse mutants. *Nat Rev Genet*, 2, 538-48.
- ROSSI, D. L., HURST, S. D., XU, Y., WANG, W., MENON, S., COFFMAN, R. L. & ZLOTNIK, A. 1999. Lungkine, a novel CXC chemokine, specifically expressed by lung bronchoepithelial cells. *J Immunol*, 162, 5490-7.
- RUSSO, R. C., ALESSANDRI, A. L., GARCIA, C. C., CORDEIRO, B. F., PINHO, V., CASSALI, G. D., PROUDFOOT, A. E. & TEIXEIRA, M. M. 2011. Therapeutic effects of evasin-1, a chemokine binding protein, in bleomycin-induced pulmonary fibrosis. *Am J Respir Cell Mol Biol*, 45, 72-80.
- SAEDERUP, N., CHAN, L., LIRA, S. A. & CHARO, I. F. 2008. Fractalkine deficiency markedly reduces macrophage accumulation and atherosclerotic lesion formation in CCR2^{-/-} mice: evidence for

- independent chemokine functions in atherogenesis. *Circulation*, 117, 1642-8.
- SALLUSTO, F., MACKAY, C. R. & LANZAVECCHIA, A. 1997. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. *Science*, 277, 2005-7.
- SALTER, B. M., JU, X. & SEHMI, R. 2021. Eosinophil Lineage-Committed Progenitors as a Therapeutic Target for Asthma. *Cells*, 10.
- SAMSON, M., LIBERT, F., DORANZ, B. J., RUCKER, J., LIESNARD, C., FARBER, C. M., SARAGOSTI, S., LAPOUMEROULIE, C., COGNAUX, J., FORCEILLE, C., MUYLDERMANS, G., VERHOFSTEDE, C., BURTONBOY, G., GEORGES, M., IMAI, T., RANA, S., YI, Y., SMYTH, R. J., COLLMAN, R. G., DOMS, R. W., VASSART, G. & PARMENTIER, M. 1996. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature*, 382, 722-5.
- SANCHEZ-ALCANIZ, J. A., HAEGE, S., MUELLER, W., PLA, R., MACKAY, F., SCHULZ, S., LOPEZ-BENDITO, G., STUMM, R. & MARIN, O. 2011. Cxcr7 controls neuronal migration by regulating chemokine responsiveness. *Neuron*, 69, 77-90.
- SARRIS, M., MASSON, J. B., MAURIN, D., VAN DER AA, L. M., BOUDINOT, P., LORTAT-JACOB, H. & HERBOMEL, P. 2012. Inflammatory chemokines direct and restrict leukocyte migration within live tissues as glycan-bound gradients. *Curr Biol*, 22, 2375-82.
- SAVINO, B., BORRONI, E. M., TORRES, N. M., PROOST, P., STRUYF, S., MORTIER, A., MANTOVANI, A., LOCATI, M. & BONECCHI, R. 2009. Recognition versus adaptive up-regulation

and degradation of CC chemokines by the chemokine decoy receptor D6 are determined by their N-terminal sequence. *J Biol Chem*, 284, 26207-15.

- SCHETTERS, S. T. T. & SCHUIJS, M. J. 2021. Pulmonary Eosinophils at the Center of the Allergic Space-Time Continuum. *Front Immunol*, 12, 772004.
- SCHMID, C. D., SCHLEDZEWSKI, K., MOGLER, C., WALDBURGER, N., KALNA, V., MARX, A., RANDI, A. M., GERAUD, C., GOERDT, S. & KOCH, P. S. 2018. GPR182 is a novel marker for sinusoidal endothelial differentiation with distinct GPCR signaling activity in vitro. *Biochem Biophys Res Commun*, 497, 32-38.
- SCHMITZ, J. M., MCCRACKEN, V. J., DIMMITT, R. A. & LORENZ, R. G. 2007. Expression of CXCL15 (Lungkine) in murine gastrointestinal, urogenital, and endocrine organs. *J Histochem Cytochem*, 55, 515-24.
- SCHNEIDER, E. H., FOWLER, S. C., LIONAKIS, M. S., SWAMYDAS, M., HOLMES, G., DIAZ, V., MUNASINGHE, J., PEIPER, S. C., GAO, J. L. & MURPHY, P. M. 2014. Regulation of motor function and behavior by atypical chemokine receptor 1. *Behav Genet*, 44, 498-515.
- SCHULZ, C., GOMEZ PERDIGUERO, E., CHORRO, L., SZABO-ROGERS, H., CAGNARD, N., KIERDORF, K., PRINZ, M., WU, B., JACOBSEN, S. E., POLLARD, J. W., FRAMPTON, J., LIU, K. J. & GEISSMANN, F. 2012. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science*, 336, 86-90.
- SCHYNS, J., BAI, Q., RUSCITTI, C., RADERMECKER, C., DE SCHEPPER, S.,

- CHAKAROV, S., FARNIR, F., PIROTTIN, D., GINHOUX, F., BOECKXSTAENS, G., BUREAU, F. & MARICHAL, T. 2019. Non-classical tissue monocytes and two functionally distinct populations of interstitial macrophages populate the mouse lung. *Nat Commun*, 10, 3964.
- SCOTT, C. L., ZHENG, F., DE BAETSELIER, P., MARTENS, L., SAEYS, Y., DE PRIJCK, S., LIPPENS, S., ABELS, C., SCHOONOOGHE, S., RAES, G., DEVOOGDT, N., LAMBRECHT, B. N., BESCHIN, A. & GUILLIAMS, M. 2016. Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells. *Nat Commun*, 7, 10321.
- SERBINA, N. V. & PAMER, E. G. 2006. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat Immunol*, 7, 311-7.
- SHAO, Z., TAN, Y., SHEN, Q., HOU, L., YAO, B., QIN, J., XU, P., MAO, C., CHEN, L.-N., ZHANG, H., SHEN, D.-D., ZHANG, C., LI, W., DU, X., LI, F., CHEN, Z.-H., JIANG, Y., XU, H. E., YING, S., MA, H., ZHANG, Y. & SHEN, H. 2022. Molecular insights into ligand recognition and activation of chemokine receptors CCR2 and CCR3. *Cell Discovery*, 8, 44.
- SHEN, H., SCHUSTER, R., STRINGER, K. F., WALTZ, S. E. & LENTSCH, A. B. 2006. The Duffy antigen/receptor for chemokines (DARC) regulates prostate tumor growth. *FASEB J*, 20, 59-64.
- SHENG, J., RUEDL, C. & KARJALAINEN, K. 2015. Most Tissue-Resident Macrophages Except Microglia Are Derived from Fetal Hematopoietic Stem Cells. *Immunity*, 43, 382-93.

- SHI, H. Z., XIAO, C. Q., LI, C. Q., MO, X. Y., YANG, Q. L., LENG, J. & CHEN, Y. Q. 2004. Endobronchial eosinophils preferentially stimulate T helper cell type 2 responses. *Allergy*, 59, 428-35.
- SHIGETA, A., HUANG, V., ZUO, J., BESADA, R., NAKASHIMA, Y., LU, Y., DING, Y., PELLEGRINI, M., KULKARNI, R. P., HSIAT, T., DEB, A., ZHOU, B., NAKANO, H. & NAKANO, A. 2019. Endocardially Derived Macrophages Are Essential for Valvular Remodeling. *Dev Cell*, 48, 617-630 e3.
- SHROKA, T. M., KUFAREVA, I., SALANGA, C. L. & HANDEL, T. M. 2023. The dual-function chemokine receptor CCR2 drives migration and chemokine scavenging through distinct mechanisms. *Sci Signal*, 16, eabo4314.
- SIERRO, F., BIBEN, C., MARTÍNEZ-MUÑOZ, L., MELLADO, M., RANSOHOFF, R. M., LI, M., WOHL, B., LEUNG, H., GROOM, J., BATTEN, M., HARVEY, R. P., MARTINEZ-A, C., MACKAY, C. R. & MACKAY, F. 2007. Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 14759-14764.
- SINGH, K., DAVIES, G., ALENAZI, Y., EATON, J. R. O., KAWAMURA, A. & BHATTACHARYA, S. 2017. Yeast surface display identifies a family of evasins from ticks with novel polyvalent CC chemokine-binding activities. *Sci Rep*, 7, 4267.
- SINGH, M. D., KING, V., BALDWIN, H., BURDEN, D., THORRAT, A., HOLMES, S., MCINNES, I. B., NICOLL, R., SHAMS, K., PALLAS, K., JAMIESON, T., LEE, K. M., CARBALLIDO, J. M.,

- ROT, A. & GRAHAM, G. J. 2012. Elevated expression of the chemokine-scavenging receptor D6 is associated with impaired lesion development in psoriasis. *Am J Pathol*, 181, 1158-64.
- SJOBERG, E., MEYRATH, M., MILDE, L., HERRERA, M., LOVROT, J., HAGERSTRAND, D., FRINGS, O., BARTISH, M., ROLNY, C., SONNHAMMER, E., CHEVIGNE, A., AUGSTEN, M. & OSTMAN, A. 2019. A Novel ACKR2-Dependent Role of Fibroblast-Derived CXCL14 in Epithelial-to-Mesenchymal Transition and Metastasis of Breast Cancer. *Clin Cancer Res*, 25, 3702-3717.
- SKOKOWA, J., DALE, D. C., TOUW, I. P., ZEIDLER, C. & WELTE, K. 2017. Severe congenital neutropenias. *Nat Rev Dis Primers*, 3, 17032.
- SPROSTON, N. R. & ASHWORTH, J. J. 2018. Role of C-Reactive Protein at Sites of Inflammation and Infection. *Front Immunol*, 9, 754.
- STENSTAD, H., ERICSSON, A., JOHANSSON-LINDBOM, B., SVENSSON, M., MARSAL, J., MACK, M., PICARELLA, D., SOLER, D., MARQUEZ, G., BRISKIN, M. & AGACE, W. W. 2006. Gut-associated lymphoid tissue-primed CD4⁺ T cells display CCR9-dependent and -independent homing to the small intestine. *Blood*, 107, 3447-54.
- STONE, M. J. 2017. Regulation of Chemokine-Receptor Interactions and Functions. *Int J Mol Sci*, 18.
- STREMMEL, C., SCHUCHERT, R., WAGNER, F., THALER, R., WEINBERGER, T., PICK, R., MASS, E., ISHIKAWA-ANKERHOLD, H. C., MARGRAF, A., HUTTER, S., VAGNOZZI, R., KLAPPROTH, S., FRAMPTON, J., YONA, S., SCHEIERMANN, C., MOLKENTIN, J. D., JESCHKE, U., MOSER,

- M., SPERANDIO, M., MASSBERG, S., GEISSMANN, F. & SCHULZ, C. 2018. Author Correction: Yolk sac macrophage progenitors traffic to the embryo during defined stages of development. *Nat Commun*, 9, 3699.
- STRIZOVA, Z., BENESOVA, I., BARTOLINI, R., NOVYSEDLAK, R., CECRDLOVA, E., FOLEY, L. K. & STRIZ, I. 2023. M1/M2 macrophages and their overlaps - myth or reality? *Clin Sci (Lond)*, 137, 1067-1093.
- SUGAYA, M., NAKAMURA, K., MITSUI, H., TAKEKOSHI, T., SAEKI, H. & TAMAKI, K. 2003. Human keratinocytes express fractalkine/CX3CL1. *J Dermatol Sci*, 31, 179-87.
- SUGIYAMA, T., KOHARA, H., NODA, M. & NAGASAWA, T. 2006. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity*, 25, 977-88.
- SWIRSKI, F. K., NAHRENDORF, M., ETZRODT, M., WILDGRUBER, M., CORTEZ-RETAMOZO, V., PANIZZI, P., FIGUEIREDO, J. L., KOHLER, R. H., CHUDNOVSKIY, A., WATERMAN, P., AIKAWA, E., MEMPEL, T. R., LIBBY, P., WEISSLEDER, R. & PITTET, M. J. 2009. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science*, 325, 612-6.
- SYEDA, M. Z., HONG, T., HUANG, C., HUANG, W. & MU, Q. 2024. B cell memory: from generation to reactivation: a multipronged defense wall against pathogens. *Cell Death Discov*, 10, 117.
- TACHIBANA, K., HIROTA, S., IIZASA, H., YOSHIDA, H., KAWABATA, K., KATAOKA, Y., KITAMURA, Y., MATSUSHIMA, K., YOSHIDA, N., NISHIKAWA, S., KISHIMOTO, T. & NAGASAWA, T. 1998. The

chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature*, 393, 591-4.

TAK, P. P., BALANESCU, A., TSELUYKO, V., BOJIN, S., DRESCHER, E., DAIRAGHI, D., MIAO, S., MARCHESIN, V., JAEN, J., SCHALL, T. J. & BEKKER, P. 2013. Chemokine receptor CCR1 antagonist CCX354-C treatment for rheumatoid arthritis: CARAT-2, a randomised, placebo controlled clinical trial. *Ann Rheum Dis*, 72, 337-44.

TAKAHASHI, K., NAITO, M., KATABUCHI, H. & HIGASHI, K. 1991. Development, differentiation, and maturation of macrophages in the chorionic villi of mouse placenta with special reference to the origin of Hofbauer cells. *J Leukoc Biol*, 50, 57-68.

TAKAYA, K., OKABE, K., ISHIGAMI, A., IMBE, Y., KANAZAWA, H., SAKAI, S., ARAMAKI-HATTORI, N. & KISHI, K. 2022. Actin cable formation and epidermis-dermis positional relationship during complete skin regeneration. *Sci Rep*, 12, 15913.

TAKENAKA, E., VAN VO, A., YAMASHITA-KANEMARU, Y., SHIBUYA, A. & SHIBUYA, K. 2018. Selective DNAM-1 expression on small peritoneal macrophages contributes to CD4(+) T cell costimulation. *Sci Rep*, 8, 15180.

TEOH, P. J., MENZIES, F. M., HANSELL, C. A., CLARKE, M., WADDELL, C., BURTON, G. J., NELSON, S. M. & NIBBS, R. J. 2014. Atypical chemokine receptor ACKR2 mediates chemokine scavenging by primary human trophoblasts and can regulate fetal growth, placental structure, and neonatal mortality in mice. *J Immunol*, 193, 5218-28.

- TERSIGNI, C., DI NICUOLO, F., MAULUCCI, G., ROLFO, A., GIUFFRIDA, D., VEGLIA, M., DE SPIRITO, M., SCAMBIA, G., TODROS, T. & DI SIMONE, N. 2016. Placental Chemokine Receptor D6 Is Functionally Impaired in Pre-Eclampsia. *PLoS One*, 11, e0164747.
- THIRIOT, A., PERDOMO, C., CHENG, G. Y., NOVITZKY-BASSO, I., MCARDLE, S., KISHIMOTO, J. K., BARREIRO, O., MAZO, I., TRIBOULET, R., LEY, K., ROT, A. & VON ANDRIAN, U. H. 2017. Differential DARC/ACKR1 expression distinguishes venular from non-venular endothelial cells in murine tissues. *Bmc Biology*, 15.
- TOURNAMILLE, C., COLIN, Y., CARTRON, J. P. & LEVANKIM, C. 1995. Disruption of a Gata Motif in the Duffy Gene Promoter Abolishes Erythroid Gene-Expression in Duffy Negative Individuals. *Nature Genetics*, 10, 224-228.
- TRIVEDI, P. J. & ADAMS, D. H. 2018. Chemokines and Chemokine Receptors as Therapeutic Targets in Inflammatory Bowel Disease; Pitfalls and Promise. *Journal of Crohns & Colitis*, 12, S641-S652.
- TU, M. M., ABDEL-HAFIZ, H. A., JONES, R. T., JEAN, A., HOFF, K. J., DUEX, J. E., CHAUCA-DIAZ, A., COSTELLO, J. C., DANKIK, G. M., TAMBURINI, B. A. J., CZERNIAK, B., KAYE, J. & THEODORESCU, D. 2020. Inhibition of the CCL2 receptor, CCR2, enhances tumor response to immune checkpoint therapy. *Commun Biol*, 3, 720.
- TUNSTER, S. J. 2017. Genetic sex determination of mice by simplex PCR. *Biology of Sex Differences*, 8.

- ULVMAR, M. H., WERTH, K., BRAUN, A., KELAY, P., HUB, E., ELLER, K., CHAN, L., LUCAS, B., NOVITZKY-BASSO, I., NAKAMURA, K., RULICKE, T., NIBBS, R. J., WORBS, T., FORSTER, R. & ROT, A. 2014. The atypical chemokine receptor CCRL1 shapes functional CCL21 gradients in lymph nodes. *Nat Immunol*, 15, 623-30.
- URAL, B. B., YEUNG, S. T., DAMANI-YOKOTA, P., DEVLIN, J. C., DE VRIES, M., VERA-LICONA, P., SAMJI, T., SAWAI, C. M., JANG, G., PEREZ, O. A., PHAM, Q., MAHER, L., LOKE, P., DITTMANN, M., REIZIS, B. & KHANNA, K. M. 2020. Identification of a nerve-associated, lung-resident interstitial macrophage subset with distinct localization and immunoregulatory properties. *Sci Immunol*, 5.
- USHACH, I. & ZLOTNIK, A. 2016. Biological role of granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) on cells of the myeloid lineage. *J Leukoc Biol*, 100, 481-9.
- VAN BERKEL, V., BARRETT, J., TIFFANY, H. L., FREMONT, D. H., MURPHY, P. M., MCFADDEN, G., SPECK, S. H. & VIRGIN, H. I. 2000. Identification of a gammaherpesvirus selective chemokine binding protein that inhibits chemokine action. *J Virol*, 74, 6741-7.
- VEGA-PEREZ, A., VILLARRUBIA, L. H., GODIO, C., GUTIERREZ-GONZALEZ, A., FEO-LUCAS, L., FERRIZ, M., MARTINEZ-PUENTE, N., ALCAIN, J., MORA, A., SABIO, G., LOPEZ-BRAVO, M. & ARDAVIN, C. 2021. Resident macrophage-dependent immune cell scaffolds drive anti-

bacterial defense in the peritoneal cavity.

Immunity, 54, 2578-2594 e5.

- VIEJO-BORBOLLA, A., MARTINEZ-MARTIN, N., NEL, H. J., RUEDA, P., MARTIN, R., BLANCO, S., ARENZANA-SEISDEDOS, F., THELEN, M., FALLON, P. G. & ALCAMI, A. 2012. Enhancement of chemokine function as an immunomodulatory strategy employed by human herpesviruses. *PLoS Pathog*, 8, e1002497.
- WANG, C., KANG, S. G., LEE, J., SUN, Z. & KIM, C. H. 2009. The roles of CCR6 in migration of Th17 cells and regulation of effector T-cell balance in the gut. *Mucosal Immunol*, 2, 173-83.
- WANG, J. & KUBES, P. 2016. A Reservoir of Mature Cavity Macrophages that Can Rapidly Invade Visceral Organs to Affect Tissue Repair. *Cell*, 165, 668-78.
- WANG, Y., LI, G., STANCO, A., LONG, J. E., CRAWFORD, D., POTTER, G. B., PLEASURE, S. J., BEHRENS, T. & RUBENSTEIN, J. L. 2011. CXCR4 and CXCR7 have distinct functions in regulating interneuron migration. *Neuron*, 69, 61-76.
- WEBER, M., HAUSCHILD, R., SCHWARZ, J., MOUSSION, C., DE VRIES, I., LEGLER, D. F., LUTHER, S. A., BOLLENBACH, T. & SIXT, M. 2013. Interstitial dendritic cell guidance by haptotactic chemokine gradients. *Science*, 339, 328-32.
- WHITEHEAD, G. S., WANG, T., DEGRAFF, L. M., CARD, J. W., LIRA, S. A., GRAHAM, G. J. & COOK, D. N. 2007. The chemokine receptor D6 has opposing effects on allergic inflammation and airway reactivity. *American Journal of Respiratory and Critical Care Medicine*, 175, 243-249.

- WILSON, G. J., FOLEY, L. K., POICALUN, Z., PITMON, E., FUKUOKA, A., LEE, K. M., FERNANDEZ, L., MATHIE, H., BARTOLINI, R., COLE, J. J. & GRAHAM, G. J. 2024. Eosinophils promote monocyte to macrophage differentiation and anti-bacterial immunity. *bioRxiv*, 2024.11.25.625164.
- WILSON, G. J., FUKUOKA, A., LOVE, S. R., KIM, J., PINGEN, M., HAYES, A. J. & GRAHAM, G. J. 2020. Chemokine receptors coordinately regulate macrophage dynamics and mammary gland development. *Development*, 147.
- WILSON, G. J., FUKUOKA, A., VIDLER, F. & GRAHAM, G. J. 2022. Diverse myeloid cells are recruited to the developing and inflamed mammary gland. *Immunology*, 165, 206-218.
- WONG, K., VALDEZ, P. A., TAN, C., YEH, S., HONGO, J. A. & OUYANG, W. 2010. Phosphatidylserine receptor Tim-4 is essential for the maintenance of the homeostatic state of resident peritoneal macrophages. *Proc Natl Acad Sci U S A*, 107, 8712-7.
- WONG, L. M., MYERS, S. J., TSOU, C. L., GOSLING, J., ARAI, H. & CHARO, I. F. 1997. Organization and differential expression of the human monocyte chemoattractant protein 1 receptor gene - Evidence for the role of the carboxyl-terminal tail in receptor trafficking. *Journal of Biological Chemistry*, 272, 1038-1045.
- WU, D., MOLOFSKY, A. B., LIANG, H. E., RICARDO-GONZALEZ, R. R., JOUIHAN, H. A., BANDO, J. K., CHAWLA, A. & LOCKSLEY, R. M. 2011. Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis. *Science*, 332, 243-7.

- WU, W., ZHANG, J., YANG, W., HU, B. & FALLON, M. B. 2016. Role of splenic reservoir monocytes in pulmonary vascular monocyte accumulation in experimental hepatopulmonary syndrome. *J Gastroenterol Hepatol*, 31, 1888-1894.
- WUYTS, A., VAN OSSELAER, N., HAELENS, A., SAMSON, I., HERDEWIJN, P., BEN-BARUCH, A., OPPENHEIM, J. J., PROOST, P. & VAN DAMME, J. 1997. Characterization of synthetic human granulocyte chemotactic protein 2: usage of chemokine receptors CXCR1 and CXCR2 and in vivo inflammatory properties. *Biochemistry*, 36, 2716-23.
- YAN, S., CUI, S., ZHANG, L., YANG, B., YUAN, Y., LV, X., FU, H., LI, Y., HUANG, C. & WANG, P. 2020. Expression of ACKR2 in placentas from different types of preeclampsia. *Placenta*, 90, 121-127.
- YANG, X., BAM, M., BECKER, W., NAGARKATTI, P. S. & NAGARKATTI, M. 2020. Long Noncoding RNA AW112010 Promotes the Differentiation of Inflammatory T Cells by Suppressing IL-10 Expression through Histone Demethylation. *J Immunol*, 205, 987-993.
- YONA, S., KIM, K. W., WOLF, Y., MILDNER, A., VAROL, D., BREKER, M., STRAUSS-AYALI, D., VIUKOV, S., GUILLIAMS, M., MISHARIN, A., HUME, D. A., PERLMAN, H., MALISSEN, B., ZELZER, E. & JUNG, S. 2013. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity*, 38, 79-91.
- YOSHIDA, T., IKUTA, K., SUGAYA, H., MAKI, K., TAKAGI, M., KANAZAWA, H., SUNAGA, S., KINASHI, T., YOSHIMURA, K., MIYAZAKI, J., TAKAKI, S. & TAKATSU, K. 1996. Defective B-1

- cell development and impaired immunity against *Angiostrongylus cantonensis* in IL-5R alpha-deficient mice. *Immunity*, 4, 483-94.
- YOUNG, V. J., BROWN, J. K., SAUNDERS, P. T. & HORNE, A. W. 2013. The role of the peritoneum in the pathogenesis of endometriosis. *Hum Reprod Update*, 19, 558-69.
- YU, C. R., PEDEN, K. W. C., ZAITSEVA, M. B., GOLDING, H. & FARBER, J. M. 2000. CCR9 is a receptor for CCL25 with two forms that show differing sensitivities to ligand and that has SIV go-receptor. *Faseb Journal*, 14, A1166-A1166.
- YU, L., CECIL, J., PENG, S. B., SCHREMENTI, J., KOVACEVIC, S., PAUL, D., SU, E. W. & WANG, J. 2006. Identification and expression of novel isoforms of human stromal cell-derived factor 1. *Gene*, 374, 174-179.
- YU, S., CRAWFORD, D., TSUCHIHASHI, T., BEHRENS, T. W. & SRIVASTAVA, D. 2011. The chemokine receptor CXCR7 functions to regulate cardiac valve remodeling. *Dev Dyn*, 240, 384-93.
- YUAN, J., LIU, Y., YU, J., DAI, M., ZHU, Y., BAO, Y., PENG, H., LIU, K. & ZHU, X. 2022. Gene knockdown of CCR3 reduces eosinophilic inflammation and the Th2 immune response by inhibiting the PI3K/AKT pathway in allergic rhinitis mice. *Sci Rep*, 12, 5411.
- ZEIDLER, C., GERMESHAUSEN, M., KLEIN, C. & WELTE, K. 2009. Clinical implications of ELA2-, HAX1-, and G-CSF-receptor (CSF3R) mutations in severe congenital neutropenia. *Br J Haematol*, 144, 459-67.
- ZEISSIG, M. N., HEWETT, D. R., PANAGOPOULOS, V., MROZIK, K. M., TO, L. B., CROUCHER, P. I., ZANNETTINO, A. C. W. & VANDYKE, K. 2021.

Expression of the chemokine receptor CCR1 promotes the dissemination of multiple myeloma plasma cells in vivo. *Haematologica*, 106, 3176-3187.

- ZHANG, Y., JIN, Y., LI, J., YAN, Y., WANG, T., WANG, X., LI, Z. & QIN, X. 2024. CXCL14 as a Key Regulator of Neuronal Development: Insights from Its Receptor and Multi-Omics Analysis. *Int J Mol Sci*, 25.
- ZHU, B., XU, D., DENG, X., CHEN, Q., HUANG, Y., PENG, H., LI, Y., JIA, B., THORESON, W. B., DING, W., DING, J., ZHAO, L., WANG, Y., WAVRIN, K. L., DUAN, S. & ZHENG, J. 2012. CXCL12 enhances human neural progenitor cell survival through a CXCR7- and CXCR4-mediated endocytotic signaling pathway. *Stem Cells*, 30, 2571-83.
- ZIEGLER-HEITBROCK, L., ANCUTA, P., CROWE, S., DALOD, M., GRAU, V., HART, D. N., LEENEN, P. J., LIU, Y. J., MACPHERSON, G., RANDOLPH, G. J., SCHERBERICH, J., SCHMITZ, J., SHORTMAN, K., SOZZANI, S., STROBL, H., ZEMBALA, M., AUSTYN, J. M. & LUTZ, M. B. 2010. Nomenclature of monocytes and dendritic cells in blood. *Blood*, 116, e74-80.
- ZLOTNIK, A. & YOSHIE, O. 2000. Chemokines: A new classification system and their role in immunity. *Immunity*, 12, 121-127.
- ZLOTNIK, A., YOSHIE, O. & NOMIYAMA, H. 2006. The chemokine and chemokine receptor superfamilies and their molecular evolution. *Genome Biology*, 7.